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Deoxynivalenol-induced cytotoxicity, cytokines and related genes in unstimulated or lipopolysaccharide stimulated primary porcine macrophages

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ABSTRACT

The cytotoxicity of deoxynivalenol (DON) as well as the induction of cytokines and related genes was investigated in porcine pulmonary alveolar macrophages (PAM) in absence or presence of lipopolysaccharides (LPS). IC_{20} values were 1.1, 0.4 and 1.0 μM DON in the MTT, neutral red and alamar blue assay, respectively, and did not differ significantly in the presence of LPS. The mRNA expression of tumour necrosis factor (TNF)-α peaked at 3 h, whereas LPS and DON showed synergistic effects resulting in an approximately 20-fold increase at 500 nM DON as compared to untreated controls. The supernatant concentrations of TNF-α showed similar synergistic effects. The expression of interleukin (IL)-1β was significantly induced by DON (except for 12 h) and LPS. An induction of the mRNA expression of IL-6 by DON was evident only at 3 h, whereas the supernatant concentrations of LPS stimulated PAM incubated with 500 nM DON were significantly decreased at most time points. Cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expression did not seem to contribute to the effects of DON in porcine macrophages. The results of the present investigation suggest a contribution of cytokines, especially TNF-α and IL-1β, induced by DON in porcine macrophages to the effects observed *in vivo*.

1. Introduction

The mycotoxin deoxynivalenol (DON), a member of the trichothecenes group which is mainly produced by fungi of the genus *Fusarium*, occurs in toxicologically relevant concentrations in cereals and grains worldwide. Among farm animals, pigs react most sensitively to the exposure to DON contaminated feed (for review see EFSA, 2004). The most typical symptoms of DON intoxication in pigs are reduced feed intake at low dietary concentrations and vomiting at extremely high concentrations, while contamination of up to 0.9 mg/kg complementary and complete feeding stuffs for pigs are considered safe (The Commission of the European Communities, 2006). In addition, DON is also known to act as a protein synthesis inhibitor and to possess immunomodulatory properties (Pestka and Smolinski, 2005). High doses of trichothecenes, like DON, may compromise actively proliferating tissues including bone marrow, lymph nodes, spleen, thymus, and the mucosa of intestines which may result in immunosuppression expressed as decrease in circulating lymphocytes, reduced resistance towards pathogens and suppression or delay of antibody response towards antigens. In contrast, low doses can increase resistance against certain pathogens, increase IgA in serum and induce a stimulation of immune-associated genes as demonstrated in model experiments in mice (for review see Bondy and Pestka, 2000). The cellular and molecular mechanisms of the immunomodulating action of DON were described in numerous studies in mice and murine cell lines (reviewed by Pestka et al., 2004). The induction of inflammatory cytokines, especially of proinflammatory cytokines like interleukin (IL)-6 in macrophages seems to play a pivotal role as they are directly linked to the differentiation of B-cells and to the stimulation of IgA secretion (Pestka, 2003). The upregulation of cytokines is a consequence of the activation of mitogen-activated protein kinases (MAPKs) followed by induction of cyclooxygenase-2 (COX-2). As these pathways are also involved in the signalling of the toll-like receptor (TLR) 4, which is stimulated by lipopolysaccharides (LPS), it is perceivable that both agents act synergistically and additively.

At the same time, cytokines secreted by activated mononuclear myeloid cells, including IL-6, tumour necrosis factor (TNF)-α and IL-1β, are known to be involved in reducing feed intake of animals in patho-physiological conditions (Johnson, 1998; Plata-Salaman, 2001), suggesting that the induction of those cytokines may also

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be involved in the reduction of feed intake of animals exposed to DON. However, mechanistic studies of the molecular and cellular actions of DON were mainly carried out in mice and cell lines. Even though the underlying mechanisms of cytokine production and inflammatory reactions belong to the most conserved mechanisms, fundamental studies in the mainly affected animals are needed to appraise the significance of the demonstrated pathways. Therefore the present investigation focused on the cytotoxicity and the gene expression and transcription of markers of inflammation such as TNFα, IL-1β and IL-6 of porcine pulmonary alveolar macrophages (PAM) exposed to DON in the presence and absence of LPS. Moreover, the contribution of iNOS and COX to the toxic effect of DON in PAMs was evaluated.

2. Material and methods

2.1. Chemicals and reagents

DON, LPS (*Escherichia coli*, 0111:B4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), gentamicin, fungizone and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Phosphate buffered saline (PBS), Roswell Park Memorial Institute (RPMI) 1640, fetal calf serum (FCS), glutamine and penicillin/streptomycin were obtained from Gibco Invitrogen (Breda, The Netherlands). Ampicillin was from Dopharma Veterinaire Farmaca BV (Raamsdonksveer, The Netherlands). Pentobarbital–sodium was from Produlab Pharma, Raamsdonkveer, The Netherlands. The mouse monoclonal antibody directed against porcine macrophages (2G6, Berndt et al., 2000) was a kind gift of Dr. A. Berndt (Friedrich Loeffler Institute, Jena, Germany). The DakoCytomation EnVision+ System-HRP (DAB) including peroxidase labelled polymer conjugated to goat anti-mouse immunoglobulins, 3,3′-diaminobenzidine (DAB) chromogen solution and substrate buffer containing hydrogen peroxide was commercially available from Dako (Uithoorn, The Netherlands). Alamar blue solution was purchased from AbD Serotec (oxford, UK). Quantakine porcine TNF-α DuoSet ELISA development system, Quantakine porcine IL-6 DuoSet ELISA development system and Quantakine porcine IL-10 DuoSet ELISA development system plus ancillary products were obtained from R&D Systems (Minneapolis, MN, USA). The SV-total RNA isolation kit was obtained from Promega (Madison, WI, USA) while iScript™ cDNA Synthesis kit and IQ™ SybrGreen Supermix were from Bio-Rad (Hercules, CA, USA). The primers were manufactured commercially (Isogen, Ijsselstein, The Netherlands).

2.2. Animals

Three castrated healthy pigs (Large white × Finnish landrace × Yorkshire) of approximately 10 weeks of age with a body weight of 24–27 kg were obtained from the breeding farm of the Faculty of Veterinary Medicine, Utrecht University. The Ethical Committee of the Faculty approved the use of the animals for the investigations.

2.3. Isolation and culture of PAM

The pigs were euthanized by injection of Pentobarbital via the jugular vein. The lungs with the trachea were removed and a silicone tube was placed in the trachea. The lung lobes were filled with ice cold PBS with 0.5% BSA and carefully massaged. The resulting cell suspension was collected and the process was repeated three to four times. The cells were washed with PBS + 0.5% BSA by centrifugation for 10 min at 50 g at 4 °C twice. Cells were resuspended in RPMI 1640 supplemented with 5% FCS, 2 mM glutamine and fungizone (2.5 μg/ml) and each 50 μg/ml gentamicin and ampicillin. For cytotoxicity experiments a seeding density of 0.2×10^6 cells/well in 96-well plates was used while for the other experiments 2.5×10^6 cells/well were seeded in 12-well plates. After incubating at 37 °C and 5% CO_2 in a humidified incubator for 3 h, unattached cells were removed with the medium, and fresh medium was added.

Twenty-four hours after isolation, the medium was replaced by RPMI 1640 supplemented with 5% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin containing the test substances or solvent control. A concentration range of 0, 0.5, 5, 50, 500, 1000, 2000, 4000 and 8000 nM DON was used for cytotoxicity experiments in the presence or absence of 0.1 μg LPS/ml. The PAMs were incubated for 48 h in triplicates. For the other experiments (on inflammatory mediators) a concentration range of 0, 0.5, 5, 50, 500 and 2000 nM DON was used in the presence or absence of 0.1 μg LPS/ml. Supernatants were harvested after 3, 12, 24 or 48 h of incubation while cell layers were used for isolation of RNA.

2.4. Immunocytochemistry

Immunohistochemical analysis on the obtained cell population was performed for the confirmation of the presence of porcine macrophages. PAM were seeded in Permanox™ Lab-Tek™ Chamber Slide™ with 8 wells/slide at a density of 0.4×10^6 cells/well. Cells were cultured as described above. Forty-eight hours past seeding, the medium was aspirated and the cells were fixed with 4% paraformaldehyde for 30 min. Cells were then incubated with mouse monoclonal antibody directed against porcine macrophages (2G6, Berndt et al., 2000) for 30 min. Immunohistochemical staining was performed using a DakoCytomation EnVision+ System-HRP (DAB) (Dako, Glostrup, Denmark) following the manufacturers instructions. In brief, peroxidase labelled polymer conjugated to goat anti-mouse immunoglobulins was applied and incubated for 30 min followed by a 5-min incubation with 3,3′-diaminobenzidine (DAB) chromogen solution with a substrate buffer containing hydrogen peroxide. Between incubation steps, slides were washed three times with PBS. After counterstaining with haematoxylin, cells were mounted with Aqua-mount and examined by light microscopy.

2.5. Cytotoxicity assays

Cytotoxicity was assayed by means of metabolic activity (MTT and Alamar blue) and Neutral red uptake by lysosomes of PAMs.

MTT was added at a concentration of 0.5 mg/ml and incubated for 2 h. After removal of medium, the cells were lysed in 0.04 M HCl in isopropanol. Absorbance was read at 595 nm on a multiwell spectrophotometer.

Alamar blue solution and neutral red were added to the PAMs to a resulting dilution of 1:10 and 1:40. After 2 h of incubation, fluorescence of the AB reduction product, resorufin, was measured at an excitation wavelength of 540 nm and an emission wavelength of 590 nm (Fluostar Optima Fluorometer, BMG Labtechnologies, Offenburg, Germany). Subsequently, the medium was discarded and the neutral red taken up by the cells was released by 20 min of incubation on an orbital shaker with 100 μl neutral red destain/well (1% acetic acid, 50% ethanol, 49% water; v,v,v). Optical density or fluorescence was read on a spectrophotometer at 546 nm.

The optical density of cells not exposed to DON or LPS was set to 100% to which all other measurements were related.

2.6. RNA isolation

RNA of PAMs was isolated to investigate the mRNA expression of iNOS, COX-2, TNF-α, IL-6, IL-1β and IL-10. Cells were scraped in lysis buffer and total RNA was isolated using the SV-total RNA isolation kit (Promega, Leiden, The Netherlands) according to the manufacturer's protocol including a DNAse treatment. The RNA was quantified spectrophotometrically at 260 nm (ND-1000, Nanodrop technologies) and stored at −70 °C.

2.7. cDNA synthesis

First strand c DNA synthesis was performed with the iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). A quantity of 1 μg total RNA was added to the mixture containing both oligo(dT) and random hexamer primers in a final volume of 20 μl. The reaction mixture was incubated at 25 °C for 5 min and 42 °C for 45 min,

Table 1
Primer sets used for the quantitative PCR analysis with the respective annealing temperatures used.

Gene	NCBI accession number	Forward primer 5′→3′	Reverse primer 3′→5′	Ta (°C)	Ref.
IL-6	NM_214399	CTGGCAGAAAACAACCTGAACC	TGATTCTCATCAAGCAGGTCTCC	60	Duvigneau et al. (2005)
TNF-α	NM_214022	CCAACGGCGTGAAGCTGAAAGAC	GATGCGGCTGATGGTGTGAGTGA	60	
iNOS	SSU59390	CTCTTCGAAATCCCTCCTGAC	AGCTCCTGGAACCACTCGT	61.2	
IL-10	NM_214041	CGGCGCTGTCATCAATTTCTG	CCCCTCTTGGAGCTTGCTA	56	
IL-1β	NM_214055	GTGCAAACTCCAGGACAAAGACCA	CACAAGCTCATGCAGAACACCAC	60	
COX-2	NM_214321	CATTGATGCCATGGAGCTGTA	CTCCCCAAAGATGGCATCTG	61	Fava et al. (2007)
Cyclophilin	NM_214353	TGCTTTCACAGAATAATTCCAGGATTTA	GACTTGCCACCAGTGCCATTA	60	Duvigneau et al. (2005)
ACTB	AY550069	GCAAATGCTTCTAGGCGGACTGT	CCAAATAAAGCCATGCCAATCTCA	64	
HPRT	NM_001032376	ATCATTATGCCGAGGATTTGGA	CCTCCCATCTCTTTCATCACATCT	63	