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Okadaic acid: A rapid inducer of lamellar bodies in small intestinal enterocytes

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ABSTRACT

Okadaic acid (OA) is a polyether fatty acid produced by marine dinoflagellates and the causative agent of diarrhetic shellfish poisoning. The effect of OA on apical endocytosis in the small intestine was studied in organ cultured porcine mucosal explants. Within 0.5–1 h of culture, the toxin caused hyper protein phosphorylation, but no detectable loss of cell polarity or cytoskeletal integrity of the enterocytes. Using a fluorescent membrane marker, FM dye, endocytosis from the brush border was affected by the toxin. Although constitutive uptake into subapical terminal web-localized early endosomes (TWEEs) occurred unimpeded in the presence of OA, FM condensed in larger subapical structures by 1 h, implying a perturbed endosomal trafficking/maturation. The fluorescent lysosomotropic agent Lysotracker revealed induction of large lysosomal structures by OA. Endocytosis from the brush border was studied at the electron microscopic level using the membrane-impermeable marker Ruthenium Red (RR). Like FM dye, RR was taken up into TWEEs and multivesicular bodies (MVBs). However, OA induced the formation of a large number of lamellar bodies (LBs), a type of lysosome-related organelles. LBs are the hallmark of phospholipidosis, a pathological condition characterized by lysosomal phospholipid accumulation. Phospholipidosis is observed in acquired lysosomal storage diseases and is induced by a large number of cationic amphiphilic drugs. Unlike the latter, however, OA does not act by accumulating in acidic organelles, implying a different toxic mechanism of action. We propose that rapid induction of LBs, an indicator of phospholipidosis, should be included in the future toxicity profile of OA.

1. Introduction

Okadaic acid (OA) and its close chemical dinophysistoxin analogs, produced by the dinoflagellates *Dinophysis* and *Prorocentrum*, are the principal toxins causing diarrheic shellfish poisoning (Daranas et al., 2001; Dickey et al., 1990; Vale and Botana, 2008). OA intoxication occurs rapidly after ingestion of contaminated seafood with symptoms such as vomiting and diarrhea, and although not considered fatal, it reportedly represents an increasing global concern, both regarding health- and economic burdens (Van Dolah, 2000). The main molecular targets of OA are the serine/threonine phosphoprotein phosphatase classes PP1- and PP2A, which are inhibited with IC_{50}'s in the nanomolar range, while other classes of protein phosphatases are being less sensitive or insensitive to the toxin (Bialojan and Takai, 1988; Cohen et al., 1990). Acting in opposition to protein kinases- and phosphorylases, protein phosphatases are an important group of enzymes involved in regulation of numerous signaling pathways, and consequently helps controlling a number of cell functions, including metabolism, cell cycle progression, gene expression, ion balance, cytoskeletal rearrangements, and cell movement (Vale and

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Botana, 2008; Van Dolah, 2000). This broad spectrum of potential targets probably explains why OA has also been termed a tumor promoter due to its ability to induce skin carcinogenesis in the mouse (Suganuma et al., 1988).

The diarrhea occurring during OA-induced intoxication is thought to result mainly from increased paracellular epithelial permeability caused by opening of the tight junctions, adherens junctions and desmosomes between neighboring enterocytes (Okada et al., 2000; Pasdar et al., 1995; Tripuraneni et al., 1997), whereas so far, OA has not been reported to affect directly the enterocyte brush border, a main barrier preventing luminal pathogens from gaining entry to the organism (Glenn et al., 2009; Pastorelli et al., 2013). This highly specialized cell membrane contains an unusually high proportion of glycolipids in its outer leaflet (Christiansen and Carlsen, 1981), and is thought to be organized into lipid raft microdomains, stably cross-linked by a number of lectins, including members of the galectin family and intelectin (Danielsen and Hansen, 2006, 2008; Simons and Ikonen, 1997). A hallmark of lipid rafts, regardless of cell type, is their relative resistance to solubilization by detergents *in vitro*, giving rise to their frequent characterization as detergent resistant membranes ("DRMs") (Brown, 2006). In case of the brush border, however, resistance to the detergent action of bile salts during absorption of fatty meals is essential for maintenance of an intact functional epithelium *in vivo*, and a constitutive apical endocytosis of predominantly non-raft membrane lipids appears to be a mechanism whereby the glycolipid enrichment of the brush border is achieved (Danielsen and Hansen, 2013; Hansen et al., 2009). Noteworthy, membrane endocytosed from the brush border remains stationary in the subapical terminal web-localized early endosomes (hence named "TWEEs") for periods > 1 h. This restriction of membrane trafficking probably occurs as a mechanism to prevent transcellular transport of pathogens from the gut lumen, via the basolateral cell surface, to the blood.

In the present work, short exposures of OA to organ cultured porcine jejunal mucosal explants were found to affect the apical endocytic system of enterocytes. By fluorescence microscopy, newly formed TWEEs condensed into larger structures, and by electron microscopy the appearance of lysosome-related lamellar bodies (LBs) was observed. Also commonly referred to as "lysosomal inclusion bodies" or "myeloid bodies", LBs constitute the morphological evidence of phospholipidosis, a pathological condition arising from a blockage of the normal lysosomal phospholipid metabolism (Anderson and Borlak, 2006; Reasor, 1989). So far, OA has not been reported to affect endocytosis in the gut, but our results suggest that rapid induction of phospholipidosis may contribute to the overall toxicity of this toxin.

2. Materials and methods

2.1. Materials

Okadaic acid (OA), potassium salt, was obtained from ICN Biomedicals (www.icnbiomed.com/). The product purchased was isolated from *Prorocentrum concavum* to a purity >98% by high performance liquid chromatography, and the salt form generated in an aqueous potassium hydroxide methanol solution. The product was certified to be a potent inhibitor of serine/threonine-specific protein phosphatases 1- and 2A (PP1 and PP2A) and of PP2B at higher concentrations. It was certified not to inhibit protein tyrosine phosphatases. Mouse monoclonal antibodies to spectrin (α-chain, nonerythroid) and rabbit antibodies to phosphoserine were obtained from Millipore (www.millipore.com), mouse monoclonal antibodies to Na^+/K^+-ATPase (α-chain) from Thermo Scientific (www.thermo.com/pierce), mouse monoclonal antibodies to acetylated α-tubulin from Santa Cruz Biotechnology (www.scbt.com), mouse monoclonal antibodies to phosphoserine/threonine, and mouse monoclonal antibodies to phosphotyrosine from BD Transduction Laboratories (www.bdbiosciences.com), secondary Alexa-conjugated antibodies, a fixable lipophilic styryl FM dye (1-43 FX), Lysotracker Red DND-99, and ProLong antifade reagent with DAPI from Life Technologies (www.lifetechnologies.com), horseradish peroxidase-conjugated secondary antibodies from DAKO (www.dako.com), an ECL Western blotting detection reagent from GE Healthcare (www.gelifesciences.com), and Ruthenium Red (RR) from Sigma–Aldrich (www.sigmaaldrich.com). Rabbit antibodies to small intestinal brush border enzymes and lactase-phlorizin hydrolase were prepared as previously described (Skovbjerg et al., 1978, 1982).

2.2. Animals

All animal experimentation in Denmark is subject to ethical evaluation by the Ministry of Justice's Council for Animal Experimentation. The present work was performed under license 2012-15-2934-00077.

Segments of jejunum, taken about 2 m from the pylorus of overnight-fasted, post-weaned pigs, were surgically removed from the anaesthetized animals by licensed staff at the department of Experimental Medicine, The Panum Institute, University of Copenhagen. After obtaining the intestinal segments, the animals were sacrified by an injection with pentobarbital/lidocaine (1 mg/kg bodyweight).

2.3. Organ culture of mucosal explants

Jejunal segments of approximately 20 cm in length were quickly removed from the animals and cooled in ice-cold RPMI medium. Within 10 min, mucosal explants (~0.1 g) were excised with a scalpel and cultured mucosal side upwards on stainless grids placed in organ culture dishes in 1 ml of RPMI medium at 37 °C for periods of 0.5–1 h, essentially as described previously (Danielsen et al., 1982). When present, OA (stock solution: 0.25 mM in dimethylsulfoxide) was added to a final concentration of 1 μM. FM dye was used at a concentration of 20 μg/ml, Lysotracker at 10 μM, and RR at 0.2% (w/v).

After culture, the explants were quickly rinsed in fresh medium and immersed in fixative at 4 °C.

2.4. Fluorescence microscopy

Mucosal explants were fixed for 2 h or overnight in 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate, pH 7.2