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Sublethal doses of dinophysistoxin-1 and okadaic acid stimulate secretion of inflammatory factors on innate immune cells: Negative health consequences

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ABSTRACT

One of the proposed mechanisms to explain why Diarrhetic Shellfish Poison (DSP) toxins are tumor promoters is founded on the capacity of these toxins to increase TNF-α secretion. Although macrophages are the principal cells in the activation of the inflammatory response, the immune profile that Okadaic acid (OA) and Dinophysistoxin-1 (DTX-1) trigger in these cells has not been fully explored. We have therefore investigated the effect of various concentrations of both toxins on the activity of several inflammatory factors. Our results demonstrate that OA and DTX-1, at sublethal doses, stimulate secretion of inflammatory factors. Nevertheless DTX-1 was more potent than OA in increasing TNF-α and IL-6 as well as their dependent chemokines KC, MCP-1, LIX, MIP-1 α, MIP-1 β and MIP-2. On the other hand, secretion of IFN-γ and the anti-inflammatory cytokines, IL-4 and IL-10, was unaffected. In addition, DTX-1 also raises matrix metalloproteinase-9 (MMP-9) activity. In this study, for the first time the effect of OA and DTX-1 over the secretion of pro-inflammatory and carcinogenic signals in macrophages are compared, showing that DTX-1 is ten times more potent that OA. The inflammatory profile produced by DTX-1 is shown for the first time. The safe limit regulation should be changed to DSP toxins zero tolerance in the shellfish to be consumed by humans.

1. Introduction

Okadaic acid (OA), dinophysistoxin-1 and 2 (DTX-1, DTX-2) and their acyl derivatives are lipophilic and thermo-stable molecules produced by dinoflagellates, which can accumulate in filter-feeding bivalves when a harmful algal bloom occurs. The ingestion of the contaminated seafood with these toxins causes Diarrheic Shellfish Poisoning (DSP), whose symptoms include diarrhea, vomiting and abdominal pain (James et al., 2010; Picot et al., 2011). While there are no records of human deaths, the DSP toxins have been widely characterized as tumor promoters in animal models, producing cancer when repeatedly applied following a mutagenic compound (Fujiki et al., 2013; Valdiglesias et al., 2013).

OA and DTX-1 are polyketide compounds with furane and pyrane-type ether rings with α-hydroxycarboxyl as a functional group. Although the difference between them is only one methyl group in the C35 position (Dominguez et al., 2010), DTX-1 is more toxic than OA, considering their capacity to disrupt actin cytoskeleton, destroy the integrity of epithelial cells (Espiña et al., 2010; Fernández et al., 2014) and generate apoptosis (Ferron et al., 2014; Soliño et al., 2015). However, in mouse bioassays, where toxins intraperitoneal are injected, both toxins have the same relative toxicity (Murata et al., 1982). They are considered equivalent in international legislation, which has set a limit in seafood of 160 μg OA equivalents/kg shellfish meat, thus protecting the population from their acute effects but permitting chronic consumption of DSP toxins (EFSA, 2008).

The molecular targets of DSP toxins are the protein phosphatases 1 and 2A (PP1, PP2A). PP2A is a regulatory enzyme of multiple

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signaling pathways, such as proliferation, survival, differentiation and apoptosis, and is considered as a tumor suppressor and a positive regulator of the intrinsic and extrinsic apoptosis pathways (Chatfield and Eastman, 2004; Westermarck and Hahn, 2008).

One proposed mechanism to explain the role of DSP toxins as tumor promoters involves an increase in the synthesis and secretion of TNF-α (Fujiki et al., 2013). The essential role of this cytokine in carcinogenesis caused by OA was described in TNF-α-deficient mice, which did not develop tumors when subjected to repeated doses of OA after application of 7,12-Dimethylbenz[a]anthracene (DMBA) as mutagenic agent, unlike wild type mice (Suganuma et al., 1999). OA and DTX-1 can stimulate translocation of NFκB from the cytosol to the nucleus (Thévenin et al., 1990; Ferron et al., 2014), and OA activates the pro-inflammatory transcription factors c-fos/c-jun (Holladay et al., 1992). Furthermore, sublethal doses of OA can increase cellular mitosis in vivo, and may activate AKT, ERK and the expression of c-Myc in several cell lines, events involved in the inflammatory response and carcinogenesis (Baharians and Schönthal, 1999; Le Hégarat et al., 2006; Guénin et al., 2008; del Campo et al., 2013).

Macrophages are the principal effectors of the inflammatory response and as one of the primary danger sensors in the host (Adams and Hamilton, 1984). They play an essential role in innate and adaptive immunity; however, in states of inflammatory or persistent chronic infectious disease, tumor microenvironment generated can promote cell transformation and neoplastic development (Erreni et al., 2011). OA in peritoneal macrophages produces augmentation of TNF-α and IP-10 mRNAs (Tebo and Hamilton, 1994), secrete prostaglandin E2 by increased activity of phospholipase A2 (PLA2) (Qiu et al., 1993) and decreases the production of NO by interfering with the regulation of PP2A acting on iNOS (Dong et al., 1995). Nevertheless, the effect of DSP toxins on inflammatory cytokines remains unresolved. We have analyzed the capacity of OA and DTX-1 to stimulate the secretion of pro-inflammatory cytokines and chemokines in primary cultures of peritoneal macrophages, and the RAW 264.7(macrophage cell line) and DC 2.4 (dendritic cell line) to describe the immune profile that these toxins trigger and also showing that both do not have the same equivalence in their toxicity and secretion of pro-inflammatory factors.

2. Materials and methods

2.1. Extraction, culture and characterization of peritoneal macrophages

Peritoneal cells of C57BL/6 mice (8–12 weeks) were obtained according to described by Zhang et al., 2008 with modifications. The use of experimental animals was approved by the ethics committee of the Faculty of Medicine, University of Chile (Protocol: CBA # 0551). Briefly, 1 mL of 3% thioglycollate was injected into the intraperitoneal cavity of mice and after 4 d, the mice were killed by cervical dislocation and the peritoneal cells were extracted from the peritoneal cavity with 5 mL of RPMI. Red blood cells were lysed with 0.88% KCl, then 3×10^4 cells per well were seeded in 96-well plates; an hour later non-adherent cells were removed and incubated with fresh medium, with or without DSP toxins, or with 10 ng/mL LPS. Adherent cells were characterized phenotypically as macrophages by the presence of the superficial molecular marker F4/80 (99.1%). In addition, we analyzed the macrophage response capacity against LPS (10 ng/mL) by the expression of surface maturation markers MHCII, CD80, CD86 and CD40 (data not shown). The antibodies were purchased from BD Biosciences Pharmingen, USA; data were acquired on a FACScan flow cytometer (Becton-Dickinson, USA) and were analyzed using WinMDI and FlowJo software (Los Angeles, CA, USA).

2.2. Cell culture

Peritoneal macrophages, RAW 264.7 and DC 2.4 cell lines were cultured in RPMI medium supplemented with penicillin (100 mg/mL), streptomycin (100 mg/mL), 1% non-essential amino acids (Invitrogen; Grand Island, New York, NY, USA), 10% heat-inactivated fetal bovine serum (FBS, HyClone; South Logan, Utah, UT, USA), and were incubated in 5% CO2 at 37 °C. RAW 264.7 cells were seeded at 2×10^5 per well, in 96 well plates, and were incubated 24 h later with or without OA and DTX-1. (purchased from National Research Council (Halifax, Nova Scotia, Canada)). The control negative cells have 1% methanol as vehicle, and positive control LPS (0111:B4 E. Coli, Sigma-Aldrich, USA) at 100 ng/mL was used.

2.3. Cell viability assay

The AlamarBlue assay was used according to manufacturer's recommendations; 10% of reagent was incubated for 2 h. Optical density of medium in each well was measured with an automatic microplate reader at a test wavelength of 570 nm and a reference wavelength of 690 nm for MTT assay and 600 nm for AlamarBlue assay. The data was expressed as the percentage of mean absorbance, relative to the negative controls (100% of viability). Results represent the mean ± SD of at three independent experiments and each measured was in quadruplicate.

2.4. Cytokine determination

2.4.1. Luminex XMap system

Protein analysis of supernatant of peritoneal macrophages cultures was performed with materials from Eve Technologies Corporation (Calgary, Canada) using a multiplex assay based on color-coded polystyrene beads. Briefly, the samples treated for 24 h with different treatment of OA and DTX-1, were pre-incubated with beads bound to Abs against 32 different cytokines, for 2 h and washed three times before incubation with the secondary Ab for 1 h. Wells were then washed and fluorescence was measured in the Luminex 200 system, determining their concentration as standards values. Results represent the mean ± SD of at four independent macrophage preparations.

2.4.2. Enzyme-linked immunosorbent assay (ELISA)

TNF−α and IL-6 were measured using commercial ELISA kits according to the manufacturer's instructions (Mouse Duoset ELISA, R&D systems, Minneapolis, MN, USA). As control, a sample of the culture medium alone was included. Results represent the mean ± SD of at three independent macrophage preparations and each measured was in quadruplicate.

2.5. MMP-2 and MMP-9 proteolytic activity

We evaluated MMP-2 and MMP-9 gelatinase activities in cell culture medium samples by zymography. 10 μl of each culture medium sample were diluted in 190 μl of sample buffer (2% SDS, 125 mM Tris–HCl; pH 6.8, 10% glycerol, and 0.001% bromophenol blue). Thereafter, 10 μl of each sample solution was loaded in each gel lane and gelatin zymography. Samples were subjected to electrophoresis on 7.5% SDS-PAGE co-polymerized with gelatin (1%) as substrate. After electrophoresis was completed, the gel was incubated for 30 s at room temperature in a 2.5% Triton X-100 solution, and incubated at 37 °C for 18 h in Tris–HCl buffer, pH 7.4, containing 10 mmol/L $CaCl_2$. These gels were stained with 0.05% Coomassie Brilliant Blue G-250, and then destained with 30% methanol and