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# Increased autophagy reduces endoplasmic reticulum stress after neonatal hypoxia–ischemia: Role of protein synthesis and autophagic pathways

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ABSTRACT

The endoplasmic reticulum (ER) stress can result from several pathological conditions that perturb ER homeostasis and is characterized by accumulation of unfolded proteins in the ER lumen. To cope with ER stress, cells activate the unfolded protein response (UPR), a protein quality control mechanism aimed at restoring homeostasis. The present study was undertaken to characterize the UPR after neonatal hypoxia/ischemia (HI) and its crosstalk with autophagy. After HI, there was a significant increase of GRP78 and Hsp70 expression, phosphorylation of eIF2α, Xbp-1 mRNA splicing and CHOP expression, revealing severe ER stress and UPR. Increasing autophagy with rapamycin (Rap) significantly reduced the UPR. Rap did not further increase the eIF2α phosphorylation and p70S6 kinase (p70S6K) inactivation induced by HI. After autophagy activation, however, there was a clear co-localization between monodansylcadaverine (MDC)-positive autophagosome-like structures and the ribosomal protein S6 (RPS6), indicating the presence of ribosomes in autophagosomes (ribophagy). We found that the autophagy inhibitor 3-methyladenine administered after Rap treatment completely reverted the increased phosphorylation of eIF2α and p70S6K inactivation, and blocked the formation of autophagosome-like structures restoring the UPR.

These results demonstrate that the UPR is strongly activated after neonatal HI. Over-activation of autophagy significantly reduces this response, highlighting the relevance of the cross-talk between ER and the autophagy machinery in this important pathological condition. Furthermore, the presence of ribosome subunits in autophagosome-like structures suggests that increased ribosome turnover through autophagy (ribophagy) may represent an additional mechanism involved in the neuroprotective effect observed after autophagy over-activation.



## Introduction

Stresses that alter endoplasmatic reticulum (ER) functions, including transient focal or global cerebral ischemia, cause the accumulation of unfolded proteins in the ER lumen, a condition referred to as ER stress (Rissanen et al., 2006). To cope with ER stress, cells activate a protein quality control mechanism known as unfolded protein response (UPR). The UPR is primarily a protective response aimed at restoring ER functions but, if the stress is severe or prolonged can result in cell death by activating ER-associated apoptotic pathways (Jager et al., 2012; Oyadomari and Mori, 2004). The UPR is carried out by three ER-transmembrane effector proteins, the PKR-like ER kinase (PERK), the inositol-requiring enzyme 1 (IRE1), and the activating transcription factor-6 (ATF-6) (Korennykh and Walter, 2012). Activated PERK phosphorylates the eukaryotic translation initiation factor 2 subunit α (eIf2α) that leads to inhibition of translation into the ER (Donnelly et al., 2013) but also causes an increased translation of the mRNA encoding the activation of translation-4 (ATF-4). The latter is a transcription factor that controls the upregulation of a subset of UPR-target genes that function in redox homeostasis and aminoacid metabolism (Rouschop et al., 2010; Rzymski et al., 2010), including pro-apoptotic effector CCAAT/enhancer-binding protein (C/EBP)

*Abbreviations:* ANOVA, analysis of variance; ATF-4, activating transcription factor-4; ATF-6, activating transcription factor-6; C/EBP, CCAAT/enhancer-binding protein; CHOP, C/EBP homologous protein; eIf2α, eukaryotic translation initiation factor 2 subunit α; ER, endoplasmic reticulum; GRP78, 78-kDa glucose regulated protein; HI, hypoxia–ischemia; Hsp70, heat shock protein 70; IRE1, inositol-requiring enzyme 1; LC3, microtubule-associated protein 1 light chain; 3MA, 3-Methyladenine; MDC, monodansylcadaverine; mTOR, mammalian target of rapamycin; NeuN, neuron-specific nuclear protein; p70S6K, 70 kDa S6 kinase; PERK, PKR-like ER kinase; PN, postnatal; RAP, rapamycin, UPR, unfolded protein response; Xbp-1, X-box binding protein-1.

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homologous protein (CHOP), that activate apoptosis (Jager et al., 2012; Oyadomari and Mori, 2004). The second effector, IRE1, is a serine/threonine protein kinase and endoribonuclease that directly regulates, through its ribonuclease domain, the unconventional splicing of the mRNA encoding the transcription for X-box binding protein-1 (XBP1). XBP1 after translocation to the nucleus causes the induction of ER-resident enzymes and chaperones (Hetz et al., 2011). Finally, activation of ATF6 leads to its translocation from the ER membrane to the Golgi apparatus where it is proteolytically processed, releasing the cytosolic domain which expresses a transcription factor that translocates to the nucleus and upregulates several ER chaperones, such as GRP78 (78-kDa glucose regulated protein), heat shock proteins (HSPs), ER-associated degradation (ERAD)-related genes and XBP1 mRNA (Yamamoto et al., 2007).

The UPR is strictly connected with macroautophagy (hereinafter referred to as autophagy), a collection of pathways by which portions of the cytosol and damaged and/or surplus cell structures are sequestered into membrane-bound compartments and the content degraded through fusion with lysosomes (Yorimitsu and Klionsky, 2005). This process is ubiquitous in eukaryotes from yeast to mammals and is essential for normal cellular development and differentiation (Mizushima, 2005). Autophagy occurs at a basal level and can be significantly induced, depending on the cell type, when necessary. Selective forms of autophagy have been recently described. Pexophagy and mitophagy, for example, are specific processes that degrade an excess of peroxysomes and mitochondria under conditions that change the requirement for these organelles (Dunn et al., 2005; Manjithaya et al., 2010; Youle and Narendra, 2011) whereas reticulophagy/ribophagy, that has been characterized in yeast cells, represents another highly selective process that sequesters and degrades portions of ER and ribosomes (Cebollero et al., 2012; Kraft et al., 2008). It has been reported that selective ER sequestration into autophagosome-like structures helps to maintain survival after severe ER stress, even without degradation by vacuolar proteases, indicating that ER sequestration may represent an important homeostatic mechanism to cope with ER stress (Bernales et al., 2006). This mechanism might also be critical for mammalian cells undergoing hypoxic/ischemic stress, since ribosome biogenesis and protein translation are among the most energy-consuming cellular processes and a rapid reduction of these processes after ischemia may prolong cell survival (DeGracia and Hu, 2007; Kraft et al., 2008).

Here we studied the UPR in a neonatal model of hypoxia/ischemia (HI)-induced brain injury and its crosstalk with autophagy. We show that the UPR is strongly activated after neonatal HI and overactivation of autophagy with rapamycin (Rap) significantly reduces this response. Moreover, we report in vivo evidence of the presence of ribosome subunits in autophagosome-like structures suggesting that increased ribosome turnover through autophagy (ribophagy) could represent an additional mechanism contributing to the neuroprotective effect of autophagy after HI.

## Materials and methods

### *Cerebral hypoxia–ischemia (HI)*

All surgical and experimental procedures were carried out in accordance with the Italian regulation for the care and use of laboratory animals (according to the EU Directive 2010/63/EU), and were approved by the Animal Care Committee of the University of Urbino “Carlo Bo”. On postnatal day 7 (PN7), Sprague-Dawley pup rats (Charles River) were anesthetized with 5% isoflurane in $N_2O/O_2$ (70/30%) mixture and subjected to permanent ligation of the right common carotid artery followed by 2.5 h hypoxia (92% nitrogen and 8% oxygen) as previously described (Balduini et al., 2001).

### *Drug administration*

The pups were anesthetized with 5% isoflurane in $N_2O/O_2$ (70/30%) mixture and placed in a stereotaxic frame. Intracerebroventricular injections were made into the right lateral ventricle using a 5 μL Hamilton syringe as described by Yin et al. (2006). Rapamycin (Rap, 0.5 ng in 0.5 μl 0.1% DMSO; Alexis Biochemicals, 380-004-M001) or the corresponding volume of vehicle was injected 30 min before HI (Yang et al., 2008); 3-methyladenine (3MA, 5 μL, 10 mM, Sigma, M9281) or the corresponding volume of vehicle was injected 20 min before HI (Carloni et al., 2010; Wang et al., 2007; Yang et al., 2008).

### *Western blot analysis*

Pups were anesthetized and euthanized by decapitation 2 or 24 h after HI. The brain was rapidly removed and the cerebral cortex sonicated in 0.4 mL lysis buffer containing 10 mM Tris, 10 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride and a complete protease inhibitor cocktail (Roche, 1 697 498) using an Ultrasonic Liquid Processor XL Sonicator (Heat SystemUltrasonic Inc.). Homogenates were centrifuged for 5 min at 18,500 *g* (4 °C) and the supernatants aspirated and stored at −80 °C until used. After mixing with sodium dodecyl sulfate gel-loading buffer and heating 4 min at 95 °C, samples (50 μg protein) were electrophoresed onto sodium dodecyl sulfate-polyacrylamide gel and proteins transferred to a PVDF membrane. ColorBurst™ eletctrophoresis marker (3 μL/gel, Sigma, C1992) was used for qualitative molecular mass determinations and for visual confirmation of blot transfer efficiency. Then, blots were blocked with non-fat dry milk in TBS-T (10 mM Tris, 150 mM NaCl, pH 7.6, plus 0.1% Tween-20) and probed with the following primary antibodies: anti-Beclin 1 (1:500, monoclonal; BD Transduction Laboratories, 612113), anti-LC3 (1:1000, polyclonal; Cell Signaling Technology, #2775), anti-CHOP (1:500, monoclonal; Santa Cruz Biotechnology, sc-7351), anti phospho(p)-eIF2α (1:500, monoclonal; Santa Cruz Biotechnology, sc-101670), anti-GRP78 (1:500, monoclonal; Santa Cruz Biotechnology, cs-166490), anti-HSP70 (1:500, monoclonal; Santa Cruz Biotechnology, sc-24) or anti phospho(p)-p70S6K (1:1000, polyclonal; Cell Signaling Technology, #9205). A monoclonal antibody against -actin (1:4000, Santa Cruz Biotechnology, sc-8432) was used as control for protein gel loading. Blots were analyzed using the J-Image software. Data were normalized to -actin and expressed as % of control.

### *RNA extraction*

Pups were anesthetized and euthanized by decapitation 2 or 24 h after HI. The brains were stored at −20 °C in RNA*later* RNA Stabilization Reagent (Qiagen, 76104) until RNA extraction. Total RNA was extracted using RNeasy plus mini kit (Qiagen, 74134). Briefly, 90–110 mg of brain tissue was initially disrupted in 200 l RLT buffer. A volume equivalent to 30 mg of brain tissue was withdrawn and completely homogenized in a final volume of 600 l RLT buffer. The purification procedure was performed following the manufacturer's instructions. Total RNA was eluted in 40 l RNase-free water and quantified using the spectrophotometer UV-2401PC (Shimadzu).

### *Detection of Xbp-1 mRNA splicing by PCR*

The cDNA was synthesized from 1 g of total RNA using M-MuLV Reverse transcription Kit (Diatheva srl, MBK0014) with oligo-dT priming. Detection of Xbp-1 mRNA splicing was done by PCR amplification of cDNA using primers designed upstream (Xbp1-rat-F 5′-GGAATG GAGTAAGGCTGGTGG-3′) and downstream (Xbp1-rat-R 5′-AGGCAACA GCGTCAGAATCC-3′) of the 26-nucleotides spliced sequence. The PCR was performed as described previously (Galluzzi et al., 2012), except