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Inhibition of N-linked Protein Deglycosylation Stimulates Autophagy

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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 Thioflavim (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 (fig. 3).

**Figure 2**: (a) Thioflavin T labelling of HEK cells increased following 48 h treatment with z-VAD-fmk (50 µM), and returned to basal levels after 72 h. 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] juices analysed by Western blotting with Grp78 (Syngene GeneTools, n = 3).

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).

**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.

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 HEK 293 cells treated with z-VAD-fmk (50 µM). Actin stained with Phalloidin 488 and nuclei with Hoechst. Scale bars = 10 µm. n=1.

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

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.

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References


Statistics

Error bars indicate SEM. * indicates P < 0.05. Data analyzed using ANDIV and receiver operating characteristic analysis.