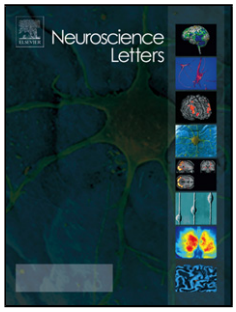

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The activation of autophagy protects neurons and astrocytes against bilirubin-induced cytotoxicity

Mohammed Qaisiya [1,2], Paula Mardešić [1], Beatrice Pastore [3], Claudio Tiribelli [1] and Cristina Bellarosa [1]

[1] Fondazione Italiana Fegato ONLUS, Trieste, Italy

[2] College of Pharmacy and Medical Sciences, Hebron University, Hebron-West Bank, Palestine

[3] International School for Advanced Studies, Trieste, Italy

Corresponding author:

Cristina Bellarosa
Fondazione Italiana Fegato ONLUS
AREA Science Park-Basovizza, Bld Q
Ss 14, km 163.5
34149 Trieste, Italy
Phone: 0039-040-3757840
e-mail: cristina.bellarosa@fegato.it

HIGHLIGHTS:
1. Unconjugated bilirubin (UCB) at high concentration leads to neurotoxicity.
2. Autophagy activation protects neuronal cells from bilirubin cytotoxicity.
3. Autophagy activation by UCB involves mTOR/ER-stress/PKC/calcium signaling.

Abstract

Unconjugated bilirubin (UCB) neurotoxicity involves oxidative stress, calcium signaling and ER-stress. The same insults can also induce autophagy, a process of "self-eating", with both a pro-survival or a pro-apoptotic role. Our aim was to study the outcome of autophagy activation by UCB in the highly sensitive neuronal SH-SY5Y cells and in the resistant astrocytoma U87 cells. Upon treatment with a toxic dose of UCB, the conversion of LC3-I to LC3-II was detected in both cell lines. Inhibition of autophagy by E64d before UCB treatment increased SH-SY5Y cell mortality and made U87 cells sensitive to UCB. In SH-SY5Y autophagy related genes ATG8 (5 folds), ATG18 (5 folds), p62 (3 folds) and FAM 129A (4.5 folds) were induced 8h after UCB treatment while DDIT4 upregulation (13 folds) started at 4h. mTORC1 inactivation by UCB was confirmed by phosphorylation of 4EBP1. UCB induced LC3-II conversion was completely prevented by pretreating cells with the calcium chelator BAPTA and reduced by 65% using the ER-stress inhibitor 4-PBA.

Pretreatment with the PKC inhibitor reduced LC3 mRNA by 70% as compared to cells exposed to UCB alone. Finally, autophagy induction by Trifluoroperazine (TFP) increased the cell viability of rat hippocampal primary neurons upon UCB treatment from 60% to 80%. In SH-SY5Y cells, TFP pretreatment blocked the UCB-induced cleaved caspase-3 protein expression, decreased LDH release from 50% to 23%, reduced the UCB-induction of HO1, CHOP and IL-8 mRNAs by 85%, 70% and 97%. Collectively these data indicate that the activation of autophagy protects neuronal cells from UCB cytotoxicity. The mechanisms of autophagy activation by UCB involves mTOR/ER-stress/PKC/calcium signaling.

Abbreviations:

Unconjugated bilirubin (UCB); trifluoroperazine (TFP); free bilirubin (Bf); 4-Phenyl-butyric acid (4-PBA); eukaryotic initiation factor 4E-binding protein (4EBP); mammalian target of rapamycin complex 1 (mTORC1); sequestosome-1 or ubiquitin-binding protein p62 **(**SQSTM1/p62); protein regulated in development and DNA damage response 1 or DNA-damage-inducible transcript 4 protein (REDD1/DDIT4); family with sequence similarity 129, member A (FAM129A/NIBAN); GABA(A) Receptor-Associated Protein Like 1(GABARAPL1/ATG8); WD repeat domain phosphoinositide-interacting protein 1 (WIPI-1/ATG18).

Keywords: bilirubin neurotoxicity, autophagy, LC-3II, ER-stress, SH-SY5Y, U87.

Introduction

Bilirubin is the end product of heme catabolism that has both cytoprotective and cytotoxic properties(1). The bilirubin circulates in the blood bound to albumin and small fraction remains free which is known as free bilirubin (Bf)(2). Unconjugated bilirubin (UCB) at higher concentration leads to neurotoxicity(3) because Bf can diffuse passively through any cell membrane(4). In brain, neurons are more susceptible to UCB induced toxicity than astrocytes through molecular mechanisms that are still unknown(5–7).

Several studies showed the involvement of mitochondrial dysfunction, oxidative stress and calcium signaling pathways in UCB-induced neurotoxicity(8). Recently, we performed an *in vitro* comparative study between neuronal cells and astrocytoma cells that indicated the endoplasmic reticulum (ER) stress as one of the earliest events associated with UCB-induced apoptosis in neuronal cells(9).

One of the central signaling pathways that lies down-stream of ER stress is autophagy(10). Chronic ER stress leads to activation of two unfolded protein degradation pathways: the ubiquitin-proteasome via ERAD and the lysosome-mediated protein degradation via autophagy(11). Autophagy is a process of "self-eating" that mediates bulk degradation of cytoplasmic proteins, cellular components and organelles. The molecular machinery of autophagy is complex and involved the formation of a double membrane vesicle called autophagosome containing unfolded proteins and damaged organelles, that fuses with lysosome(12). Different sets of genes are involved in autophagy regulation such as the core ATG proteins(13) and SQSTM1/p62(14). Confirmation of autophagy activation is monitored by the conversion of the microtubule-associated protein 1 light chain (LC3-I or Atg8) to phospholipid associated proteins (LC3-II) which is recruited in the autophagosome and then degraded together with its cargo in the lysosome(15–17). The accumulation of autophagosomes is not always indicative of autophagy induction and may represent either the increased generation of autophagosomes and/or a block in autophagosomal maturation and the completion of the autophagy pathway. It is necessary to distinguish whether autophagosome accumulation is due to autophagy induction or rather a block in downstream steps, by performing "autophagic flux" assays that distinguish between these two possibilities. One of the principal methods in current use to measure autophagic flux is the monitoring of LC3 turnover, which is based on the observation that LC3-II is degraded in autolysosomes. If cells are treated with inhibitors of lysosomal proteases such as E64d, the degradation of LC3-II is blocked, resulting in the accumulation of LC3-II (18). In addition to ER stress-induced autophagy, several extra and intra-cellular stress signals can independently induce autophagy or integrate together to determine autophagy outcome (12,19,20).

mTORC1 regulates many major cellular processes. Once inactivated, under conditions such as hypoxia, nutrient deprivation, energy stress and increase of intracellular calcium (that also affects ER integrity), it will induce autophagy(21,22). Well-known mTORC1 targets include ribosomal p70S6, eukaryotic initiation factor 4E-binding protein (4EBP)(22)and REDD1 (also known as DDIT4) which in turn is regulated by UPR signaling(23). FAM129A (also known as NIBAN) has been demonstrated to be involved in the ER stress response and to positively affect the protein translation machinery by regulating the mTOR pathway(24). ER stress may induce autophagy also *via* a mTOR- independent mechanism through the increase

of intracellular calcium and activation of PKC(25). Stress response kinases, such as PI3K, MAPK and JNK, are also involved in the regulation of autophagy- related genes(26).

Since toxic doses of UCB causes ER-stress, mitochondrial dysfunction and an increase in intracellular calcium levels, autophagy activation may play a central role in determining cell fate. Until now, few studies have investigated the role of UCB in inducing autophagy. The up-regulation of some autophagy- related genes such as GABARAPL1/ATG8 and WIPI1/ATG18 in human neuroblastoma SH-SY5Y cells by UCB suggested autophagy involvement (27). Only two studies showed the conversion of LC3-I to LC3-II by UCB, the first in a model of human brain microvascular endothelial cells(28) and the second in the Ugt1-/-mouse model of neonatal hyperbilirubinemia[28]. Both works showed that autophagy is activated at high bilirubin levels and at later stages. In the Ugt1-/- mouse model, autophagy activation follows ER-stress and is suggested as the latest pro-survival mechanisms activated by cells to overcome bilirubin toxicity(29). In the brain microvascular endothelial cells, it was suggested that autophagy may be among the mechanisms involved in UCB-induced cell death(28).

Because autophagy may have both pro-survival or pro-apoptotic role, we extended our previous work on cell signaling activation to analyze further the role of autophagy in the highly sensitive SH-SY5Y cells and in the resistant U87 cells. The outcome of autophagy activation by UCB and the signaling pathways involved in this process was investigated.

Methods

Cell culture

SH-SY5Y human neuroblastoma cells (ATCC CRL-2266) were maintained in EMEM/F12 1:1 medium supplemented with 15% fetal bovine serum (FBS) and 1% nonessential amino acids (Sigma–Aldrich, USA). U87 astrocytoma cells, kindly provided by Dr. Maria Elisabetta Ruaro, Department of Medical and Biological Sciences (DSMB), University of Udine, Udine,Italy, were maintained in DMEM medium supplemented with 10% FBS. All media were supplemented with 1% penicillin/streptomycin solution (100 U/mL penicillin, 100 mg/mL streptomycin), 2mM l –glutamine.

Primary cell cultures were prepared from rat hippocampal neurons, according to the method described by Andjus et al.(30) with slight modifications. All procedures were approved by the local veterinary authorities and performed in accordance with the Italian law (decree 26/2014) and the EU guidelines (2007/526/CE and 2010/63/UE). Hippocampi were dissected from 3day-old postnatal animals. The isolated tissue was quickly sliced and digested in a digestion solution containing trypsin and DNAse (Sigma-Aldrich). The reaction was stopped with trypsin inhibitor (Sigma-Aldrich) and cells were mechanically dissociated in a dissection medium containing DNAse. After centrifugation, the cell pellet was resuspended in the culture medium and distributed in a 24 well Multiwell (Falcon), previously coated with polyornithine (50 µg/ml, Sigma-Aldrich) and Matrigel (2% (w/v), BD). Plating was carried out at a density of 100.000 cells per well. Cultures were incubated at 37°C in a humidified atmosphere with 5% CO2, in the culture medium consisting of Minimum Essential Medium with GlutaMAX (Invitrogen, Life Technologies) supplemented with 10% dialyzed foetal bovine serum (Invitrogen, Life Technologies), 0.6% D-glucose, 15 mM Hepes, 0.1 mg/ml

apo-transferrin, 30 μ g/ml insulin, 0.1 μ g/ml D-biotin, 1 μ M vitamin B12 (Sigma-Aldrich), and 2.5 μ g/ml gentamycin (Life Technologies). Two days after plating, 2 μm cytosine-β-d-arabinofuranoside (Sigma-Aldrich) was added to the culture medium, to inhibit the growth of glial cells, and the concentration of FBS was decreased to 5% (31).

Treatments

UCB toxicity is related to the amount of the free bilirubin (Bf)(32) and the threshold value of toxicity *in vitro* occurs at Bf of 70 nM(33). Cells at 80 % of confluence were treated with toxic Bf concentration (90 or 140 nM) or DMSO-controls. UCB dissolved in DMSO (3 μg/μL) was added to complete cell medium, and its concentration was verified spectrophotometrically at 468 nm. The Bf concentration was calculated by the peroxidase method as previously described (34).

E64d (Santa Cruz Biotechnology, SC 201280), an inhibitor of lysosomal proteases (35), was dissolved in DMSO. SH-SY5Y cells and U87 cells were exposed to 100μM E64d or 0.3% DMSO for 24h and then exposed to140nM Bf for additional 24h in the presence of E64d. Trifluoroperazine (TFP) (Santa Cruz Biotechnology, SC 201498),an autophagy inducer (36), was dissolved in water. SH-SY5Y cells were pre-treated with 10μM TFP while primary cultures of rat hippocampal neurons were pre-treated with either 1μMor 5μM TFP. After 24h, SH-SY5Y cells were exposed to 140 nM Bf for additional 24h, while primary cultures of rat hippocampal neurons were exposed to 90 nM Bf for 24h. 4-Phenyl-butyric acid (Sigma Aldrich, P21005) was prepared by titrating equimolecular amount of 4-PBA with sodium hydroxide to pH 7.4. SH-SY5Y cells were pre-treated with 2 mM of 4-PBA for 2h and then treated with 0.6% DMSO or 140 nM Bf for additional 24 h in the presence of 4-PBA. Specific signaling pathway inhibitors (Sigma Aldrich) were melted and applied to SH-SY5Y cells as previously described (37). They are: PI3K inhibitor LY-294002 (10 μM), MEK1/2 inhibitor PD 98059 (20 μM), c-Jun NH2-terminal kinases (JNK) inhibitor SP 600125 (10 μM), p38α inhibitor SB 203580 (10 μM) and PKC inhibitor GF 109203X (10 μM).

Assessment of autophagy flux

To measure autophagic flux we both monitored LC3 turnover by Western Blot and measured the fluorescence of accumulated autophagic vacuoles in lysosomally inhibited live cells by fluorescence microplate assay.

Cyto-ID Green autophagy detection kit (Enzo Life Sciences, Farmingdale, NY, USA) was used to detect acid autophagic vacuoles as recommended by manufacturer. Briefly, SH-SY5Y cells were seeded in a 96-well plate at a confluence of 12500 cells/cm^2. The day after cells were pretreated with 100μM E64d or 0.3% DMSO for 24h and then exposed to140nM Bf or 0.6% DMSO for additional 24h in the presence of E64d. After treatment, cells were stained with Cyto-ID Green and Hoechst 33342 dyes for 30 min at 37 °C, and then washed. The plate was then analyzed by a fluorescent microplate reader (Perkin Elmer, EnSpire Multimode Plate Reader, Hamburg, Germany). The CYTO-ID Green detection reagent was read with a FITC filter (Excitation ~480 nm, Emission ~530), and the Hoechst 33342 Nuclear Stain read with a DAPI filter set (Excitation ~340, Emission ~480). The green autophagy signal was then normalized with the blue signal and the results expressed as relative to control cells.

LDH and MTT assay

Cells toxicity was determined by the mean of LDH release using CytoTox-one™ homogenous membrane integrity assay (Promega, USA) following the manufacturer's instructions. Percentage of dead cells was expressed as relative to control cells treated with lysis buffer in order to obtain 100 % of LDH release. Cells viability were determined by 3(4,5-dimethylthiazolyl-2)-2,5 diphenyltetrazolium (MTT, Sigma Aldrich) assay as previously described(38).

RNA extraction, reverse transcriptase and Real time-PCR

RNA extraction, cDNA synthesis and real-time PCR (qRT-PCR) were performed as previously described(9). Primers used were listed in **Table 1**. Expression was normalized to housekeeping genes and expressed as relative to cells treated with DMSO, in the absence or presence of inhibitors when applied. Results are obtained from at least three independent experiments.

Gene	Accession number	Forward primers 5' - 3'	Reverse primers 5' - 3'
GABARAPL1	NM_031412.2	ACGCCTTATTCTTCTTTG	AACCACTCATTTCCCATA
WIPI1	NM_017983.5	GAGAAGTTATTCCTGAACAT	CACGGCACAAGATTATAG
SQSTM1	NM_001142298.1	GGACTTGGTTGCCTTTTC	TTAATGTAGATTCGGAAGATGTC
FAM129A	NM_052966.4	GAATGAAGTCAGCCAGAA	TTAGTATAACAAGGTTCCATCT
DDIT4	NM_019058.2	GTGGAGGTGGTTTGTGTA	CAGGTCAGTAGTGATGCT
HO-1	NM_002133.2	ATGCCCCAGGATTTGTCA	CCCTTCTGAAAGTTCCTCAT
CHOP	NM_001195056	CACTCTCCAGATTCCAGTCAG	AGCCGTTCATTCTCTTCAGC
IL-8	NM_000584	GACATACTCCAAACCTTTCCAC	CTTCTCCACAACCCTCTGC
GAPDH*	NM_002046.4	TCAGCCGCATCTTCTTTTG	GCAACAATATCCACTTTACCAG
HPRT*	NM_000194	ACATCTGGAGTCCTATTGACATC	CCGCCCAAAGGGAACTGATAG

Table 1. List of human primer sequences used for qRT-PCR analysis **Housekeeping genes used to normalize the expression of target genes.*

Total protein extraction and Western blot

Total protein extraction, quantification and Western blot analysis were performed as described in previous work(9). First antibodies, used at 1:2000 dilution, include LC3 (Thermo Fisher, PA1-16930), p-4EBP1 (Cell Signaling Technology, #9451), caspase-3 (Cell Signaling Technology, #9662) and α-actin (Sigma–Aldrich, A2066). The relative intensities of protein bands were measured in three independent experiments, analyzed by using the NIH Image software (Scion Corporation Frederick, MD, USA), normalized to α-actin and represented as relative to controls.

Statistical analysis

GraphPad Prism software was used to perform statistical analysis. Data were obtained from at least three independent experiments and are expressed as mean ± SD. Analysis was performed using student's t test. $P < 0.05$ was considered as significant.

Results

UCB induces autophagy as a pro-survival mechanism both in SH-SY5Y neuronal cells and U87 astrocyte cells

In the present work we studied the effects of toxic doses of UCB on autophagy signaling pathways using the two extremes of cell lines: the highly sensitive SH-SY5Y neuronal cells versus the highly resistant U87 astrocytoma cells(9). We monitored autophagy by analyzing the conversion of LC3-I to LC3-II taking the latter as a protein marker (39). LC3-II proteins were not detected in DMSO-exposed controls while it was induced in SH-SY5Y and U87 cell lines upon 140nM Bf exposure **(Fig 1A and 1B)**. To confirm that the induction of LC3-II proteins was related to Bf-induced autophagy, we measured "autophagic flux" by treating cells with the lysosomal inhibitor E64d. In SH-SY5Y cells the accumulation of LC3-II proteins by UCB were further increased (1.9 folds) in the presence of E64d compared to cells treated only with UCB (1 fold) **(Fig 1C and 1D)**. This results was confirmed also by the increase of the Cyto-ID Green autophagy signal measured in cells treated with both 140nM Bf and 100 µM E64d (4 fold) compared to cells in cells treated with 140nM Bf alone (2 fold) **(Fig 1E)**.

E64d is commonly used to block autophagy at later stage by inhibiting the autolysosome activity and have been used in SH-SY5Y cells (35). SH-SY5Y and U87 cells viability were analyzed upon UCB exposure in the presence or absence of E64d. UCB reduced SH-SY5Y cells viability by 40% and reduced further by 60% in the presence of E64d. U87 cells did not show reduction in viability by UCB exposure. Interestingly U87 cells become sensitive and reduced cells viability by 60% in the presence of E64d **(Fig 1F)**.

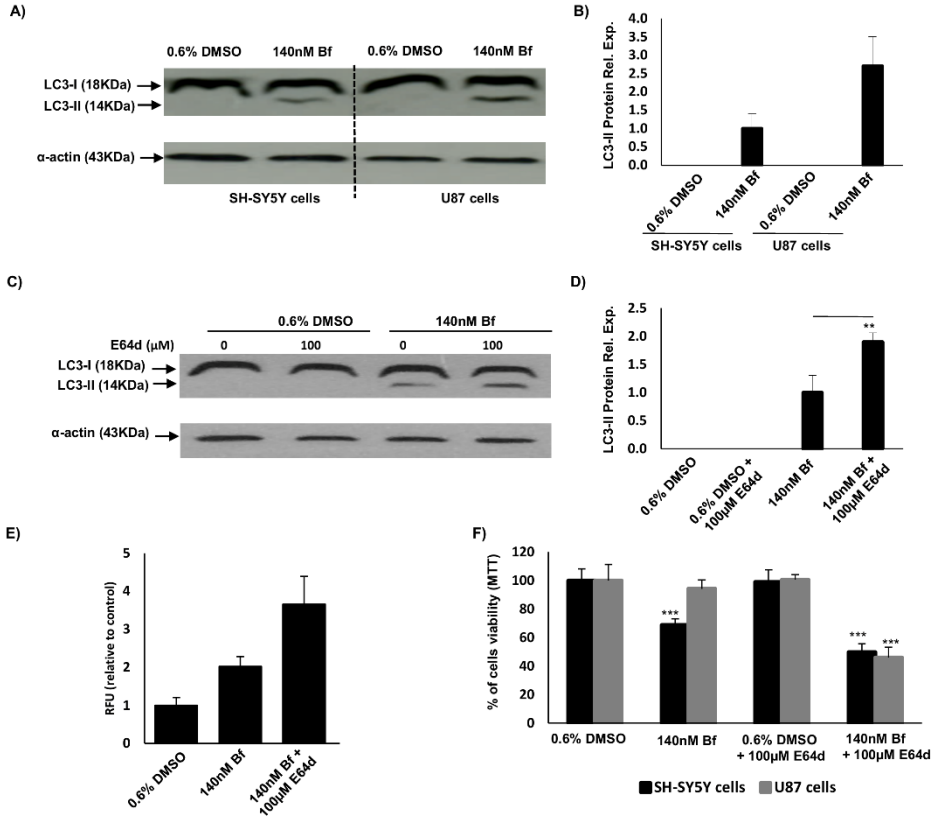

Figure 1: Effects of UCB on autophagy and cell survival in both neuron and astrocyte cell lines.

A) Representative Western blot for LC3 proteins expression in SH-SY5Y cells and U87 cells treated for 24h with 0.6% DMSO or 140nM Bf.

B) The optical density of LC3-II protein from three independent experiments was normalized to α-actin and represented as relative to Bf treated SH cells.

C) Representative Western blot for LC3 proteins expression in SH-SY5Y cells exposed to 0.6% DMSO or 140nM Bf for 24h in the presence or absence of 100μM E64d

D) The optical density of. LC3-II protein from three independent experiments was normalized to α-actin and represented as protein expression relative to control.

E) Detection of autophagy by microplate reader. SH-SY5Y cells were exposed to 0.6% DMSO(control), 140nM Bf or 140nM Bf in the presence of 100 μM E64d for 24h. Cells were then stained with CYTO-ID® Green Detection Reagent and with Hoechst 33342 for cell number normalization. The data indicate the relative green fluorescence normalized by blue fluorescence and expressed as relative to control. Data represent the mean of four wells.

*F) Cells viability (MTT) of SH-SY5Y cells and U87 cells exposed to 0.6% DMSO or 140nM Bf in the presence of absence of 100μM E64d. Results obtained from four independent experiments and significance of Bf- treated cells is relative to DMSO -treated cells in the absence or presence of E64d inhibitor (***P < 0.001)*

UCB induces mRNA expression of autophagy related genes and activates mTOR signaling pathways in SH-SY5Y cells

To confirm autophagy activation we analyzed some of autophagy related genes previously detected by microarray performed in SH-SY5Y cells exposed to 140nM Bf for 24h (27).ATG8, ATG18, p62, FAM 129A and DDIT4 mRNAs were analyzed by qRT-PCR in SH-SY5Y cells treated with 140nM Bf or 0.6% DMSO for 4h, 8h and 24h to identify early and late response. Compared to DMSO-treated cells, SH-SY5Y cells induced the mRNA expression of DDIT4 which starts at 4h (13 folds) followed by the induction of ATG8 (5 folds), ATG18 (5 folds), p62 (3 folds) and FAM 129A (4.5 folds) at 8h. Induction continues for 24h **(Table 2)**.

Genes	140nM Bf (4 h)	140nM Bf (8 h)	140nM Bf (24 h)
GABARAPL1/ ATG8	1.1 ± 0.3	5.4 ± 0.9**	11.3 ± 2.7**
WIPI1/ATG18	1.9 ±0.1***	4.7 ± 0.2***	27.1 ± 4.6**
SQSTM1/p62	1.1 ±0.2	2.7 ± 0.1***	21.4 ± 6.0**
FAM129A/NIBAN	1.0 ±0.6	4.6 ± 0.4**	30.3 ± 9.8*
DDIT4 /REDD1	12.8 ± 3.9**	45.8 ±11**	23.1 ± 3.7***

Table 2. mRNA relative expression of Autophagy related genes in SH-SY5Y cells *mRNA expression of the autophagy-related genes in SH-SY5Y cell line exposed to 140nM Bf or 0.6% DMSO. Expression is relative to cells treated with 0.6% DMSO for each indicated time (considered as 1). Data are representative of the mean ± SD of at least three independent experiments (*P < 0.05, **P < 0.01, ***P < 0.001).*

The induction of FAM 129A and DDIT4 suggests mTORC1 inactivation by UCB. To further confirm this process, we performed Western blot analysis, which detected p-4EBP1 (mTORC1 target) in both SH-SY5Y and U87 cells. Compared to DMSO-treated cells, UCB induced phosphorylation of 4EBP1 in SH-SY5Y cells while no change was detected in U87 cells. SH-SY5Y cell starved for 24h were used as positive control. It is worth to note that the protein basal expression of 4pEBP1 is higher in U87 cells compared to the undetected expression in SH-SY5Y cells **(Fig 2A and 2B)**.

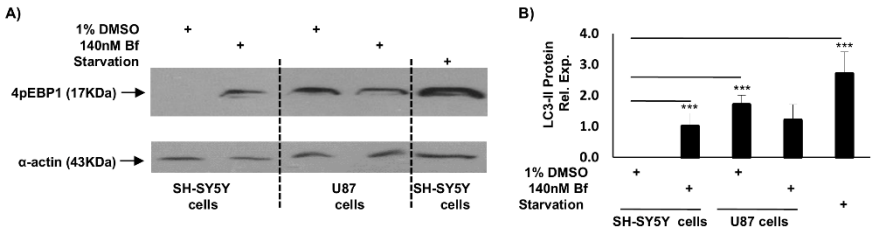

Figure 2: Effects of UCB on 4pEBP1 phosphorylation (mTOR signaling) in SH-SY5Y cells.
A) Representative Western blot for 4pEBP1 protein expression in SH-SY5Y cells and U87 cells exposed to 0.6% DMSO or 140nM Bf for 24h. B) The optical density for 4pEBP1 protein from three independent experiments was normalized to α-actin and represented relative to DMSO-treated cells. SH-SH5Y cells starved for 24h were used as positive control.

Intracellular Calcium, PKC and ER-stress pathways are involved in LC3-II conversion by UCB in SH-SY5Y cells

The intracellular mechanisms that control autophagy are complex. Our previous studies demonstrated that the mechanisms of UCB- induced cell injury are a multifaceted complex process involving oxidative stress, ER-stress and calcium-PKC signaling pathways. We performed qRT-PCR in order to analyze LC3 mRNA expression in SH-SY5Y cells after exposure to UCB in the presence of different kinases inhibitor. Cells exposed to UCB in the presence of PKC inhibitors reduced LC3 mRNA by 70% as compared to cells exposed to UCB alone. No significant change was detected in the presence of inhibitors of MEK1/2, p38α, JNKor PI3K signaling pathways **(Fig 3A)**. PKC activation suggests calcium signaling. The treatment of SH-SY5Y cells with 140nM Bf in the presence of intracellular Ca^{+2}– chelator (BAPTA) completely prevented the formation of LC3-II proteins **(Fig 3B and 3C)**.

As we previously observed the activation of ER-stress signaling by UCB and since autophagy induction under ER-stress is fundamental, SH-SY5Y cells were treated with UCB in the presence of 2mM 4-PBA (ER-stress inhibitor) to assess the contribution of ER-stress to autophagy. Cells treated with 140nM Bf in the presence of 4-PBA reduced the UCB-

induction of LC3-II proteins by 65% compared to cells treated only with 140nM Bf **(Fig 3D and 3E)**. Similar effect was also detected at the mRNA level of LC3-II which is reduced by 70% in cells treated with 140nM Bf in the presence of 4-PBA compared to cells treated only with 140nM Bf **(Fig 3F)**.

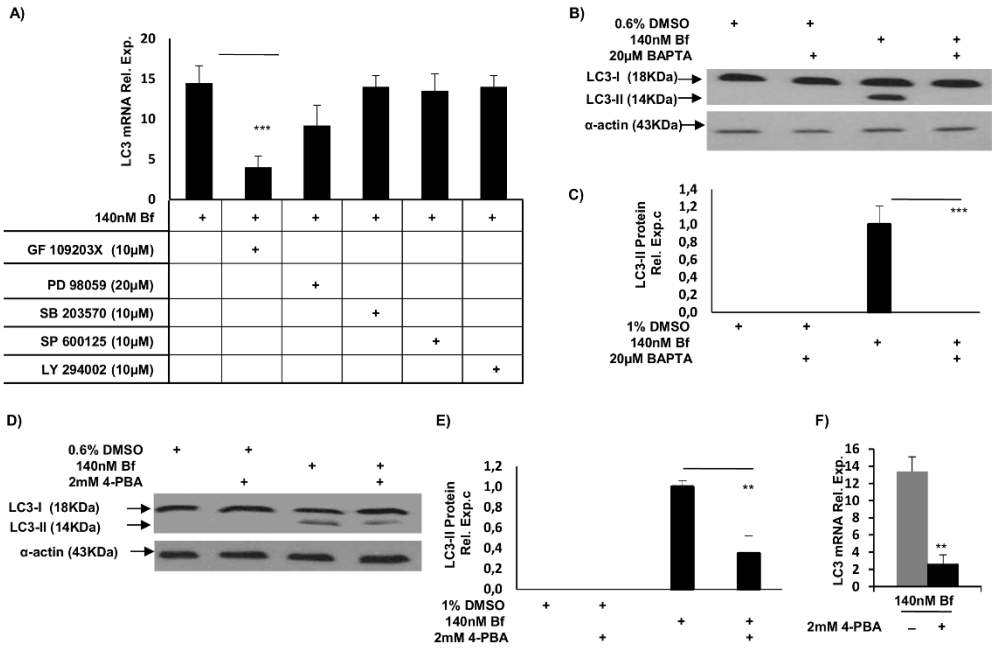

Figure 3:Effects of PKC, Intracellular Calcium and ER-stress on UCB-induced LC3-II expression.
*A) LC-3 mRNA expression in SH-SY5Y cells treated with 140 nM Bf for 24h in the absence or presence of specific signaling pathway inhibitors used at the indicated concentrations: PKC (GF 109203X), MEK1/2 (PD 98059), p38α (SB 203570), JNK (SP 600125), and PI3K (LY-294002). Relative expression was normalized to cells incubated with specific chemical inhibitors alone or with 0.6% DMSO for Bf treated cells. Significance of Bf-GF 109203X-treated cells is relative to Bf-treated cells (***P < 0.001). Data is obtained from three independent experiments for each treatment.*
B) Representative Western blot for LC3 proteins expression in SH-SY5Y cells treated with 0.6% DMSO or 140nM Bf in the absence or presence of intracellular calcium chelator (20μM BAPTA).
*C) Optical density for LC3-II protein from three independent experiments was normalized to α-actin and represented relative to Bf-treated cells in the absence of BAPTA. (***P < 0.001).*
D)Representative Western blot for LC3 proteins expression in SH-SY5Y cells treated with 0.6% DMSO or 140nM Bf for 24h in the absence or presence of 2mM 4-PBA.
E) Optical density for LC3-II protein from three independent experiments was normalized to α-actin and represented relative to Bf-treated cells in the absence of 4-PBA
*F) LC3 mRNA expression in SH-SY5Y cells treated with 140nM Bf for 24h in the absence or presence of ER-stress inhibitor (2mM 4-PBA). Expression is relative to UCB-treated cells. Significance of 4PBA-Bf treated cells is relative to Bf-treated cells (**P < 0.01).Data is obtained from three independent experiment for each treatment.*

Autophagy induction prevents UCB-induced oxidative stress, ER-stress, inflammation and neuronal cell death

To understand the role of autophagy, SH-SY5Y cells were pre-exposed to the autophagy activator (10µM TFP) before exposure to 140nM Bf. TFP induced LC3-II proteins was observed after 24h of treatments (data not shown). Compared to cells treated only with UCB, TFP reduced the UCB-induction of HO1 (marker of OS), CHOP (pro-apoptotic factor of ER-stress signaling) and IL-8 (marker of inflammation) mRNAs by 85%, 50% and 97%, respectively **(Fig 4A)**. SH-SY5Y cells exposed to UCB showed 50% increase in LDH release, which was decreased to 23% in the presence of TFP **(Fig 4B)**. To confirm this results, cleaved caspase-3 was analyzed in SH-SY5Y cells exposed to 140nM Bf in the absence or presence of TFP. TFP blocked the UCB-induced cleaved caspase-3 proteins to more than 95% compared to cells treated only with UCB **(Fig 4C and 4D)**. Furthermore, we exposed rat hippocampal primary neurons to 1µM and 5µM TFP for 24h before the exposure to 90nM Bf. 90nM Bf treatment reduced cells viability to 60% which was further increased to 80% in the presence of TFP **(Fig. 4E)**.

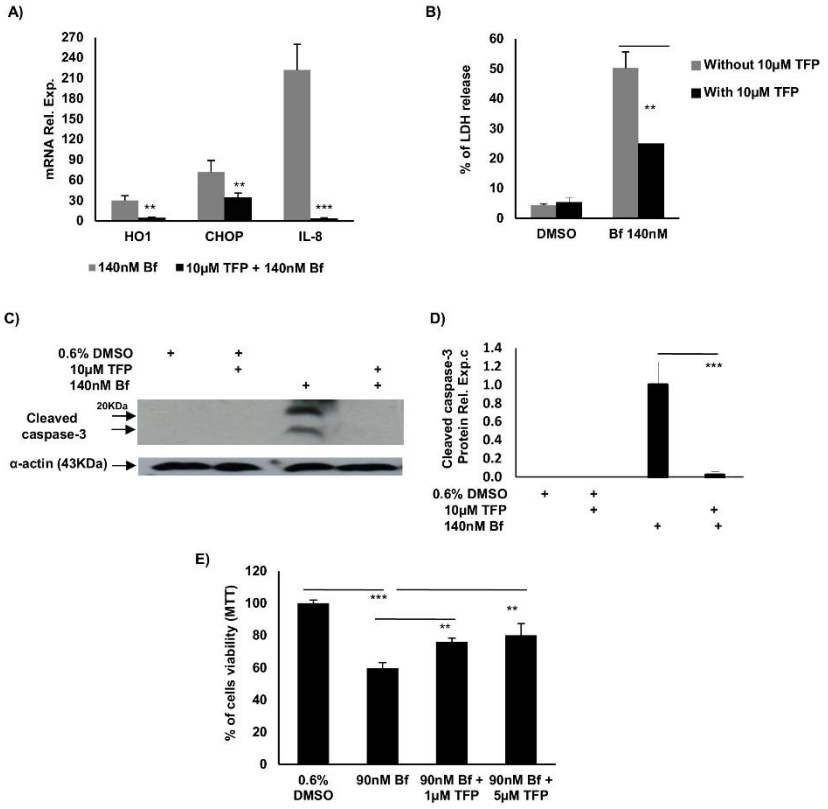

Figure 4: Effects of TFP (autophagy activator) on UCB-induced oxidative stress, ER-stress, inflammation and apoptosis in SH-SY5Y cells and primary neuronal cells

*A) HO1, CHOP and IL-8 mRNA expression in SH-SY5Y cells treated with 140nM Bf in the absence or presence of 10µM TFP. Expression is relative to cells treated with 0,6% DMSO which was considered as one. TFP alone does not change gene expression of the analyzed genes. Significance of TFP-Bf treated cells is relative to Bf-treated cells (**P < 0.01, ***P < 0.001).Data is obtained from three independent experiments for each treatment.*

*B) LDH release in cells exposed to 0.6%DMSO or 140nM Bf in the absence or presence of 10μM TFP. Significance of TFP-Bf treated cells is relative to Bf-treated cells (**P < 0.01). Data is obtained from three independent experiments.*
C) Representative Western blot of cleaved caspase-3 protein expression in SH-SY5Y cells exposed to 0.6% DMSO or 140nM Bf in the presence or absence of 10μM TFP.
D) The optical density of cleaved caspase-3 protein from three independent experiments was normalized to α-actin and represented relative to Bf-treated cells in the absence of TFP.
*E) Cells viability (MTT) performed in primary neuronal cells exposed to 90nM Bf in the absence or presence of TFP (1μM or 5μM). Significance of Bf treated cells is relative to 0,6% DMSO treated cells, while significance of TFP-Bf treated cells is relative to Bf-treated cells (**P < 0.01, ***P < 0.001). Data is obtained from three independent experiments.*

Discussion

In principle, autophagy activation during chronic ER stress is an essential pro-survival response that is activated physiologically to remove long-lived protein aggregates and damaged organelles in order to replenish ER(11,20). Induction of autophagy could have clinical applications in neurodegenerative diseases associated with accumulation of misfolded proteins such as Alzheimer disease and Multiple Sclerosis (40). However, continuous activation of autophagy itself might lead to cell death (41). We have previously demonstrated the role of UCB in the induction of several autophagy-associated stress events such as oxidative stress, inflammation and ER-stress (9,37). Two studies showed the conversion of LC3-I to LC3-II by UCB, one suggesting that autophagy may be among the mechanisms of UCB-induced cell death(28), the second that it is a pro-survival mechanism(29) . Because autophagy may has both pro-survival or pro-apoptotic role we extended our previous work on cell signaling activation by toxic doses of UCB to further analyze the role of autophagy in the highly sensitive SH-SY5Y cells and the resistant U87 cells.

We observed that toxic levels of UCB induce autophagy (through LC3-II conversion) in both SH-SY5Y neuronal and U87 astrocytoma cells **(Fig 1A)**. Autophagy activation in SH-SY5Y cells was confirmed by analyzing the mRNA of some of autophagy related genes such as GABARAPL1/ATG8, WIPI1/ATG18, SQSTM1/p62, FAM129A/NIBAN and DDIT4/REDD1(13,14,23). mRNA levels of all genes were induced starting at early time (4h-8h), **(Table 2)** which suggest the activation of autophagy as an early response to UCB toxicity.

To study the role of autophagy, SH-SY5Y and U87 cells were exposed to UCB in the presence of autolysosome inhibitor (E64d)(35) which was able to sensitize both cell lines to UCB toxicity and decreases cells viability **(Fig 1F)**. Interestingly, the U87 astrocytoma cells that do not exhibit any UCB toxicity becomes sensitive to UCB toxicity in the presence of E64d suggesting an important role for autophagy to maintain cell survival. The results obtained by E64d were confirmed by using the autophagy inducer TFP. Pre-activation of autophagy using TFP reduced the SH-SY5Y cells death (as shown by measuring LDH release) **(Fig 4B)** and increased cells viability (MTT test) of rat primary hippocampal neurons **(Fig 4E)**. As expected, neuronal primary culture was more sensitive to Bf treatment than SH-SY5Y cell line. A 24-hour Bf 90nM treatment reduced the cell viability to 60%. Accordingly, the TFP effect was evaluated under these conditions. As shown by Tsvetkov et al, 1 μM TFP is enough to induce autophagy in neuronal primary culture (36). When we

assesses the effects of TFP on the cleaved form of the pro-apoptotic caspase-3, we observed that TFP reduces the UCB-induced pro-caspase-3 levels in SH-SY5Y cells **(Fig 4C and 4D).**

We previously observed that UCB- induced SH-SY5Y cell death involves oxidative stress, inflammation and ER-stress/CHOP signaling pathways (9,37). Previous studies demonstrated that autophagy activation blocks oxidative stress, inflammation, ER-stress and apoptosis (42–44). qRT-PCR performed in SH-SY5Y cells showed that TFP decreased the UCB-induction of HO-1, CHOP and IL-8 **(Fig 4A)**. All together, these results indicate the pro-survival role of autophagy in protecting neuronal cells against UCB induced cytotoxicity and suggest its role in reducing markers of oxidative stress, inflammation, ER-stress and apoptosis.

Autophagy signaling activation is complex process that involve multifactorial processes and include activation by several stress response kinases, oxidative stress, mTOR, ER-stress and calcium signaling pathways (12,26). To understand the contribution of these signaling pathways we first analyzed the effects of UCB on mTOR signaling pathway as the induction of DDIT4 and FAM129A in SH-SY5Y neuronal cells suggest mTOR inactivation. Indeed the phosphorylated form of 4EBP1 was enhanced by UCB indicating the inactivation of mTOR signaling **(Fig 2A and 2B)**. It is worth to note that the protein basal expression of 4pEBP1 is higher in U87 cells compared to the undetected expression in SH-SY5Y cells. Autophagy may be necessary for neuronal protection (45). We can speculate that the higher level of 4pEBP1 protein present in the U87 cell line contributes to make astrocytes resistant against UCB-induced oxidative stress, through a higher basal autophagy. The inactivation of mTOR by UCB could be attributed to either oxidative stress, ER-stress and/or amino acid deprivation (27,46). Recently we reported that ER-stress is an early event in UCB- mediated neurotoxicity, able to induce important signaling(9). ER-stress occurred at the early stages of the damage also in the in the Ugt1-/- mouse model of hyperbilirubinemia, and it is followed by autophagy activation(29). The inhibition of ER-stress by the chemical chaperon 4-PBA reduced the UCB induction of LC3-II proteins **(Fig 3Dand 3E)** and LC3 mRNA in SH-SY5Y cells **(Fig 3F)** indicating that ER-stress contributes to autophagy activation by UCB. Chronic ER-stress leads to intracellular calcium increase and PKC activation which are involved in regulation of autophagy activation(25). To analyze this pathway, SH-SY5Y cells were exposed to different kinases inhibitors and intracellular calcium chelator (BAPTA). PKC inhibitor and BAPTA were able to reduce the UCB-induction of LC3 mRNA and LC3-II proteins **(Fig 3A and 3B)**, respectively, suggesting calcium/PKC/autophagy activation.
In conclusion, we demonstrated that UCB induces autophagy in SH-SY5Y neuronal and U87 astrocyte cells. Autophagy activation has a pro-survival role in both cell lines and that pre-activation of autophagy protects SH-SY5Y neuronal cell line, primary hippocampal neurons and U87 astrocytoma cell line against UCB cytotoxicity. The mechanisms of autophagy activation in SH-SY5Y cells by UCB are complex and involves mTOR/ER-stress/PKC/calcium signaling **(Fig 5)**. Collectively these data suggest that the activation of autophagy may be a potential mechanism to overcome UCB induced cytotoxicity.

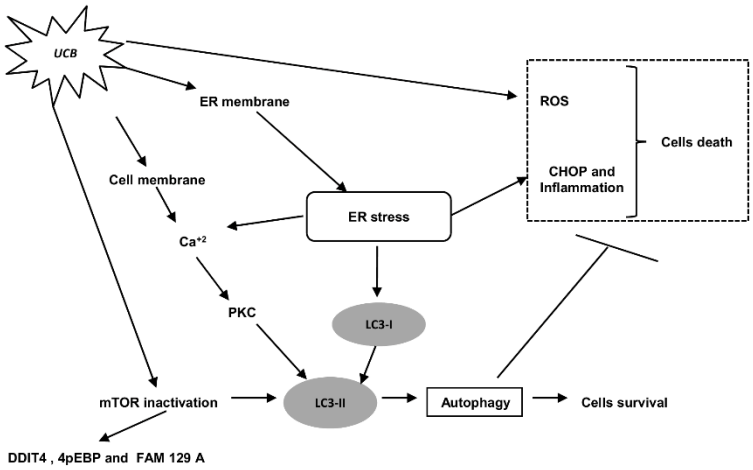

Figure 5: Proposed model of UCB induced autophagy in SH-SY5Y cells.

UCB induces calcium/PKC signaling, ER stress, mTOR inactivation. All these mechanisms contribute to autophagy activation, which reduces inflammation, oxidative stress, CHOP induction and apoptosis.

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