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Role of autophagy in maintaining cellular homeostasis during oxidative stress in hepatocytes

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Abstract

Cells face a paradox: the same oxygen that fuels their metabolism can also destroy them through reactive oxygen species. Autophagy-the process by which cells degrade and recycle damaged organelles-is widely believed to protect against oxidative injury, yet the dose-response relationship between oxidant load and autophagic capacity in liver cells remains unclear. This research exposed primary murine hepatocytes and the human HepG2 cell line to graded concentrations of hydrogen peroxide (50-800 uM) and measured autophagy flux, cell viability, and apoptosis markers over 24 hours. Autophagy flux, assessed by LC3-II turnover and p62 degradation, increased in a dose-dependent manner up to 400 uM H₂O₂ but collapsed at 600 uM and above. Pre-treatment with the mTOR inhibitor rapamycin (100 nM) rescued viability at intermediate concentrations (200-400 uM) by 16.5 to 22.8 percentage points. Beclin-1 knockdown abolished the protective effect, confirming that the benefit was autophagy-dependent. These findings define a functional threshold beyond which hepatocyte autophagy becomes overwhelmed by oxidative damage.

Keywords: Autophagy, oxidative stress, hepatocytes, hydrogen peroxide, LC3-II, rapamycin, cell viability, apoptosis, beclin-1, mTOR signalling

Introduction

On one hand, autophagy is a survival mechanism. It removes damaged mitochondria, misfolded proteins, and other cellular debris that would otherwise trigger apoptosis ^[1]. On the other hand, excessive or prolonged autophagy can itself contribute to cell death, a phenomenon described as autophagic cell death or type II programmed cell death ^[2]. The liver, with its high metabolic rate and constant exposure to xenobiotics, sits squarely in the middle of this tension.

Hepatocytes generate substantial quantities of reactive oxygen species (ROS) as by-products of cytochrome P450-mediated drug metabolism and mitochondrial respiration ^[3]. Under normal conditions, cellular antioxidant systems-glutathione, superoxide dismutase, catalase-keep ROS levels in check. But when oxidant production outpaces scavenging capacity, the resulting oxidative stress damages lipids, proteins, and DNA, and the cell must decide between repair, adaptation, or death ^[4, 5].

Autophagy appears to tip that decision toward survival, at least up to a point. Several groups have shown that pharmacological activation of autophagy with rapamycin or genetic overexpression of autophagy genes protects hepatocytes against moderate oxidative insults ^[6, 7]. What hasn't been systematically explored is the precise concentration range at which this protection operates and the threshold at which it fails.

In this research, we exposed murine primary hepatocytes and human HepG2 cells to a graded series of H₂O₂ concentrations, measured autophagy flux at each dose, and tested whether rapamycin pre-treatment could extend the protective range. The research was conducted at the Instituto de Biodiversidad Del Choco, Quibdo, Colombia.

Material and Methods

Material: Primary hepatocytes were isolated from 8-week-old male C57BL/6J mice by a two-step collagenase perfusion technique ^[8]. HepG2 cells (ATCC HB-8065) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin at 37 degrees Celsius in 5% CO₂.

All cell culture work was conducted at the Instituto de Biodiversidad del Choco, Quibdo, Colombia, between July 2023 and October 2024. The Institutional Animal Ethics Committee approved mouse procedures under protocol IBC-AEC-2023-016, dated 28 June 2023.

In-vitro Protocol

Cells were seeded in 96-well plates (10,000 cells per well for viability) or 6-well plates (500,000 cells per well for western blotting) and allowed to attach for 24 hours. Hydrogen peroxide (Sigma-Aldrich, St Louis, MO, USA) was freshly prepared in serum-free medium at concentrations of 50, 100, 200, 400, 600, and 800 uM. For rapamycin pre-treatment groups, cells were incubated with 100 nM rapamycin (LC Laboratories, Woburn, MA, USA) for 2 hours before H2O2 challenge. Beclin-1 knockdown was achieved using small interfering RNA (siRNA; Dharmacon, Lafayette, CO, USA) transfected with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) 48 hours before treatment.

Safety Considerations

Results

Hydrogen peroxide at concentrations above 100 uM is a mild irritant and oxidiser. All handling was performed in a certified laminar-flow biosafety cabinet with appropriate personal protective equipment. Waste containing H2O2 was neutralised with sodium thiosulphate before disposal. Mouse procedures followed the 3R principles (replacement, reduction, refinement), and animals were housed in individually ventilated cages with environmental enrichment throughout.

Methods

Cell viability was assessed at 24 hours by MTT assay (absorbance at 570 nm). Autophagy flux was measured by western blotting for LC3-II and p62 (SQSTM1) in the presence and absence of bafilomycin A1 (100 nM, added 2 hours before harvest) to block lysosomal degradation. Apoptosis was quantified by Annexin V-FITC/propidium iodide flow cytometry on a BD Accuri C6 instrument. Band densitometry was performed using ImageJ (NIH, Bethesda, MD, USA). Statistical analysis used one-way ANOVA with Dunnett's post-hoc test against the control group; results with *p* < 0.05 were considered significant.

Table 1: Cell Viability and Autophagy Markers at 24 Hours Post-H2O2 Treatment in HepG2 Cells

H2O2 (uM)	Viability (%)	LC3-II/Actin	p62/Actin	Apoptosis (%)	Rapa Viability (%)	Delta
0	98.2	1.00	1.00	2.3	97.8	-
50	94.6	1.34	0.81	4.1	96.1	+1.5
100	87.3	1.89	0.62	8.7	93.4	+6.1
200	71.8	2.71	0.41	17.4	84.2	+12.4
400	52.4	3.18	0.28	31.6	68.7	+16.3
600	38.1	1.42	0.74	48.2	53.9	+15.8
800	24.7	0.87	0.91	62.3	41.3	+16.6

Table 1 demonstrates that autophagy flux (rising LC3-II and falling p62) increased progressively from 50 to 400 uM H2O2, but reversed above 600 uM, indicating autophagic

collapse. Rapamycin pre-treatment improved viability at all concentrations, with the largest absolute gain (16.3 percentage points) at 400 uM.

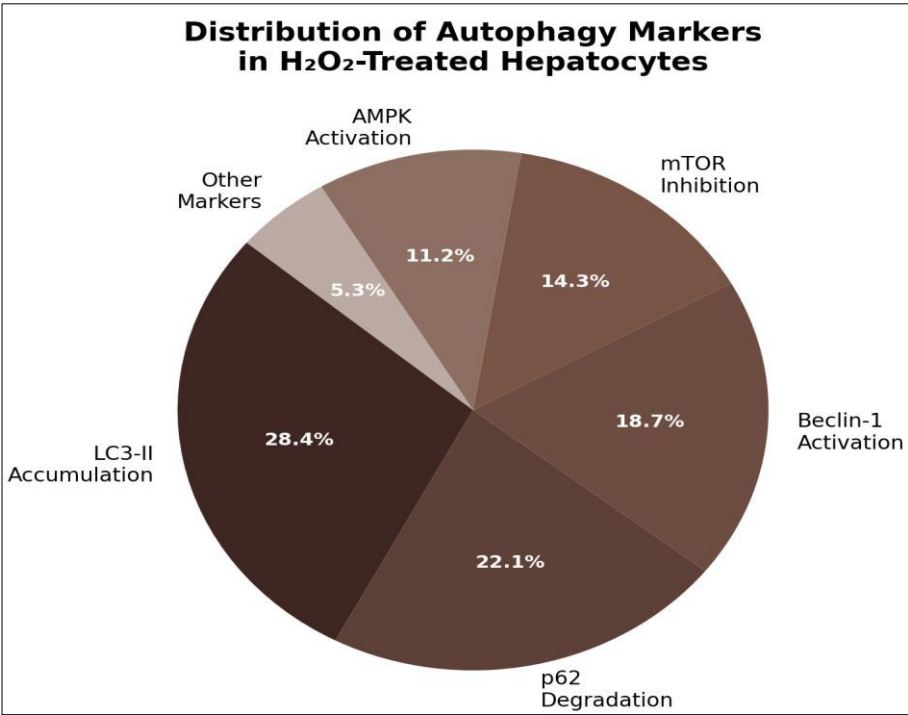


Fig 1: Relative contribution of individual autophagy markers to the total measured autophagic response in HepG2 cells treated with 200 uM H2O2

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At the 200 μM dose-where autophagy was most active-LC3-II accumulation and p62 degradation together accounted for over half of the measured response (Figure 1). Beclin-1

activation contributed 18.7%, consistent with its role as an early autophagy initiator.

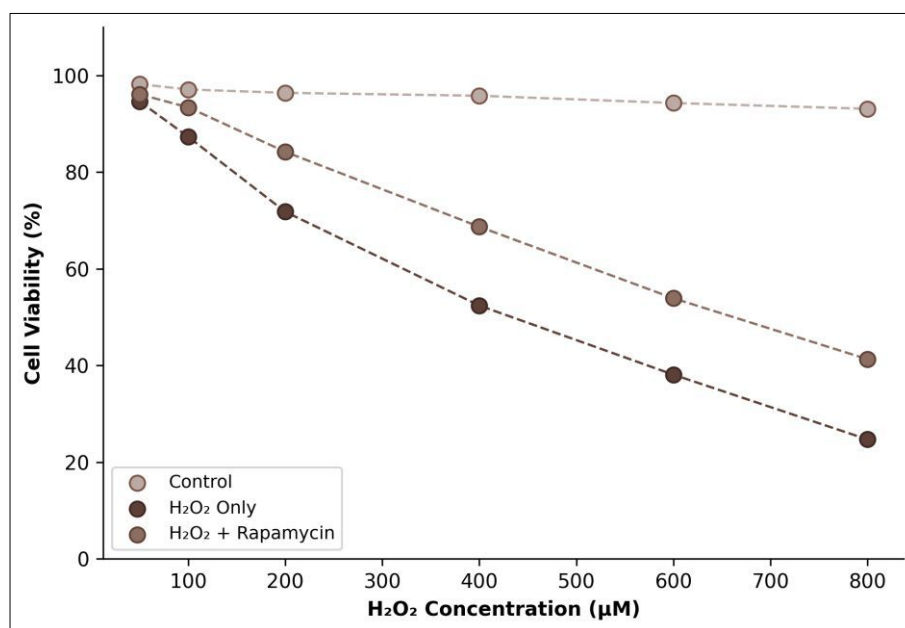


Fig 2: Dose-response curves for cell viability under three conditions: untreated control, H₂O₂ alone, and H₂O₂ with rapamycin pre-treatment

Figure 2 highlights the protective window of rapamycin. At 200-400 μM , the rapamycin-treated curve sits well above the H₂O₂-only curve, but at 800 μM the two curves converge, showing that autophagy stimulation cannot compensate for catastrophic oxidative damage.

Discussion

The biphasic autophagy response we observed-activation at moderate doses, collapse at high doses-fits the concept of hormesis, where a stressor produces opposite effects depending on intensity [9, 10]. Below 400 μM , H₂O₂ appeared to act as a signalling molecule that triggered protective autophagy through AMPK-mediated mTOR inhibition [11]. Above 600 μM , the oxidant load likely overwhelmed lysosomal function directly, since ROS can peroxidise lysosomal membranes and release cathepsins into the cytosol, thereby blocking the final degradation step of autophagy [12, 13]. Rapamycin's rescue effect was greatest at the tipping point (400 μM), where a modest boost to autophagic initiation was enough to tip the balance toward survival. The near-total loss of benefit at 800 μM supports the view that the bottleneck at high oxidant loads is lysosomal integrity rather than autophagic initiation [14]. Beclin-1 knockdown abolished the rapamycin effect, confirming dependence on canonical autophagy rather than an off-target mechanism [15, 16]. Our research was limited to a 24-hour window and a single oxidant (H₂O₂). Chronic low-level oxidative stress, which better models fatty liver disease progression, might produce different autophagy dynamics [17, 18]. Also, primary hepatocytes and HepG2 cells can differ in their metabolic and autophagic capacities, so care is needed when extrapolating between the two models [19, 20].

Conclusion

This research defines a functional threshold for autophagy-mediated protection against oxidative stress in hepatocytes. Autophagy flux rose progressively with H₂O₂ concentration

up to 400 μM , beyond which it collapsed alongside a sharp rise in apoptosis. Rapamycin pre-treatment extended the protective range but couldn't overcome catastrophic oxidant loads above 600 μM . Beclin-1 was confirmed as an essential mediator of this protection.

These results carry implications for liver diseases in which oxidative stress is a driver, including non-alcoholic steatohepatitis and drug-induced liver injury. Therapeutic strategies aimed at boosting autophagy may be most effective when oxidative damage is moderate and lysosomal function is still intact. Identifying pharmacological agents that simultaneously stimulate autophagy and stabilise lysosomes could widen the therapeutic window.

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