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Inhibition of N-linked Protein Deglycosylation Stimulates Autophagy

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Abstract

Glycosylation is the process by which sugars are added to biological molecules such as proteins. It regulates a myriad of biochemical processes, such as protein folding, adhesion, targeting and recognition events. Conditions resulting from in-born defects of glycosylation manifest with wide-ranging pathologies impacting nearly every organ system and result in severe multi-system diseases. These disorders are challenging to diagnose and difficult to map onto the clinical presentations, as a single defect in a sugar processing pathway may result in complex and varied downstream effects [1].

Protein folding and degradation

In a healthy cell, N-glycanase removes N-linked glycans from misfolded proteins prior to proteosomal degradation (figure 1).

NGU1 disorder is a rare congenital disease caused by mutations in the gene that encodes N-glycanase resulting in:

- Loss/reduction of N-glycanase activity
- Build up of misfolded proteins which aggregate in the cell [1,2].

If N-glycanase is inhibited, autophagy is stimulated to compensate for the accumulation of misfolded proteins. N-glycanase inhibition was studied using z-VAD-fmk, an irreversible inhibitor of N-glycanase. As z-VAD-fmk is also a caspase inhibitor, Q-VD-OPh was employed as a control as it has a similar caspase inhibition profile but does not inhibit of N-glycanase.

N-glycanase inhibition causes transient ER stress

Inhibition of N-glycanase using z-VAD-fmk increases protein aggregation and expression of Grp78 Cellular ThioflavinT (ThT) labelling provides a convenient assay for ER stress and protein misfolding [3] (fig. 2) Grp78 is upregulated during ER stress and the unfolded protein response (figure 3).

N-glycanase inhibition activates autophagy

Autophagosome density was quantified using a GFP-LC3 reporter (fig. 4). The number of autophagosomes increased significantly after 48 h, coinciding with a reduction in ER stress (fig 5a). Treatment with z-VAD-fmk and Q-VD-OPh does not interfere with autophagic flux (fig 5b).

Autophagy deficient cells cannot recover from cellular stress caused by N-glycanase inhibition

Cells lacking ATG13 are unable to form autophagic vesicles (fig 6).

Autophagy-deficient ATG13⁻/⁻ MEFs incubated with 50 µM z-VAD-fmk for 72 h showed a significant reduction in cell viability (fig 7a).

Wild type MEFs showed no reduction in cell viability (fig 7b). ATG13⁻/⁻ MEFs showed no reduction in cell viability when treated with Q-VD-OPh, indicating cell toxicity is due to N-glycanase inhibition and not caspase inhibition (fig 7c).

Future work

- CRISPR generated knockouts and clinical mutations of N-glycanase to study long-term inhibition on global/aggregate glycosylation
- How aggregates are targeted to autophagy network
- Analysis of proteosomal degradation machinery included in aggregates

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References


Statistics

Error bars indicate SEM. * indicates P < 0.05.

Data analysed using ANOVA and respective post hoc test

Figure 1: Glycoprotein synthesis, quality control and degradation. Adapted from Hirayama, H., Suzuki, T (2013) Glycobiol. 23(10): 1341

Figure 2: (a) Thioflavin T labelling of HEK cells increased following 48h treatment with z-VAD-fmk (50 µM), and returned to basal levels after 72h. Live cell images were captured following incubation with ThT (5 µM) (b) Fluorescence intensity was determined using ImageJ. n = 3.

Figure 3: (a) HEK 293 cells were treated with z-VAD-fmk (50 µM) (b) Brutes analysed by Western blotting for GRP78 (Syngene GeneTools, n = 3).

Figure 4: Images of live cells expressing GFP-LC3 construct: Scale bar = 10 µm

Figure 5: (a) The number of GFP-LC3 puncta per cell in control cells, or in cells incubated with 50 µM z-VAD-fmk or Q-VD-OPh. n = 3. (b) Cells treated as before and incubated for 3 hr in 0.1 µM Bafilomycin A1 or 5 mM 3-MA to determine if flux was disturbed. n=3.

Figure 6: ATG13⁻/⁻ MEFs showed a reduced level of basal autophagy. AAT Bio autophagy assay kit. n = 2.

Figure 7: (a) Viability of ATG13⁻/⁻ and matched control MEFs following incubation with (a) z-VAD-fmk (50 µM) 72 h. n = 6 for ATG13⁻/⁻ cells (b) wild type MEFs. n = 3 (c) Q-VD-OPh (50 µM) 72 h n = 6 for ATG13⁻/⁻ cells

Summary

- Peptide N-glycanase inhibition causes simultaneous ER stress and the accumulation of autophagic vesicles.
- Autophagy enables cells to degrade misfolded proteins and reduce ER stress
- Cells die if they are unable to trigger autophagy during N-glycanase inhibition.

N-glycanase inhibition does not disrupt ER or actin cytoskeletal structure

Figure 8 (a) Confocal images of HEK293 cells treated with z-VAD-fmk and transfected with GFP-ER. (b) Fluorescent images of HEK293 cells treated with z-VAD-fmk (50 µM). Actin stained with Phalloidin 488 and nucleus with Hoechst. Scale bars = 10 µm. n=1.