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# Polyunsaturated fatty acids regulate Shiga toxin transport

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**Abstract**

Shiga toxin (Stx) is internalized by receptor-mediated endocytosis and transported retrogradely to the endoplasmic reticulum from where the enzymatically active part of the toxin is translocated to the cytosol. In this study, we have investigated the effect of polyunsaturated fatty acids (PUFA) on intoxication and retrograde transport of Stx. In HEp-2 cells, PUFA treatment inhibited Stx intoxication by a factor of 10. Moreover, both Stx internalization and endosome-to-Golgi transport were reduced by PUFA and these reductions can together explain the reduced toxicity. Also cholera toxin internalization was reduced by PUFA treatment. Finally, ricin and *Pseudomonas* exotoxin 1 cytotoxicity were not reduced by PUFA, demonstrating that PUFA do not cause a general block in retrograde transport to the endoplasmic reticulum. In conclusion, these results clearly demonstrate the importance of PUFA for Stx and cholera toxin trafficking.


*Keywords:* Shiga toxin; Polyunsaturated fatty acids; PUFA; Eicosapentaenoic acid; Docosahexaenoic acid; Endocytosis; Retrograde transport; Cholera toxin; *Pseudomonas* exotoxin 1; Ricin

Protein toxins are produced both by plants and bacteria and are often composed of two domains, an A-chain with toxic enzymatic activity and one or several B-chains that bind to certain receptors at the cell surface of target cells. Shiga toxin (Stx) is a bacterial toxin consisting of a single A-chain and 5 identical B-chains [1]. Stx binds specifically to the glycosphingolipid globotriaosylceramide (Gal(α1-4)Gal(β1-4)glucosyl ceramide; Gb3) at the cell surface. Surface-bound Stx is internalized and transported retrogradely through the Golgi apparatus to the endoplasmic reticulum (ER) from where the toxic part of the toxin is translocated to the cytosol. The A-chain of Stx has RNA *N*-glycosidase activity and removes a specific adenine residue from ribosomes, impairing protein synthesis and causing cell death [1].

Binding of Stx to its receptor is a complex process modulated both by the receptor itself and by the local membrane environment. The presence of Gb3 in the plasma membrane is necessary but not sufficient for Stx intoxication. For instance, macrophages and A431 cells express Gb3 and internalize Stx, but are not sensitive to Stx [1,2]. Stx binds to the carbohydrate part of Gb3, but it has been shown that the length of the fatty acid chains in Gb3 is important not only for the affinity of Stx for Gb3 [3] but also for the retrograde sorting of the toxin from endosomes to the Golgi apparatus [4]. Both Gb3 alone and in complex with Stx are found in detergent resistant membranes (DRM) after extraction with cold non-ionic detergents, and it has been reported that Stx localization in DRM is important for efficient intoxication [2]. Stx bound to cells at low temperature is distributed evenly on the cell surface. However, the toxin can be rapidly clustered in clathrin-coated pits on the plasma membrane after heating to 37 °C [5]. Furthermore, receptor-bound Stx appears to be able to signal across the plasma membrane and in that way increase its own uptake [6].

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Dietary polyunsaturated fatty acids (PUFA) have long been known to have health beneficial effects, and several large studies describe these effects on severe conditions like rheumatoid arthritis [7] or in cardiovascular diseases [8]. Several mechanisms for the action of PUFA have started to emerge [9]. In T-cells it has been shown that addition of PUFA causes the removal of Src family kinases from DRM and abolishes T-cell activation [10]. Also studies performed with liposomes suggest that PUFA can affect lipid rafts [11], and when lipids are extracted from whole cells with cold non-ionic detergents a fraction of PUFA is found in the DRM fraction [12].

Since it has been shown that Stx and its receptor are associated with DRM fractions after detergent extraction [2] and PUFA have been reported to incorporate into cellular membranes and affect lipid rafts [12], we reasoned that PUFA treatment could affect Stx interaction with cells. Our results show that this is the case and that Stx internalization, transport to the Golgi apparatus, and cytotoxicity were reduced in PUFA-treated cells. Since PUFA containing fish oils are established dietary supplements with few side effects, they might be considered to be used as supportive treatment during infection with Stx producing bacteria.

## Materials and methods

*Materials.* Eicosapentaenoic acid (5*Z*, 8*Z*, 11*Z*, 14*Z*, 17*Z*-eicosapentaenoic acid; EPA) and docosahexaenoic acid (4*Z*, 7*Z*, 10*Z*, 13*Z*, 16*Z*, 19*Z*-docosahexaenoic acid; DHA) were from Cayman (Ann Arbor, MI, USA). The following antibodies were used: mouse anti-Stx (3C10, Toxin Technology, Sarasota, FL, USA), rabbit anti-giantin (Babco, Berkeley, CA, USA), and anti-rabbit CY2 (Jackson Immunoresearch Laboratories, West Grove, PA, USA). All the other chemicals were from Sigma (St. Louis, MO, USA) unless otherwise stated.

*Cell culture and modification of cellular lipids.* Stock cultures of HEp-2 cells were maintained in DMEM (Gibco, Paisley, UK) supplemented with 10% heat inactivated foetal calf serum (Paa, Pasching, Austria), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin under standard cell culture conditions. Cells were seeded out in tissue culture plates (Falcon, Franklin Lakes, NJ, USA) in complete medium and grown overnight. The cells were then treated with 50 μM EPA, DHA or stearic acid for 2 days as described by Stulnig et al. [13]. EPA, DHA, and stearic acid were allowed to equilibrate with the albumin supplemented medium for 30 min in glass beakers before addition to cells. When lipids are fed to cells it is difficult to determine whether they incorporate into a membrane or just stick to the cell surface. In this study, we have fed cells with EPA, DHA or stearic acid as albumin complexes since it has been shown with this protocol that lipids are incorporated into metabolic products, strongly indicating that the lipids are taken up and correctly integrated into cellular membranes [13]. As a control for EPA and DHA we have used stearic acid.

*Toxicity assay.* Stx, ricin or *Pseudomonas* exotoxin 1 was added to lipid treated cells and incubated as described in the figure legends. Protein synthesis was measured as we have described previously [14]. The values are plotted as percent of controls that had not been treated with toxins. Neither EPA, DHA, nor stearic acid affected protein synthesis in HEp-2 cells (data not shown).

*Production and purification of Shiga-B with a sulfation tag.* The B-chain of Stx with a tandem sulfation site added (STxB-Sulf$_2$) was produced in bacteria, and a periplasmic extract was prepared as we have previously described [6]. Periplasmic extract was incubated with Glycosorb-6 beads (Glycorex, Lund, Sweden) with gentle agitation for 10 min at 4 °C and the beads were washed twice with PBS. STxB-Sulf$_2$ was eluted with 0.1 M glycine, pH 2.5, into 2 ml 1 M Tris–HCl, pH 7.5. The eluate was applied to a 1 ml resource Q column (GE Healthcare, Bucks, UK) and STxB-Sulf$_2$ was eluted with a 0–1.0 M NaCl gradient in 20 mM Tris–HCl, pH 7.5, using an ËKTA Explorer system (GE Healthcare, Bucks, UK).

*Sulfation of ricin sulf-1 and STxB-Sulf$_2$.* A ricin variant containing a sulfation tag, ricin sulf-1, was produced and purified as described by Rapak et al. [15]. HEp-2 cells were seeded out in 50 mm tissue culture dishes and treated with EPA or stearic acid. Sulfation of ricin sulf-1 [14] and STxB-Sulf$_2$ [6] was performed as we have described earlier. Briefly, each dish was incubated with 0.3 mCi/ml $Na_2{}^{35}SO_4$ for 3 h before STxB-Sulf$_2$ (1 μg/ml) or ricin sulf-1 (200 ng/ml) was added and the incubation was continued for 2 h. EPA treatment did not affect total sulfation relative to stearic acid treatment (data not shown).

*Confocal fluorescence microscopy.* HEp-2 cells were seeded out on glass coverslips, treated with EPA or stearic acid, and prepared for confocal microscopy as previously described [6] using an anti-giantin primary antibody and an anti-rabbit CY2 secondary antibody.

*TAG- and biotin-labelling of toxins and antibody.* Stx was labelled with a reducible ImmunoPure NHS-SS-Biotin (Pierce, Rockford, IL, USA). An anti-Stx antibody and cholera toxin were labelled with *N*-hydroxysuccinimide ester-activated Tris(bipyridine) chelated ruthenium(II) (BV-TAG; BioVeris, Gaithersburg, MD, USA) according to the manufacturer's instructions.

*Binding of TAG-labelled Stx.* HEp-2 cells were seeded out in 24-well tissue culture plates and treated with EPA or stearic acid. Each well was washed twice with cold HMEM and incubated for 15 min on ice before 3.7, 6.2, 12.5, 25 or 50 ng/ml biotin-labelled Stx and the incubation was continued for 1 h. Bound biotin-labelled Stx was detected as described in the endocytosis assay. Background was found to be less than 10% of the measured binding.

*Binding of $^{125}$Iodine-labelled STxB-Sulf$_2$.* HEp-2 cells were seeded out in 24-well tissue culture plates and treated with EPA or stearic acid. STxB-Sulf$_2$ was labelled with $^{125}$Iodine and binding was measured as previously described [14].

*Endocytosis of Stx.* HEp-2 cells were seeded out in 24-well tissue culture plates and treated with EPA or stearic acid. Endocytosed biotin-labelled Stx was measured as previously described [6].

*Endocytosis of cholera toxin.* HEp-2 cells were seeded out in 24-well tissue culture plates and treated with EPA or stearic acid. Each well was washed twice with HMEM and 1 μg/ml TAG-labelled cholera toxin in HMEM supplemented with 2 mg/ml albumin was added for 15 min at 37 °C. Endocytosed TAG-labelled cholera toxin was detected as described for endocytosis.

*Endocytosis of $^{125}$Iodine-labelled transferrin.* HEp-2 cells were seeded out in 24-well tissue culture plates and treated with EPA or stearic acid. The cells were incubated with 0.2 μg/ml $^{125}$I-transferrin (iron saturated; specific activity: 28,000 cpm/ng) for 5 min at 37 °C in the presence of EPA or stearic acid. The cell-associated (internalized) and the supernatant (surface-bound) radioactivity was counted separately. Internalized transferrin was calculated as percentage cell-associated radioactivity relative to the total radioactivity. Lipid treatment did not change the total associated radioactivity.

## Results and discussion

### *PUFA protect cells against Stx intoxication*

To investigate the role of PUFA on intracellular transport of Stx we have incubated HEp-2 cells with the two most common ω-3 fatty acids, EPA (20:5 ($n-3$)) and DHA (22:6 ($n-3$)), for 2 days and then measured the effect of increasing concentrations of Stx on protein synthesis. As shown in Fig. 1A, treatment with 50 μM EPA protected HEp-2 cells against Stx by approximately a factor of