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Role of host xanthine oxidase in infection due to enteropathogenic and Shiga-toxigenic *Escherichia coli*

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Xanthine oxidase (XO) has been recognized as an important host defense enzyme for decades. In our recent study in Infection and Immunity, we found that enteropathogenic and Shiga-toxigenic *E. coli* (EPEC and STEC) were far more resistant to killing by the XO pathway than laboratory *E. coli* strains used in the past. Although XO plus hypoxanthine substrate rarely generated enough H_2O_2 to kill EPEC and STEC, the pathogens were able to sense the H_2O_2 and react to it with an increase in expression of virulence factors, most notably Shiga toxin (Stx). H_2O_2 produced by XO also triggered a chloride secretory response in T84 cell monolayers studied in the Ussing chamber. Adding exogenous XO plus its substrate in vivo did not decrease the number of STEC bacteria recovered from ligated intestinal loops, but instead appeared to worsen the infection and increased the amount of Stx2 toxin produced. XO plus hypoxanthine also increases the ability of Stx2 to translocate across intestinal monolayers. With regard to EPEC and STEC, the role of XO appears more complex and subtle than what has been reported in the past, since XO also plays a role in host-pathogen signaling, in regulating virulence in pathogens, in Stx production and in toxin translocation. Uric acid produced by XO may also be in itself an immune modulator in the intestinal tract.

We recently reported our findings on the role of the host enzyme xanthine oxidase in enteropathogenic (EPEC) and Shiga-toxigenic *Escherichia coli* infection (STEC; also known as enterohemorrhagic *E. coli*, EHEC).[1] Xanthine oxidase (XO) has been the subject of biochemical study for more than 60 y and is considered an important host defense molecule. XO is expressed in high amounts in the intestinal epithelium and liver and is also very abundant in human and animal milk. As shown in **Figure 1A**, xanthine oxidase catalyzes two sequential steps in the purine catabolic pathway, converting hypoxanthine and xanthine to uric acid, with the production of H_2O_2. XO is the only enzyme capable of producing uric acid in mammalian cells.

In our article we found that EPEC and STEC infection triggered the release of XO into the intestinal lumen and that the flux of purines through the pathway shown in **Figure 1A** was greatly stimulated. We also noted that EPEC and STEC strains were not very susceptible to killing or inhibition by XO, but that anaerobic bacteria were very susceptible to killing by this pathway. Since the anaerobic microbiota play an important role in defense against exogenously acquired enteropathogens, such as EPEC, STEC, Salmonella and others, activating the XO pathway might be a "back-door" way to wipe out the competition, allowing the pathogen to initiate infection. While intermediate amounts of XO activity (0.1 to 0.5 U/mL) were not sufficient to kill or inhibit EPEC or STEC, those amounts were sufficient to stimulate Stx toxin production from STEC, by activation of the SOS stress response. H_2O_2 produced by XO activates the SOS response in *E. coli* bacteria, and the SOS response is a strong inducer of Stx and of Stx-encoding bacteriophage.[2-4]

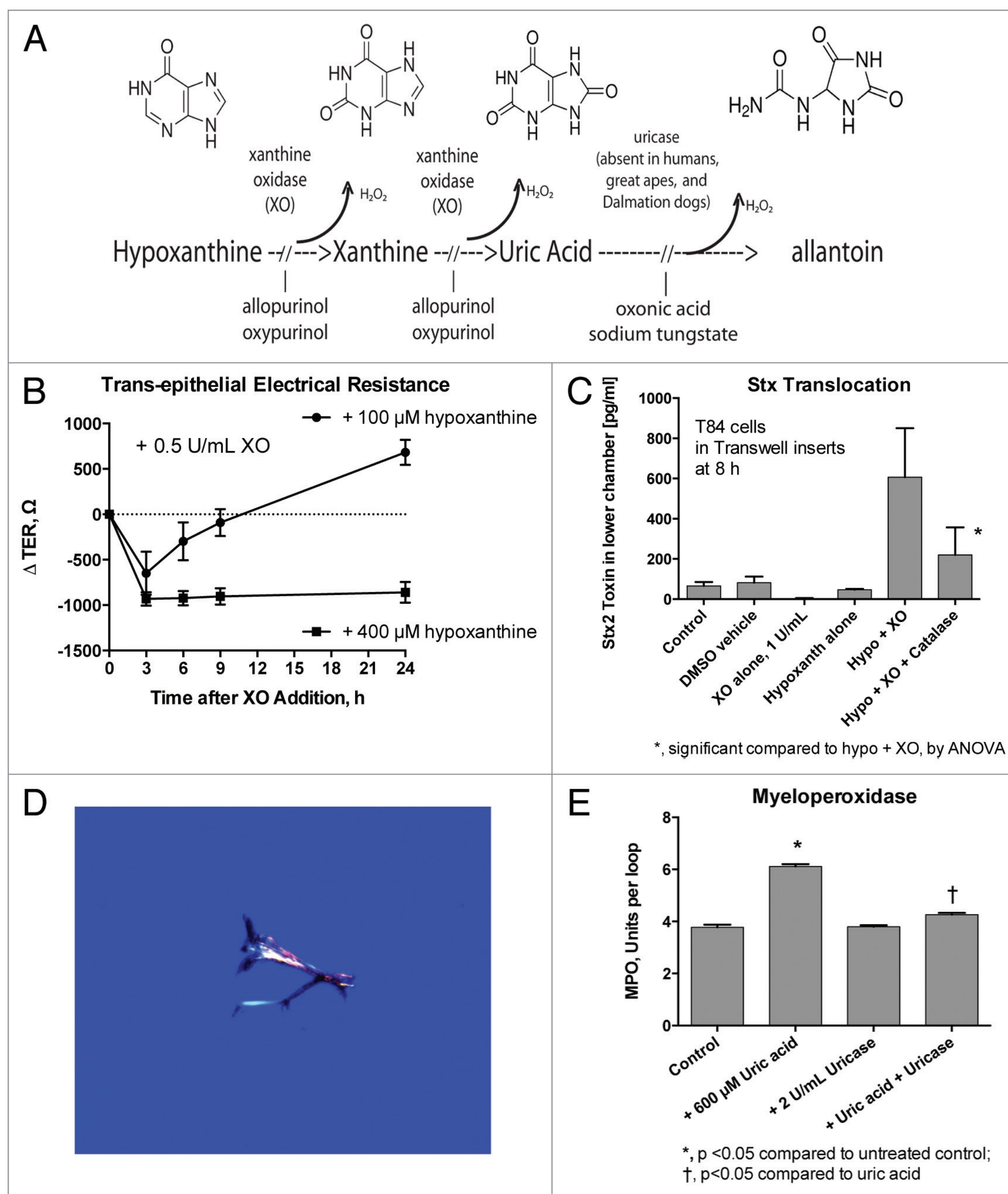

Figure 1. Roles of xanthine oxidase and uric acid in host responses to EPEC and STEC infection. (**A**) Terminal phase of purine catabolism in the intestinal tract in response to EPEC and STEC infection. EPEC- and STEC-induced damage to intestinal cells results in a large release of nucleotides and nucleosides into the lumen of the gut, where they are broken down to uric acid. Uricase is non-functional in humans but is present in most mammals, birds and in gut microbes. (**B**) Importance of the concentration of hypoxanthine substrate on xanthine oxidase (XO)-mediated damage to host cells. In the presence of XO but a low concentration of hypoxathine substrate, T84 cell monolayers show a drop in trans-epithelial electrical resistance (TER) followed by a recovery. In the presence of 400 μM hypoxanthine, however, the drop in TER is sustained and the monolayers do not recover within 24 h. (**C**) Role of XO in translocation of Shiga toxin 2 (Stx2) across T84 cell monolayers. T84 cell monolayers in Transwell inserts were treated with either XO alone, 400 μM hypoxanthine alone, or XO plus hypoxanthine, followed by addition of Stx2 to the upper chamber. Treatment with XO + hypoxanthine resulted in a large increase in the amount of Stx2 recovered in the lower chamber. This increase was reversed by the addition of catalase, showing that it is indeed the H_2O_2 generated by XO that triggers the monolayer damage. (**D**) Formation of uric acid crystals in response to XO plus hypoxathine treatment of T84 cells. Supernatant medium was collected from the upper chamber of an experiment similar to that shown in (**C**), showing birefringent crystals in the wells receiving XO + hypoxanthine; uric acid crystals were photographed using two polarizing filters and 100× magnification. (**E**) Effect of exogenous uric acid on host inflammatory response in ligated rabbit ileal segments. Ten cm intestinal segments (loops) were treated with 600 μM (10 mg/dL) uric acid, or 2 U/mL uricase, or both, but without addition of pathogenic bacteria. Influx of neutrophils (in the rabbit, they are called heterophils) into the gut was quantitated by measuring myeloperoxidase (MPO) activity; uric acid triggered a modest increase in MPO in the loop fluid which was reversed by uricase.

This led us to hypothesize the existence of an "uncanny valley" of XO activity, in which intermediate amounts of XO activity would be associated with a worse outcome of infection compared with high XO activity, or to no XO at all. Our concept of the uncanny valley, however, may have overemphasized the amount of XO present without proper consideration of the amount of substrate (hypoxanthine or xanthine) that is also present. **Figure 1B** shows that the concentration of hypoxanthine present plays a critical role in damage inflicted by XO on monolayers of T84 cells grown in polarized fashion in Transwell inserts. **Figure 1B** shows the change in the trans-epithelial electrical resistance (TER) of the monolayers, a measure of epithelial barrier function. In the presence of a lower concentration of hypoxanthine, 100 μM, TER drops transiently in response to XO, but then is able to recover and even rebounds to a level above that of the starting resistance by 24 h after exposure to XO (**Fig. 1B**, top curve). In contrast, with a somewhat higher concentration of hypoxanthine, 400 μM, the monolayer does not recover in 24 h and the TER remains low (**Fig. 1B**, lower curve). The change in TER observed in **Figure 1B** is accompanied by an increase in the translocation of Stx2 across the monolayer. **Figure 1C** shows that neither XO alone nor hypoxanthine alone triggered much translocation of Stx2 across T84 cell monolayers, but that XO + hypoxanthine triggered Stx2 translocation more than 10 times that observed in the control wells. XO-mediated Stx2 translocation was reversed by the addition of catalase, indicating that it is the peroxide produced by XO that is triggering the increased translocation.

The amount of uric acid generated in response to EPEC and STEC infection is high, exceeding 200 μM (3.4 mg/dL) in culture medium in experiments in cultured cells and sometimes even higher levels in the fluid that accumulates in ligated intestinal segments ("loops") infected with EPEC or STEC (over 400 μM, or > 6.7 mg/dL). These high uric acid concentrations are at or above the solubility limit of this compound, i.e., concentrations associated with precipitation of uric acid crystals in the tissues or in urine in diseases such as gout or tumor lysis syndrome.[5] We examined whether uric acid crystals ever formed in intestinal tissues or in the lumen of the gut as well as in the joints of gout sufferers. **Figure 1D** shows that crystals presumptively identified as monosodium urate (MSU) crystals did appear in the medium of T84 cells treated with XO + hypoxanthine; these crystals show the birefringence characteristic of uric acid under a polarizing microscope; similar crystals were also seen in the unfiltered loop fluids from intestines infected with EPEC and STEC.[6] Until about 10 y ago uric acid was not felt to have any role in host defense or immune regulation, but in the last decade it has been recognized that uric acid can act as a danger signal to the immune system.[7-10] Uric acid crystals affect multiple aspects of innate immunity and can activate neutrophils,[11] stimulate antibody production,[12] act as immune adjuvants[13] and activate the NALP3 inflammasome pathway[14] in toll-like receptor-independent fashion. **Figure 1E** shows that uric acid can have inflammatory effects in the gut as well. Addition of exogenous uric acid in the absence of pathogenic bacteria modestly stimulated neutrophil (heterophil) accumulation in vivo in ligated rabbit intestinal loops and this increase was reversed by addition of uricase. Human cells are not capable of producing uricase (**Fig. 1A**), since this gene suffered a nonsense (stop codon) mutation sometime in primate evolution, but this does not mean that uricase is irrelevant to human biology, since many phylogenetic types of gut microbes do produce this enzyme, including gram-negative enteric bacteria, anaerobes, archaea and yeasts.

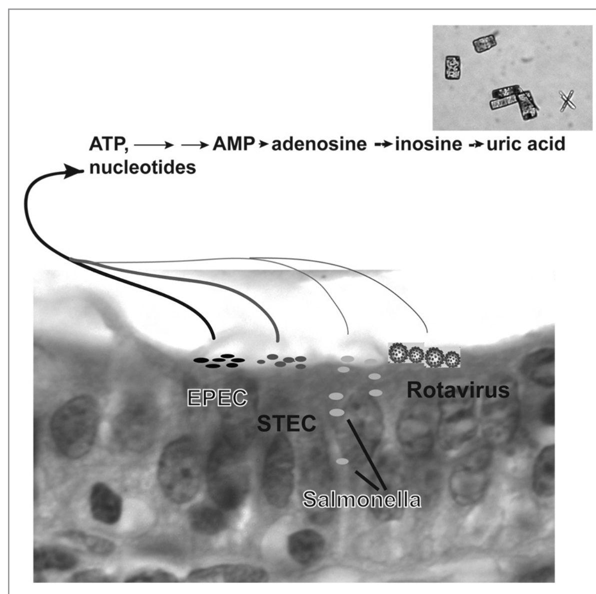

Figure 2. Cartoon showing the pathways for generation of uric acid via XO in response to infection with enteric pathogens. Damaged enterocytes release nucleotides and nucleosides in response to infection with rotavirus, EPEC, STEC and Salmonella. Breakdown of nucleic acids (DNA and RNA) may contribute to the flux of nucleotides released. Host enzymes catalyze the catabolism of the extracellular purines to uric acid (photo of uric acid crystals at top right), some of which may be reabsorbed into the bloodstream, since hyperuricemia can result from these infections. Bacteria and rotaviruses are obviously not drawn to scale, since rotavirus is smaller than bacteria.

The immune stimulating properties of uric acid might be harnessed as an oral adjuvant to increase the effectiveness of oral vaccines, such as the available oral cholera vaccines which are currently not highly protective and not very long lasting.[15] Alternatively, would inhibition or removal of uric acid be a strategy to reduce inappropriate gastrointestinal inflammation, as for example in inflammatory bowel disease? Drugs now available for human use, including rasburicase, a recombinant form of uricase, make the uric acid metabolic pathway more amenable to therapeutic manipulation than ever. Although our experimental study dealt primarily with EPEC and

STEC, our preliminary results and work from other laboratories suggest that the XO-uric acid pathway is also activated in response to infection with rotavirus and Salmonella (see cartoon in **Fig. 2**).

While more research is needed in many of these areas, there are things that can be implemented now based on our work and the available literature. First, serum uric acid levels should be measured more often in patients with infectious diarrhea, since high uric acid levels not only serve as a clue to the etiology of the diarrheal illness[16,17] but also may function as a biomarker of more severe disease[18] and of a greater chance of developing renal failure in those infected with STEC. Those individuals might be candidates for more intensive monitoring, more aggressive hydration and possibly even uric acid-lowering treatments.[19]

The role of xanthine oxidase in response to enteric infections appears more complex than what has been emphasized in the literature,[20,21] since this enzyme can serve in a signaling role and enhance pathogen virulence as well as merely killing bacteria. Research in this area seems likely to produce both more insight into basic aspects of XO biology as well as possible practical and therapeutic strategies.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

Acknowledgments

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