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Oxidative stress induced by palytoxin in human keratinocytes is mediated by a H^+-dependent mitochondrial pathway

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ABSTRACT

In the last decades, massive blooms of palytoxin (PLTX)-producing *Ostreopsis* cf. *ovata* have been observed along Mediterranean coasts, usually associated to human respiratory and cutaneous problems. At the molecular level, PLTX induces a massive intracellular Na^+ influx due to the transformation of Na^+/K^+ ATPase in a cationic channel. Recently, we have demonstrated that Na^+ overload is the crucial step in mediating overproduction of reactive oxygen species (ROS) and cell death in human HaCaT keratinocytes, tentatively explaining PLTX-induced skin irritant effects. In the present study the molecular mechanisms of ROS production induced by PLTX-mediated Na^+ intracellular overload have been investigated. In HaCaT cells, PLTX exposure caused accumulation of superoxide anion, but not of nitric oxide or peroxynitrite/hydroxyl radicals. Even if RT-PCR and western blot analysis revealed an early NOX-2 and iNOS gene and protein over-expressions, their active involvement seemed to be only partial since selective inhibitors did not completely reduce O_2^- production. A significant role of other enzymes (COX-1, COX-2, XO) was not evidenced. Nigericin, that counteracts Na^+-mediated H^+-imbalance, dissipating ΔpH across mitochondrial inner membrane, and the uncouplers DNP significantly reduced O_2^- production. These inhibitions were synergistic when co-exposed with complex-I inhibitor rotenone. These results suggest a novel mechanism of O_2^- production induced by PLTX-mediated ionic imbalance. Indeed, the H^+ intracellular overload that follows PLTX-induced intracellular Na^+ accumulation, could enhance ΔpH across mitochondrial inner membrane, that seems to be the driving force for O_2^- production by reversing mitochondrial electron transport.

Introduction

Palytoxin (PLTX) is a non-protein marine toxin identified in *Palythoa* zoanthid corals and *Ostreopsis* dinoflagellates as well as in *Trichodesmium* cyanobacteria. In the last few years, concomitantly with massive blooms of *Ostreopsis* cf. *ovata* along the Mediterranean coasts, several cases of human intoxications have been reported, mainly consisting in respiratory and cutaneous distresses (Durando et al., 2007; Kermarec et al., 2008; Tichadou et al., 2010; Tubaro et al., 2011). Furthermore, symptoms like skin irritation and general malaise have been associated to PLTX cutaneous exposure after handling zoanthid corals (Deeds and Schwartz, 2010).

The molecular mechanism through which PLTX can induce cell toxicity primarily relies on a massive intracellular influx of Na^+ ions that follows the transformation of the Na^+/K^+ ATPase (NaKa) in a cationic channel. Under normal conditions, intracellular Na^+ levels are strictly controlled and, in turn, the maintenance of Na^+ homeostasis is essential for the preservation of physiological cytoplasmic concentrations of Ca^{2+} and H^+ ions. Indeed, in presence of a sustained overload of Na^+ ions, the functioning of Na^+/H^+ (NHE) and Na^+/Ca^{2+} (NCX) exchangers can be reversed, therefore affecting also H^+ and Ca^{2+} ions balance (Rossini and Bigiani, 2011).

One of the possible consequences of a sustained imbalance of Na^+, Ca^{2+} or H^+ ions is the induction of oxidative stress. The role for Na^+ overload as an upstream signal for the production of reactive oxygen species (ROS) and cell death has been established in many cell types (Yang et al., 2004), in diabetic (Babsky et al., 2001) and in ischemia/reperfusion cardiomyocytes models (Murphy and Eisner, 2009; Szabo et al., 1992). However, the intracellular sequence of events that links Na^+ overload to ROS production is still not completely understood. With regard to epithelial cells, a crucial role in mediating ROS production has been demonstrated in skin keratinocytes only for Ca^{2+} (Goldman et al., 1998), whereas very few data are presently available regarding the role of Na^+ in oxidative stress induction at the skin level.

We have recently demonstrated that in human HaCaT keratinocytes, Na^+ influx is essential for PLTX to induce both ROS production and cytotoxicity (Pelin et al., 2011), and thus it represents the first step for conveying the irritant and cytotoxic properties of PLTX. However, the

Abbreviations: COX, cyclooxygenase; DNP, 2,4-dinitrophenol; DPI, diphenyleneiodonium chloride; NaKa, Na^+/K^+ ATPase; NBT, nitro blue tetrazolium, NHE, Na^+/H^+ exchanger; NMMA, N^G-Methyl-L-arginine acetate; NOS, nitric oxide synthase; NOX, NADPH oxidase; PLTX, palytoxin; ROS, reactive oxygen species; XO, xanthine oxidase.

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issues that remain to be clarified are the molecular mechanisms by which PLTX-dependent Na^+ imbalance leads to ROS production and the role of oxidative stress in causing cytotoxicity and cell death. In the present study, we investigated in human skin keratinocytes the ROS species produced during PLTX exposure, their putative formation pathways and their role in cytotoxicity. Our results indicate that PLTX-induced intracellular accumulation of Na^+ causes ROS production through intracellular overload of H^+ and the consequent alteration of mitochondrial electron transport chain activity. The increased O_2^- levels, however, do not appear to be primarily responsible for PLTX-induced cell toxicity.

Material and methods

Chemicals

Palytoxin, isolated from *Palythoa tuberculosa*, was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan; lot number WKL7151, purity > 90%). Nitro Blue Tetrazolium (NBT), 2′,7′-Dichlorofluorescin diacetate (DCFDA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), nigericin sodium salt, rotenone, 2,4-dinitrophenol (DNP), 4′-hydroxy-3′-methoxyacetophenone (apocynin), allopurinol, indomethacin, Kodak® BioMax light film and Kodak® processing chemicals were purchased from Sigma-Aldrich (Milano, Italy). Diphenyleneiodonium chloride (DPI) and N^G-Methyl-L-arginine acetate salt (NMMA) were purchased from Vinci-Biochem (Firenze, Italy). PAGEr Gold precast gels were purchased from Lonza (Milano, Italy) and all primary and secondary antibodies were from Millipore. All the other reagents of analytical grade were purchased from Sigma-Aldrich (Milan, Italy).

Cell culture

HaCaT cells (DKFZ, Eppelheim, Germany) were kept in DMEM medium (Euroclone, Milano, Italy) supplemented with 10% foetal bovine serum, L-glutamine (1.0×10^{-2} M), penicillin (1.0×10^{-4} g/ml) and streptomycin (1.0×10^{-4} g/ml) at 37 °C in a humidified 95% air/5% CO_2 atmosphere. Cell passage was performed 2 days post-confluence, once a week. All the experiments were performed between passages 48 and 60.

Colorimetric assays

NBT reduction assay. Cells were plated in 96 multi-well plates at a concentration of 15×10^3 cells/well. After 5 days, medium was removed and wells refilled with 200 μl of 0.5 mg/ml NBT in PBS containing 25 mM Hepes. Cells were then exposed to PLTX for 1 h and the reduced NBT was solubilized with 140 μl/well DMSO and 120 μl/well 2 M KOH. The absorbance was measured by an Automated Microplate Reader EL 311s (Bio-Tek Instruments, Winooski, VT) at 630 nm. Data are the means ± SEM of 6 independent experiments performed in quintuplicate and are reported as % increase with respect to negative controls (cells not exposed to the toxin) applying the following equation:

$$\%\,\text{increase} = ((\text{Abs treated} - \text{Abs controls})/\text{Abs controls}) * 100).$$

For experiments performed in the presence of ROS inhibitors, cells were pre-exposed for 1 h to 5.0×10^{-6} M DPI, 1.0×10^{-5} M apocynin, 1.0×10^{-4} M NMMA, 5.0×10^{-6} M rotenone, 1.0×10^{-4} M indomethacin or 1.0×10^{-4} M allopurinol and then treated with the toxin.

Griess assay. Cells (10×10^3 cells/well) were plated for 3 days in 96 multi-well plates. Wells were refilled with PBS containing 5×10^{-2} M Hepes and exposed to PLTX for 1 h. Griess assay was then performed on 85 μl of medium following manufacturer's instructions (Bioxytech® Nitric Oxide Assay, OxisResearch™, USA). Optical density was measured by an Automated Microplate Reader EL 311s (Bio-Tek Instruments, Winooski, VT) at 540 nm. Data are the means ± SEM of 4 independent experiments performed in quadruplicate and are reported as % increase with respect to negative controls as described above.

MTT assay. Cells were seeded in 96 multi-well plates at a density of 5×10^3 cells/well and after 3 days pre-exposed for 1 h to the ROS inhibitors and then to PLTX (10^{-8}–10^{-10} M) for 4 h. Cells were then washed and wells refilled with fresh culture medium containing 0.5 mg/ml MTT. After 4 h the insoluble crystals were solubilized by 200 μl/well DMSO and the absorbance was measured by an Automated Microplate Reader EL 311s (Bio-Tek Instruments, Winooski, VT) at 540/630 nm. Data are the means ± SEM of 4 independent experiments performed in quadruplicate and are reported as % increase with respect to negative controls as described above.

Fluorometric analysis (DCFDA). Cells were plated in 96 multi-well plates at a density of 10×10^3 cells/well and after 3 days exposed for 1 h to PLTX (10^{-8}–10^{-12} M). Cells were then refilled with PBS containing 1.0×10^{-4} M DCFDA and cells maintained for 30 min at 37 °C in the dark. Cells were then washed with PBS and fluorescence read by a fluorocount microplate fluorometer (Packard, Germany) with excitation length of 485 nm and emission length of 570 nm. Data are the means ± SEM of 4 independent experiments performed in quintuplicate and are reported as % increase with respect to negative controls as described above.

Real time qPCR. HaCaT were seeded for 48 h in 25 cm^2 flask at a density of 5×10^5 cells/flask and then treated for 1, 2, 4, 12 and 24 h with 1.0×10^{-11} M PLTX. The cells were collected, washed with ice-cold PBS, and total RNA was extracted by High Pure RNA Isolation kit (Roche, Mannheim, Germany) following the manufacturer's instructions. Total RNA (2 μg) was retro-transcribed by PCR with SuperScript II 200U (Invitrogen). To quantify the mRNA expression of specific genes, SYBR green real time qPCR assay was performed by LightCycler technology (Roche, Mannheim, Germany) in 20 μl PCR mixture volume consisting of 10 μl of 2× Quantitect SYBR Green PCR Master Mix containing HotStarTaq DNA polymerase (Qiagen, Hilden, Germany), 400 nM of each oligonucleotide primer and 100 ng of retrotranscribed total RNA extracted from each sample per reaction. The amplification was performed with initial activation of HotStar Taq DNA Polymerase at 95 °C for 15 min and 40 cycles in three steps: 94 °C for 10 s, 60 °C for 15 s, and 72 °C for 30 s for all tested genes. Following cycling, to ensure specificity, melting curve analysis was carried out to verify the amplification of PCR products starting at 60 °C and ramping to 95 °C at 0.1 °C/s. The relative quantifications were performed by specific standard external curves. The normalisation was performed by parallel amplification of beta actin as described previously (Kim, 2001). The specific oligo pairs to amplify cyclooxygenase (COX-1 and COX-2), nitric oxide synthetase (iNOS, eNOS and nNOS), beta actin and NADPH oxidase (NOX-2) were already published (Dodd et al., 2000; Fink et al., 2008; Gibellini et al., 2008; Houliston et al., 2002; Pierzchalska et al., 2007). The oligo primer sequences for xanthine oxidase (XO) were: forward 5′-GCATATCATTGGTGCTGTGG-3′, reverse 5′-GGTCCCCTTTCTCG ATCTTC-3′.

Western blot analysis. Cells (1.5×10^6) were seeded for 4 days in 75 cm^2 culture flask. Cells were then exposed to 1.0×10^{-11} M PLTX for 1, 2, 4, 12 and 24 h, collected, and washed with ice-cold PBS and total protein extracted by 200 μl lysis buffer (Tris HCl 10 mM, EDTA 100 mM, NaCl 100 mM and SDS 0.1%) containing 10% of protease inhibitor. Samples were then run on 10% acrylamide gels in a Tris-Glycine buffer in a PAGEr™ Mini-gel Chamber (Lonza, Milano, Italy) and then semi-dry blotted for 2 h with 50 mA current on PVDF membrane. Membranes were blocked for 1 h with 3% not-fat milk in PBS and incubated overnight at 4 °C with primary antibodies (anti-actin 1:10,000,