Human and non-human intestinal NHE3: Human NHE3 demonstrates increased susceptibility to inhibition and unique regulation by ubiquitin

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Introduction: The Na^+/H^+ Exchanger 3 (NHE3) is responsible for the majority of the electroneutral sodium absorption occurring in the intestine. As such, NHE3 has a major role in controlling electrolyte and fluid balance and is a frequent target of inhibition in many diarrheal diseases. While mice and rabbits have been used to investigate the mechanisms of diarrhea, they are less prone to develop diarrhea than humans. Recently, we have shown that human NHE3, but not non-human NHE3s, interacts with the ubiquitin E3 ligase Nedd4-2. We hypothesize that this property of human NHE3 contributes to the increased severity of diarrhea.

Method and Materials: To investigate this hypothesis, we determined human and non-human NHE3 activities and ubiquitination levels in response to the NHE3 inhibitors forskolin (FSK), cholera toxin (CTX) or Enteropathogenic E.coli (EPEC). *In vitro*, we generated Caco-2/bbe cells transfected with human or rabbit NHE3, and *in vivo* we generated transgenic mice expressing human NHE3 in the intestine ($hNHE3^{int}$). For *in vitro* experiments, inhibitor treatments of 30 and 90 min were directly applied to cells diluted with normal growth media. NHE3 activity was measured by Na^+ dependent intracellular pH recovery. NHE3 ubiquitination was evaluated by immunoprecipitation of NHE3 followed by western blot of ubiquitin. Nedd4-2 was knocked down in cells *via* electroporation. For *in vivo* experiments, mice were anesthetised with a ketamine/xylazine cocktail and a 2 - 5 cm section of ileum was tied off and injected with inhibitor treatment or Hanks Buffered Saline Solution (HBSS) vehicle buffer. Mice were allowed to recover for 5h post-injection before cervical dislocation was performed. Closed loops were removed, measured and weighed, and villi were dissected and used for NHE3 activity analysis.

Results: *In vitro*, we found that 10uM CTX significantly increased human NHE3 ubiquitination. Both CTX (1-10 uM) and EPEC treatments induced significantly more inhibition of human NHE3 activity in Caco-2/bbe cells than what was observed in rabbit NHE3 transfected cells. Nedd4-2 knockdown blunted the inhibitory effect on human NHE3, demonstrating the importance of Nedd4-2 in regulating human NHE3. *In vivo*, NHE3 knockout mice (NHE3-/-) have previously been shown to display symptoms of diarrhea. However, our model of $hNHE3^{int}$ mice did not show any signs of diarrhea, indicating that the transgenic hNHE3 is functional. In anesthetised $hNHE3^{int}$ mice, we found that both 5h closed-loop intestinal treatment with inhibitors EPEC (2×10^8 CFU) and CTX (10ug) significantly increased water accumulation in the small intestine and significantly reduced NHE3 activity compared to wild type mice.

Conclusion: These findings demonstrate that human and non-human NHE3s are differentially regulated, suggesting that the characteristics of human NHE3 regulation may contribute to increased diarrhea severity in humans.