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UNUSUAL ASTROCYTE REACTIVITY CAUSED BY THE FOOD MYCOTOXIN OCHRATOXIN A IN AGGREGATING RAT BRAIN CELL CULTURES

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Abstract—Ochratoxin A (OTA), a mycotoxin and widespread food contaminant, is known for its patent nephrotoxicity and potential neurotoxicity. Previous observations *in vitro* showed that in the CNS, glial cells were particularly sensitive to OTA. In the search for the molecular mechanisms underlying OTA neurotoxicity, we investigated the relationship between OTA toxicity and glial reactivity, in serum-free aggregating brain cell cultures. Using quantitative reverse transcriptase–polymerase chain reaction to analyze changes in gene expression, we found that in astrocytes, non cytotoxic concentrations of OTA down-regulated glial fibrillary acidic protein, while it up-regulated vimentin and the peroxisome proliferator-activated receptor-γ expression. OTA also up-regulated the inducible nitric oxide synthase and the heme oxygenase-1. These OTA-induced alterations in gene expression were more pronounced in cultures at an advanced stage of maturation. The natural peroxisome proliferator-activated receptor-γ ligand, 15-deoxy-$\Delta^{12,14}$ prostaglandin J2, and the cyclic AMP analog, bromo cyclic AMP, significantly attenuated the strong induction of peroxisome proliferator-activated receptor-γ and inducible nitric oxide synthase, while they partially reversed the inhibitory effect of OTA on glial fibrillary acidic protein. The present results show that OTA affects the cytoskeletal integrity of astrocytes as well as the expression of genes pertaining to the brain inflammatory response system, and suggest that a relationship exists between the inflammatory events and the cytoskeletal changes induced by OTA. Furthermore, these results suggest that, by inducing an atypical glial reactivity, OTA may severely affect the neuroprotective capacity of glial cells.

Key words: gliosis, glial fibrillary acidic protein, brain inflammation.

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Abbreviations: ANOVA, analysis of variance; Br-cAMP, 8-bromoadenosine-3′,5′-cyclic monophosphate.Na; DIV, day *in vitro*; GFAP, glial fibrillary acidic protein; GS, glutamine synthase; HO-1, heme oxygenase-1; iNOS, inducible nitric oxide synthase; ISH, *in situ* hybridization; LDH, lactate dehydrogenase; NO, nitric oxide; OTA, ochratoxin A; PBS, phosphate-buffered saline; PPARγ, peroxisome proliferator-activated receptor γ; RT, reverse transcriptase; RT-PCR, reverse transcriptase–polymerase chain reaction; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$ prostaglandin J2.

Reactive gliosis is a complex cellular process involving astrocytes in response to various types of CNS injury. Reactive astrocytes are classically characterized by the increased expression of glial fibrillary acidic protein (GFAP), a constituent of the intermediate filaments. GFAP is specifically expressed by astrocytes (Eng and Ghirnikar, 1994) and represents the prototypic marker of astrocytes and astrogliosis. Astrogliosis is usually detected prior to any toxic effect on neurons and is therefore regarded as an early marker of neurotoxicity (Monnet-Tschudi et al., 1995; O'Callaghan, 1991). Besides GFAP, reactive astrocytes alter the expression of a variety of proteins, including the intermediate filament protein vimentin, the enzyme glutamine synthase (GS), and several cytokines (for review, see Eddleston and Mucke, 1993). As a potent source of immunologically relevant cytokines and chemokines, astrocytes play a pivotal role in CNS inflammatory responses (for review, see Aschner, 1998; Dong and Benveniste, 2001; Eddleston and Mucke, 1993), and in the modulation of neurotoxic injury (Aschner et al., 2002). Numerous genes, including peroxisome proliferator-activated receptor-γ (PPARγ), inducible nitric oxide synthase (iNOS), and heme oxygenase-1 (HO-1), are regulated and participate in the inflammatory responses.

The mycotoxin ochratoxin A (OTA) (van der Merwe et al., 1965), a widespread food contaminant, was found to accumulate in the brain (Belmadani et al., 1998) and to be neurotoxic (Belmadani et al., 1998, 1999; Dortant et al., 2001; Hong et al., 2000; Miki et al., 1994; Monnet-Tschudi et al., 1997), in addition to its well-known nephrotoxicity in several animal species, and its carcinogenicity in rodent kidneys (Kuiper-Goodman and Scott, 1989). One of the mechanisms responsible for OTA toxicity is thought to be the inhibition of protein synthesis by competition with phenylalanine for phenylalanine-dependent enzymes (Creppy et al., 1984, 1986, 1990; Zanic-Grubisic et al., 2000). However, Bruinink and Sidler (1997) failed to show a protection by phenylalanine of OTA-induced neurotoxic effects in brain and retina cell cultures. In the brain, OTA was found to modify the levels of the free amino acids, phenylalanine and tyrosine (Belmadani et al., 1996), and to modify the activity of several membrane bound enzymes (Zanic-Grubisic et al., 1996). Continued treatment of three-dimensional brain cell cultures with low concentrations of OTA caused an increase in GS activity, suggesting astrogliosis (Monnet-Tschudi et al., 1997). Other reported cellular actions of OTA include oxidative damage (Gautier et al., 2001), enhancement of lipid peroxidation (Omar et al., 1991; Rahimtula et al., 1988), binding to proteins and DNA

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(Obrecht-Pflumio et al., 1996; Schwerdt et al., 1999), disturbance of calcium homeostasis, and impairment of mitochondrial oxidation reactions (Dopp et al., 1999; Eder et al., 2000). Interestingly, the anti-inflammatory prostaglandin and PPARγ ligand 15-deoxy-$\Delta^{12,14}$ prostaglandin J2 (15d-PGJ_2) has recently been shown to prevent OTA-induced cytotoxicity in cultured rat embryonic midbrain cells (Hong et al., 2002), suggesting a role for inflammatory events in OTA neurotoxicity.

The aim of the present work was to further investigate OTA adverse effects in the brain, and their relationship with brain inflammatory reactions and astrocyte reactivity. To this end, we used serum-free aggregating brain cell cultures, a versatile and relevant *in vitro* model for neurotoxicological studies (Zurich et al., 2004a), enabling the detection of reactive gliosis (Monnet-Tschudi et al., 1995; Zurich et al., 2000).

EXPERIMENTAL PROCEDURES

Cell cultures

Rotation-mediated aggregating cell cultures of fetal rat telencephalon were prepared as previously described in detail (Honegger and Monnet-Tschudi, 2001). In brief, the pooled forebrains of 16-day fetal rats (Charles River Laboratories, L'Arbresle, France) were dissociated mechanically using nylon sieves of 200- and 115-μm pores. The dissociated and washed cells, resuspended in serum-free modified DMEM (Honegger and Monnet-Tschudi, 2001) were incubated at a density of 6×10^7 cells per flask, and maintained under constant gyratory agitation at 37 °C in an atmosphere of 10% CO_2 and 90% humidified air. These rotation-mediated cultures form even-sized spherical structures composed of all the different types of neural cells (neurons, astrocytes, oligodendrocytes and microglial cells) and are able to differentiate in a histotypic fashion.

Treatments

For experiments, cultures were randomized and aliquoted 16–20 h prior to treatments. The aggregates from several flasks were pooled and then redistributed into flasks containing pre-equilibrated medium. Each of the culture replicates thus obtained (each corresponding to one sample) contained about 500 aggregates. Cultures were treated at day *in vitro* (DIV) 5 (DIV5) or at DIV18. Cultures were harvested 24 h, 48 h and 9 days after treatment. OTA (Sigma, St. Louis, USA) was added to the cultures using 1000-fold stock solutions, prepared in sterile $NaHCO_3$ (0.1 M). 8-Bromoadenosine-3′,5′-cyclic monophosphate.Na (Br-cAMP, Alexis Corporation, Lausen, Switzerland) was added to the cultures using 250-fold stock solutions, prepared in H_2O. 15d-PGJ_2 (Cayman Chemical, Ann Arbor, MI, USA) was added to the cultures using 32-fold stock solutions, prepared in the culture medium. Experiments were performed according to the rules of the 3Rs. Animals were treated according to Swiss national laws and international guidelines for protection of animals in science.

Biochemical analyses

Aggregating cell cultures were washed twice with 5 ml of ice-cold phosphate-buffered saline (PBS) and homogenized in 0.5 ml of 2 mM potassium phosphate containing 1 mM EDTA (pH 6.8), using glass-glass homogenizers (Bellco, Vineland, NJ, USA). The homogenates were briefly sonicated, divided into aliquots for the different assays and stored at −80 °C.

Protein content in homogenates was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Intracellular lactate dehydrogenase activity (LDH; EC 1.1.1.27) was determined by a conventional photometric assay method (Koh and Choi, 1987).

GS (EC 6.3.1.2) activity was assayed by a modification (Patel et al., 1982) of the method of Pishak and Phillips (1979). L-[1-^{14}C] glutamic acid was used as precursor, and phosphoenolpyruvate/pyruvate kinase as the ATP-regenerating system (Patel et al., 1982).

Data analysis. The units of the raw data were: mg/flask for protein content, U/flask for total LDH activity and U/mg protein for GS activity (1 U is defined as the amount of enzyme that catalyzes the formation of 1 μmol of substrate per minute). Results are expressed as percentage of untreated control cultures. They are the mean of six replicate cultures of two independent experiments. Results were statistically evaluated for significance by using one-way analysis of variance (ANOVA) followed by the Dunnett post-test (* $P<0.05$; ** $P<0.001$).

Immunostaining

Aggregating cell cultures were washed twice in pre-warmed PBS, embedded in cryoform and frozen in isopentane cooled with liquid nitrogen. Cryosections (10 μm) were fixed for 10 min in acetone at room temperature and dried overnight at 4 °C (Honegger and Monnet-Tschudi, 2001). Sections were double-labeled with the polyclonal antibody anti-GFAP (1:125, Chemicon, Temecula, CA, USA) and with the monoclonal antibody anti-vimentin (1:20, Sigma) overnight at 4 °C. Thereafter, the sections were incubated at room temperature with a biotinylated secondary antibody (horse anti-mouse IgG, 1:100, Vector) followed by avidin DCS (1:50, Vector, Burlingame, CA, USA), and with a Cy3-conjugated secondary antibody (donkey anti-rabbit IgG, 1:250, Jackson Immuno Research Laboratories, West Grove, PA, USA). Sections were mounted with FluorSave Reagent (Calbiochem, Darmstadt, Germany), and analyzed with a Leica confocal microscope.

In situ hybridization

Rat PPAR-γ cDNA and probes used for in situ hybridization. A cDNA comprising nucleotides 139–454 of the sequence of rat PPAR-γ1 (Guardiola-Diaz et al., 1999, gene bank AF156666) was subcloned into the *Eco*RI and *Sma*I sites of the pBluescript KS^- vector (Stratagene, Heidelberg, Germany), yielding pBS-PPARγ. Digoxigenin-labeled PPARγ riboprobes were transcribed *in vitro* as described (Braissant, 2004). The antisense probe was transcribed from pBS-PPARγ linearized with *Hind*III, while the sense probe was synthesized from pBS-PPARγ linearized with *Xba*I.

In situ hybridization analysis (ISH). Aggregating cell cultures were washed twice with pre-warmed PBS, embedded in cryomatrix and frozen in isopentane cooled with liquid nitrogen. Cryosections (12 μm) were prepared and analyzed by ISH, as described (Braissant, 2004). Briefly, hybridization with antisense and sense riboprobes for rat PPAR-γ was carried out at 58 °C in 5× SSC and 50% formamide for 40 h. Then, washes (30 min in 2× SSC at room temperature, 1 h in 2× SSC at 65 °C, 1 h in 0.1× SSC at 65 °C) and alkaline-phosphatase staining (15 h at room temperature) were performed. The specificity of hybridization was ascertained by the use of a sense probe having the same length, GC content and specificity as the antisense probe. Sections were further processed for immunohistochemistry as described (Braissant, 2004). Neurons were labeled using a monoclonal anti-Neu-N antibody (MAB377, Chemicon), while astrocytes were labeled with a monoclonal anti-vimentin antibody (Sigma). Briefly, after rehydration, the ISH-stained sections were fixed 1 h in 4%