TOXICOLOGICAL SCIENCES **110(1)**, 84–94 (2009)
doi:10.1093/toxsci/kfp090
Advance Access publication May 4, 2009

Ochratoxin A–Mediated DNA and Protein Damage: Roles of Nitrosative and Oxidative Stresses

Christophe Cavin,*[,1,2] Thierry Delatour,*[,2] Maricel Marin-Kuan,* François Fenaille,† Daisy Holzhäuser,* Gabrièla Guignard,* Claudine Bezençon,* Dominique Piguet,* Véronique Parisod,* Janique Richoz-Payot,* and Benoît Schilter*

**Quality and Safety Department, Nestlé Research Center, Vers-chez-les-Blanc, CH-1000 Lausanne 26, Switzerland; and †Laboratoire d'Etude du Métabolisme des Médicaments, DSV/iBiTec-S/SPI, CEA/Saclay, 91191 Gif-sur-Yvette Cedex, France*

Received January 8, 2009; accepted April 27, 2009

Ochratoxin A (OTA) is a mycotoxin occurring in a variety of foods. OTA is nephrotoxic and nephrocarcinogenic in rodents. An OTA-mediated increase of the inducible nitric oxide synthase (iNOS) expression was observed in normal rat kidney renal cell line and in rat hepatocyte cultures, suggesting the induction of nitrosative stress. This was associated with an increased nuclear factor kappa-light chain enhancer of activated B cells activity. The potential consequences of iNOS induction were further investigated. A significant increase in the levels of protein nitrotyrosine residues was observed with OTA. In addition, OTA was found to increase the level of DNA abasic sites in both cell cultures system. This end point was used as an indirect measure of 8-nitroguanine formation. Treatment of the cells with L-N^6-(1-iminoethyl) lysine, a specific inhibitor of iNOS activity, inhibited the OTA-mediated overnitration of proteins but did not reduce the level of DNA abasic sites. It was found previously that nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) activators were able to restore the cellular defense against oxidative stress and could prevent DNA abasic sites in cell cultures. In the present study, pretreatment of the cells with activators of Nrf2 prevented OTA-mediated increase in lipid peroxidation, confirming the potential of Nrf2 activators to confer protection against OTA-mediated oxidative stress. In addition, it was found that Nrf2 activators could also prevent OTA-induced protein nitration and cytotoxicity. In conclusion, the present data further confirm oxidative stress as a key source of OTA-induced DNA damage and provide additional evidence for a role of this mechanism in OTA carcinogenicity. The exact role of nitrosative stress still remains to be established.

Key Words: **mycotoxin; ochratoxin A; inducible nitric oxide synthase; nuclear factor-erythroid 2 p45-related factor 2; oxidative and nitrosative stress; nephrotoxicity; carcinogenicity.**

Ochratoxin A (OTA), characterized as (*R*)-N-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1*H*-2-benzopyran-7-yl)carbonyl)-L-phenylalanine, is a secondary metabolite of some filamentous fungi species belonging to genera *Aspergillus* and *Penicillium* (Pitt, 1987). It is a worldwide natural contaminant of human food and animal feed which mainly occurs in grains, cereals, and coffee and remains detectable in processed foodstuffs such as fermented beverages (Clark and Snedeker, 2006; Jörgensen *et al*., 1996; Zimmerli and Dick, 1996). It is well documented that OTA is a potent nephrotoxic compound which induces renal tumors (EFSA, 2006; O'Brien and Dietrich, 2005; WHO, 2001, 2007). In human, several studies have associated OTA exposure with the Balkan endemic nephropathy (BEN) and the etiology of urinary tract tumors (UTTs) (Castegnaro *et al*., 2006; Pfohl-Leszkowicz and Manderville, 2007; Pfohl-Leszkowicz *et al*., 2007). However, international experts of the World Health Organization/Food and Agriculture Organization Joint Expert Committee on Food Additives and Contaminants (WHO, 2001), the International Life Sciences Institutes, and the European Food Safety Authority concluded that there is no convincing evidence from human epidemiological studies confirming any association between OTA exposure and the prevalence of BEN or UTT (EFSA, 2006; Fink-Gremmels, 2005; WHO, 2001, 2007). An international symposium held in Zagreb brought together scientists and clinical investigators engaged in research on BEN and UTT. Their final conclusion was that OTA is not involved in these diseases (Grollman and Jelakovic, 2007). Instead, recent studies indicated that aristocholic acid is likely to be a causal factor in the development of BEN and subsequent occurrence of transitional cell cancer (Grollman and Jelakovic, 2007; Grollman *et al*., 2007).

In absence of adequate human data, chemical risk assessment has to rely on toxicological data obtained in experimental animals. For OTA, such an approach is limited by the uncertainties regarding its mechanism of carcinogenicity. It is now widely acknowledged that OTA is genotoxic, causing DNA damage (EFSA, 2006; WHO, 2007). However, the exact molecular events involved in the production of DNA damage are still not fully elucidated. Using ^{32}P-postlabelling, some authors have raised the hypothesis that OTA is activated

[1] To whom correspondence should be addressed at Nestle Research Center, PO Box 44, Vers-chez-les Blanc, CH-1000 Lausanne 26, Switzerland. Fax: +41-21-785-85-53. E-mail: christophe.cavin@rdls.nestle.com.

[2] These authors contributed equally to this study.

into reactive metabolites which then react with DNA to form adducts (Faucet *et al*., 2004; Manderville, 2005; Manderville and Pfohl-Leszkowicz, 2008; Pfohl-Leszkowicz and Manderville, 2007). However, several laboratories have failed to detect any OTA-DNA adducts in treated animals, either through radiolabeled OTA-DNA binding or analytical chemistry using mass spectrometry (Delatour *et al*., 2008, 2009; EFSA, 2006; Mally *et al*., 2004, 2005; Pfohl-Leszkowicz *et al*., 2009; Turesky, 2005; WHO, 2001, 2007) did not consider this evidence as sufficient to demonstrate the actual formation of OTA-DNA adducts in rats *in vivo*. In this context, the OTA-mediated DNA damage may be associated to other, indirect mechanisms, such as oxidative stress and cytotoxicity (Arbillaga *et al*., 2007; Cavin *et al*., 2007; Kamp *et al*., 2005; Petrik *et al*., 2003; Schaaf *et al*., 2002).

In a previous study conducted in our laboratory, a selective induction of heme-oxygenase 1 (HO-1) was observed in the kidney of rats administered OTA (Gautier *et al*., 2001). This was interpreted as a strong biological evidence of an OTA-mediated induction of oxidative stress. In addition, since HO-1 is known to be regulated by the transcription factor nuclear factor kappa-light chain enhancer of activated B cells (NF-κB), the data on HO-1 indicated the possibility that OTA could also induce the expression of inducible nitric oxide synthase (iNOS), an enzyme responsible for the production of nitric oxide (NO). Under normal conditions, NO presents a broad range of biological activities including blood flow regulation, neurotransmission, antimicrobial defense mechanisms, and immunomodulation (Oldreive and Rice-Evans, 2001; Radi, 2004; Taylor *et al*., 1998). In excess, NO may behave as a toxic radical producing nitrosative stress. NO is known to react with the oxygen anion radical superoxide ($O_2^{\bullet -}$) to form the prooxidant peroxynitrite $ONOO^-$ (Oldreive and Rice-Evans, 2001). Under physiological conditions, peroxynitrite rapidly decomposes to generate the nitro radical ($^{\bullet}NO_2$) resulting in protein and DNA damage (nitrosative stress). Indeed, the nitro radical has been shown to react with the aromatic ring of tyrosine residues to produce the stable 3-nitrotyrosine (NY) or with the DNA guanine nucleobase to give rise to 8-nitroguanine (Yermilov *et al*., 1995). Due to the strong electron withdrawal effect of the nitro group on the N-glycosidic bond, 8-nitroguanine is rapidly depurinated from DNA, yielding apurinic sites which have been described as potentially mutagenic (Gentil *et al*., 1992).

Although some data have suggested that OTA may induce iNOS expression in various experimental systems (Ferrante *et al*., 2008; Zurich *et al*., 2005), to our knowledge, no information was available on the potential toxicological consequences (e.g. DNA damage) of such an induction. Therefore, the potential of OTA to produce nitrosative stress was investigated in cell culture *in vitro*. OTA was found to stimulate the formation of NO through a NF-κB–dependent induction of iNOS. The consequences of the increased NO production, together with oxidative stress, were then studied using markers of protein and DNA damage.

MATERIALS AND METHODS

Chemicals

OTA was obtained from Sigma (St Louis, MO) and α,β,γ,1,2,3,4,5,6-$^{13}C_9$-tyrosine from Cambridge Isotopes Laboratories (Andover, MA). 2,5,6-d_3-3- and α,β,γ,1,2,3,4,5,6-$^{13}C_9$-3-nitrotyrosine were prepared as reported elsewhere (Delatour *et al*., 2002a).

Cafestol and kahweol (C+K) were prepared from coffee oil according to the procedure of Bertholet (1987). The mixture contained C+K in the proportion of 52.5:47.5, and its purity was greater than 98%. Other chemicals were of the highest grade and readily available commercially. Coumarin (Cou) and L-N^6-(1-iminoethyl) lysine (L-NIL) were purchased from Sigma-Aldrich and Co. (St Louis, MO).

Cell Cultures

Primary isolated hepatocytes were obtained by perfusion of the liver in adult male Sprague-Dawley rats (230 ± 20 g, fed *ad libitum* with Nafag diet) with a collagenase solution as described previously (Sidhu *et al*., 1993). Cell viability, estimated by Trypan blue exclusion test, was found to range between 90 and 95%. Cells were seeded at a density of 10^6 cells/ml on 60-mm plastic tissue culture dishes in 3 ml William's E medium supplemented with 2mM L-glutamine, 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 1% ITS+ (insulin, transferrin, selenious acid, bovine serum albumin (BSA), linoleic acid) (BD Biosciences, Bedford, MA), 100 U/ml penicillin/streptomycin, 100nM dexamethasone, and fetal bovine serum 5% (Hi-clone). Hepatocytes were allowed to attach for 2 h and then washed with Earle's balanced salt solution to remove debris and unattached cells. Fresh serum-free medium containing 250nM dexamethasone was added followed by application of an overlay of Matrigel (233 μg/ml). Fresh Matrigel was added to the cultures every other day following medium change. To study the effects of OTA on specific markers, the component was added to culture media containing ITS+ 0.2% 24 h after cells seeding over periods of 24 or 48 h.

Normal rat kidney (NRK) cells were purchased from the American Tissue Culture Collection (ATCC CRL-6509). NRK cells are adherent proximal tubule epithelial cells isolated from normal kidney of Osborne Mendel rats. NRK cells were cultivated in minimal essential medium (MEM) containing 2mM L-glutamine supplemented with MEM nonessential amino acids, 100 units/ml penicillin/streptomycin and with 10% foetal bovine serum (FBS) for cell growth or 1% FBS for treatments with OTA.

Cytotoxicity Assay

Cytotoxicity of OTA in cell cultures (NRK cell line and primary hepatocytes) was determined by the amount of lactate dehydrogenase (LDH) leakage into the medium after 24 h of treatment. In protection experiments with the coffee diterpenes C+K and Cou, cells were initially treated with 2–4 μg/ml C+K for 24 h before OTA treatment (1.5–6μM) for 24 h. LDH activity was measured spectrophotometrically by analyzing the appearance of lactate at 340 nm using the Biomérieux kit containing the substrate pyruvate and the cofactor nicotinamide dinucleotide dehydrogenase. The procedure was performed using a Cobas Mira S plus robot.

Electrophoretic Mobility Shift Assay

Nuclear proteins were extracted from hepatocyte cultures by adding 300 μl of cold hypotonic buffer containing proteases inhibitors (10mM HEPES, pH 8, 1.5mM $MgCl_2$, 10mM KCl, 200mM sucrose, 0.5% Nonidet P-40, 0.5mM PMSF, 0.5mM 1,4-dithiotreitol (DTT), 10 μg/ml protein inhibitors E64, chymostatin, leupeptin, aprotinin, pepstatin). After incubation on ice for 30 min, cell pellets were centrifuged at 3000 × g for 30 min at 4°C. The supernatant formed the cytosolic fraction, and the pellets were resuspended in

50 μl of cold hypertonic buffer (20mM HEPES, pH 8, 1.5mM $MgCl_2$, 420mM NaCl, 0.2mM EDTA, 20% glycerol, 0.5mM PMSF, 0.5mM DTT, 10 μg/ml protein inhibitors E64, chymostatin, leupeptin, aprotinin, pepstatin). Cell debris were removed by centrifugation at 12,000 × g for 30 min at 4°C. The supernatant fraction containing the nuclear proteins was stored at − 80°C. Binding reactions were performed on ice for 40 min with 15 μg of nuclear protein in 20 μl of binding buffer (10mM Tris-HCl, pH 7.5, 20mM NaCl, 1mM EDTA, 5% glycerol, 1mM DTT), 1 μg poly(dI-dC) (Roche, Basel, Switzerland), 30,000 cpm ^{32}P-labeled oligonucleotides (Amersham Pharmacia Biotech, Piscataway, NJ) labeled with T4 polynucleotide kinase (Roche, Basel, Switzerland), and γ^{32}P-ATP (5000 Ci/mmol). DNA-protein complexes were separated from the unbound DNA probe on a 5% polyacrylamide gel. The gels were vacuum dried and exposed to Konica film at − 80°C for 6–16 h. The rat NF-κB 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ sequence was synthesized by MWG-Biotech AG (Ebersberg, Germany) as double-stranded oligonucleotides.

Western Blot Analysis

Cytoplasmic or whole-cell protein fractions were prepared from cells according to standard methods. Protein contents were measured using the method of Bradford, BSA serving as standard. Protein expression was analyzed by Western blot. Using the MiniCell XCellSureLock system (Invitrogen Ltd, Paisley, UK), 10–20 μg of protein/lane was loaded in the NuPAGE Bis-Tris Pre-Cast Gel 4–12% System (Invitrogen). Protein was transferred to a transfer membrane (Invitrogen) for 1 h at 30 V. Transferred membranes were probed with antibodies specific for iNOS and HO-1 enzymes (Stressgen, Ann Arbor, MI). For immunodetection of nitrated proteins, blots were incubated 90 min with mouse anti-NY antibody (1:1400 dilution) in the blocking solution threefold diluted in 0.15% Tween/PBS. Each blot was subsequently probed with horseradish peroxidase (HRP) linked to the specific secondary antibody. Proteins were detected indirectly by chemiluminescence using enhanced chemiluminescence (ECL) solution (Pierce, Rockford, IL) and exposed to Kodak films.

Western blotting for hydroxynonenal-protein adducts. Sample preparation was performed as described elsewhere (Fenaille *et al*., 2003) (dilution in lithium dodecyl sulfate buffer with 10% mercaptoethanol and 10-min incubation at 70°C), and 15 μg of proteins was loaded onto a 4–12% Bis-Tris Nupage gel (Novex, San Diego, CA). Migration was performed at a potential of 180 V for 45 min, and separated proteins were transferred electrophoretically at 25 V for 1 h and 30 min. Membranes were blocked for 1 h with 2% BSA in TBST (Tris 0.05M, NaCl 0.15M, Tween 0.1%, pH 7.5) and then washed threefold with TBST (20 min). Blots were incubated overnight at 4°C with a polyclonal rabbit anti-hydroxynonenal (HNE) antibody (1:5000 dilution; Alpha Diagnostic, San Antonio, TX) in 0.5% BSA in TBST and then for 1 h at room temperature with HRP-conjugated goat anti-rabbit antibody (1:15,000 dilution; Dako, Zug, Switzerland) in TBST. Detection was performed by chemiluminescence.

Detection of HNE-Modified Proteins by Matrix-Assisted Laser Desorption Ionization Mass Spectrometry

The analysis of protein-bound HNE was adapted from Fenaille *et al*. (2002). Briefly, The HNE-modified peptides were loaded onto the anti-HNE-bound sorbent of a cartridge after a preliminar dilution in 5 ml of PBS. After sample loading, the immunosorbents were washed twice with 5 ml of bidistilled water. The modified peptides were then eluted with 2 ml of 0.1% trifluoroacetic acid (TFA). This solution was further concentrated to ~50 μl using a SpeedVac centrifuge (Savant, Farmingdale, NY). In all, 1 μl of this mixture was mixed with 1 μl of a 10 mg/ml solution of sinapinic acid in 50% acetonitrile containing 0.1% TFA, and 1 μl of the resulting mixture was deposited on the target and air-dried at room temperature. Matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOFMS) experiments were carried out on an Autoflex instrument (Bruker Daltonik, Bremen, Germany). Desorption/ionization experiments were performed with a nitrogen laser pulse (337 nm), and the ions were analyzed under the linear mode after acceleration at 20 kV, with an extraction delay of 330 ns.

Quantification of Protein-Bound NY by Liquid Chromatography-Tandem Mass Spectrometry

The quantification of NY was performed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) on an HP series 1100 HPLC system (Hewlett-Packard, Palo-Alto, CA) coupled to an electrospray ionization tandem mass spectrometer using a Finnigan MAT TSQ7000 equipped with the API 2 interface (San Jose, CA). The isotope dilution method was employed, and 2,5,6-d_3-3-nitrotyrosine was used as internal standard. The potential artifactual nitration of tyrosine was controlled by supplementing samples with α,β,γ,1,2,3,4,5,6-$^{13}C_9$-tyrosine and monitoring α,β,γ,1,2,3,4,5,6-$^{13}C_9$-3-nitrotyrosine in the extracts. To ensure an adequate sensitivity, the detection was carried out in the multiple reaction-monitoring mode, and a good selectivity was achieved by recording two characteristic transitions per compound. Additional details regarding the method can be found in (Delatour *et al*., 2002a,b).

Analysis of Abasic Sites by Aldehyde Reactive Probe-Slot Blot

The analysis of abasic sites in DNA was adapted from Nakamura *et al*. (1998). Typically, DNA was extracted from rat hepatocytes or kidney cells with a kit purchased from Dojindo Molecular Technologies (Gaithersburg, MD). DNA (15 μg) was incubated with the aldehyde reactive probe reagent (1mM, total volume 150 μl) at 37°C for 15 min in the TE buffer (Tris-HCl 10mM, EDTA 1mM, pH 7.2). Then, DNA was precipitated with 45 μl of sodium acetate 1M and 1 ml of ethanol prior to centrifugation. DNA pellets were washed with 1.5 ml of ethanol 70% and dried *in vacuo*. Pellets were dissolved in 500 μl of TE buffer (Tris 10mM, EDTA 1mM, pH 7.2), and DNA was quantified (for 1 ml, 1 absorbance unit at 260 nm corresponds to 50 μg DNA). Samples were diluted in the TE buffer to reach a concentration of 10 μg/ml and further incubated for 10 min at 100°C. Samples were cooled down on ice, and an equal volume of ammonium acetate 2M was added. The BAS-85 NC membrane was soaked in water (5 min) prior to ammonium acetate 1M (5 min). DNA (1 μg) was applied into slots which were subsequently run dry. Slots were washed with 250 μl of ammonium acetate 1M and run dry for 10 min. The membrane was soaked with the saline sodium citrate buffer (0.75M NaCl, 75mM trisodium citrate) at 37°C for 15 min prior to irradiation with the FLX-20C device (Vilber Lourmat, Marne-la-Vallée, France) for 1 min at 0.364 J/cm^2 maximum. Then, the membrane was preincubated at room temperature during 1 h in 50 ml of Tris-NaCl buffer (20mM Tris-HCl, pH 7.5, 0.1M NaCl, 1mM EDTA, 0.1% Tween) containing 2% BSA. The membrane was washed with 30 ml of the Tris-NaCl buffer and incubated overnight in the Tris-NaCl buffer containing 0.6% BSA with streptavidin-conjugated HRP (dilution 1600×). The membrane was soaked for 5 min with the washing buffer (20mM Tris-HCl, pH 7.5, 0.26M NaCl, 1mM EDTA, 0.1% Tween) and the enzymatic activity on the membrane was visualized with the ECL reagents (Amersham, Buckinghamshire, UK).

Statistical Analyses

Statistical significance was determined by one-way ANOVA using the Newman-Keuls multiple comparison test with a 5% critical value.

RESULTS

Effects of OTA on HO-1 and iNOS Expression

The effects of OTA on protein expression of the NF-κB–dependent genes HO-1 and iNOS were investigated in cell culture *in vitro* using Western blot analysis. In both NRK kidney cells and rat primary hepatocytes, a dose-dependent increase in HO-1 and iNOS expression was observed after 24 h of OTA treatment (Fig. 1). A drastic decrease was observed in kidney cells at 9μM OTA probably due to OTA cytotoxicity. The OTA-mediated induction of iNOS (1.5–6μM) in cell culture was significant but quantitatively lower than the

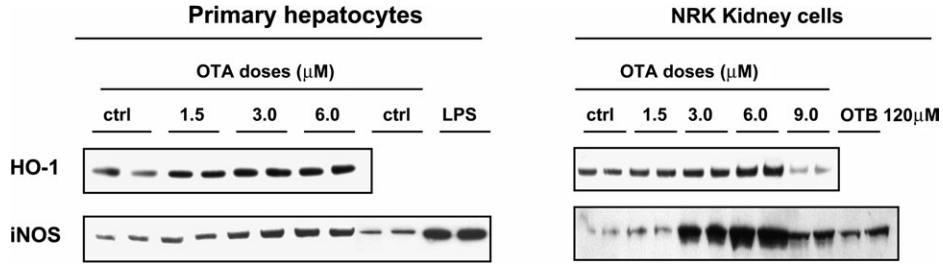

FIG. 1. Western blot analysis of the dose-dependent expression of HO-1 and iNOS in rat primary hepatocytes and NRK kidney cells treated with OTA for 24 h. LPS (20 μg/ml) was used as positive control and OTB (120 μM) as a negative control.

response produced by prototypical inducers such as lipopolysaccharide (LPS) (30 μg/ml) or interleukin-1beta (IL-1β) (data not shown). To address the specificity of OTA-mediated induction of iNOS expression, Ochratoxin B (OTB), the dechlorinated OTA analogue, was employed as comparator. OTB induced iNOS expression, but this effect was weaker and observed at much higher concentrations (120μM).

OTA-Mediated Activation of NF-κB

Both HO-1 and iNOS are known to be regulated at transcriptional level, and their gene promoters possess binding sites for multiple transcription factors, including NF-κB (Hecker *et al*., 1997). We performed gel retardation assays to study the effects of OTA on the activity of NF-κB (Fig. 2). In both cell types, an activation of NF-κB was observed, already after 6 h of OTA treatment. This activity was further enhanced with longer exposure period.

Effect of OTA on Protein Nitration

The consequence of the induction of iNOS on protein nitration was investigated by measuring the level of NY residues in proteins of cells treated with OTA. Based on the result of Figure 1, OTA concentrations up to 6μM were selected to limit cytotoxicity. For quantitative determination of NY, protein extracts from rat hepatocytes and kidney cells were analyzed by LC-MS/MS. A Western blot approach using antibodies directed against NY was applied as confirmation. In NRK kidney cells treated 24 h with OTA, the levels of protein-bound NY were significantly increased at 1.5, 3, and 6μM OTA as compared to control samples. The maximum level of protein nitration by OTA was observed at 3.0μM (76%) (Fig. 3A). At 6.0μM, protein nitration was found to be slightly lower than at 3.0μM. Similar pattern of effects was observed in hepatocyte cultures.

A Western blot approach using NY-specific antibodies was then employed to study the potential protein selectivity of OTA-mediated nitration. Four independent samples per dose were analyzed. Representative data are presented in Figure 3B, using samples from hepatocytes treated with 1.5μM OTA. In samples from treated cells, some proteins displayed increased NY immunoreactivity. Interestingly, the proteins within a mass range of 10–25 kDa appeared somehow more prone to OTA-mediated nitration. Similar data were observed in NRK kidney cells (data not shown)

Effect of OTA on DNA Damage

It has been shown that in DNA, peroxynitrite was able to react with guanine nucleobase to give rise to 8-nitroguanine (Yermilov *et al*., 1995). Due to the strong electron withdrawal effect of the nitro group on the N-glucoside bond, 8-nitroguanine is rapidly depurinated from DNA, yielding apurinic sites. The depurination process of 8-nitroguanine makes the measurement of this marker difficult to perform (no accumulation in DNA, loss during DNA isolation). The

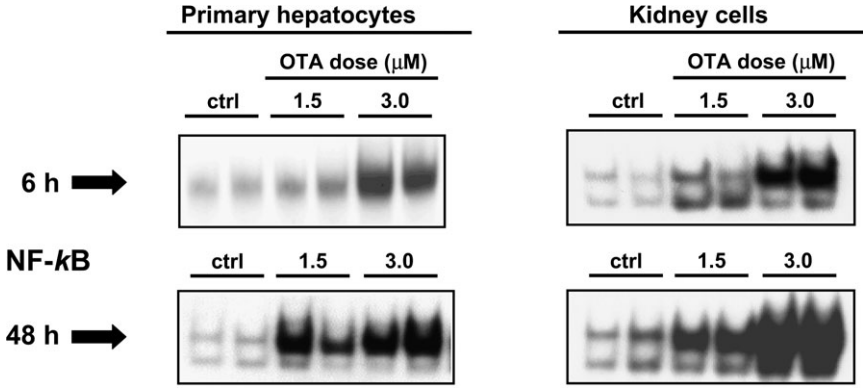

FIG. 2. Dose-dependent NF-κB activation in rat primary hepatocytes and kidney cells exposed to OTA. Formation of the NF-κB-DNA complex was analyzed by electrophoretic mobility shift assay in cells treated with 1.5 or 3μM OTA for either 6 or 48 h.

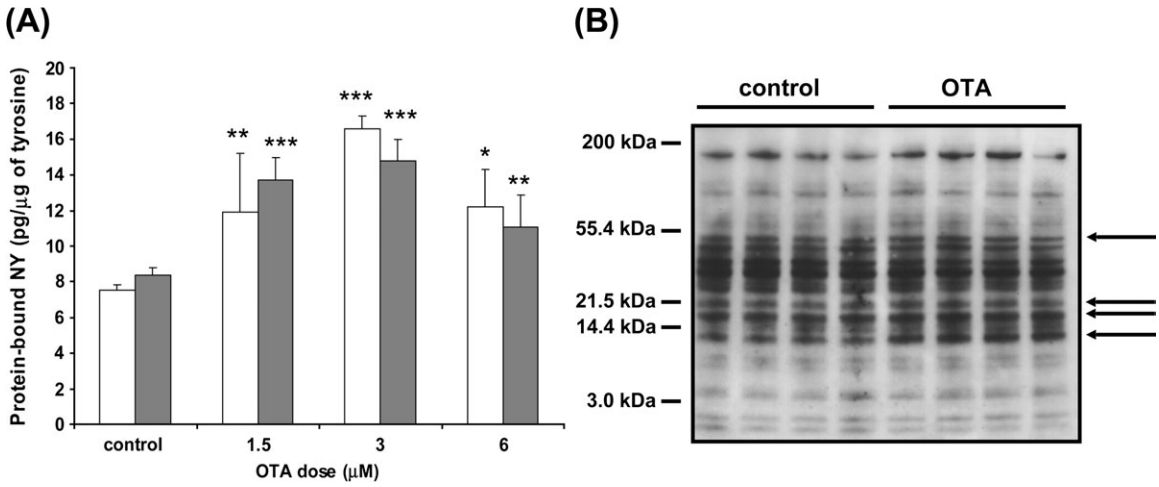

FIG. 3. (A) Level of protein-bound NY in rat primary hepatocytes (white) and NRK kidney cells (gray) treated with OTA (24 h) as measured by LC-MS/MS (mean ± SD, $n = 4$). Significantly different (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) from control values, using the Newman-Keuls multiple comparison test. (B) Western blot analysis of protein-bound NY in rat hepatocytes. Black arrows indicate proteins sensitive to OTA-mediated nitration.

determination of abasic sites in DNA by slot blot was therefore applied as an alternative for 8-nitroguanine analysis. The approach was first verified using 3-morpholinosyndnonimine (SIN-1, 5mM), which is known to decompose at physiological pH to give rise simultaneously to $^{\bullet}NO$ and anion radical superoxide ($O_2^{\bullet -}$) (Spencer *et al*., 1996). These radicals further react to form $ONOO^-$, which then binds to guanine nucleobases in DNA to form 8-nitroguanine. With this reagent, a significant increase in the level of abasic site was observed confirming the link between abasic sites and 8-nitroguanine formation (Fig. 4A). Then the effects of OTA on abasic sites were studied in NRK kidney cells and in rat primary hepatocytes. After 24 h, increases in abasic sites levels were measured in OTA-treated cultures as compared to controls. The effect was similar in size at 3 and 6μM OTA (Fig. 4B). Moreover, similar increase in abasic sites formation was observed after 48 h of OTA treatment (data not shown).

Role of iNOS-Mediated NO Formation in OTA-Induced Protein and DNA Damage

To ascertain the role of iNOS-mediated NO formation in OTA-induced protein and DNA damage, L-NIL, a specific inhibitor of iNOS activity, was used. Since no cell selectivity was observed for these end points, hepatocytes were used for these mechanistic studies.

L-NIL was able to prevent the NY formation in primary hepatocytes treated with the proinflammatory agent IL-1β (10 ng/ml) serving as a positive control for iNOS induction (Fig. 5). In this set of experiments, the OTA-mediated increase in NY was confirmed. When L-NIL (250μM) was added together with OTA

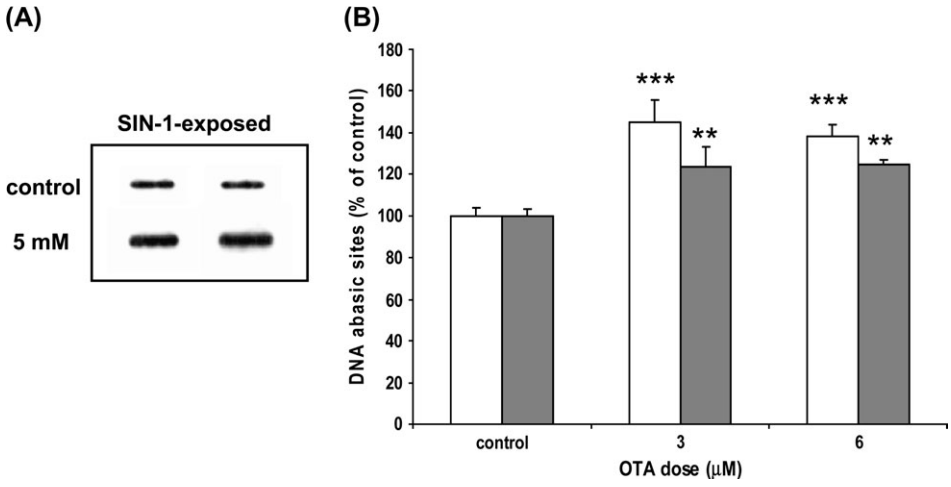

FIG. 4. (A) Slot blot analysis of DNA abasic sites from rat primary hepatocytes exposed 24 h to SIN-1 (5mM). (B) Dose-response effect of OTA on the formation of abasic sites in rat primary hepatocytes (white) and NRK kidney cells (gray) treated with OTA (3 and 6μM) for 24 h. Significantly different (**$p < 0.01$, ***$p < 0.001$) from control values, using the Newman-Keuls multiple comparison test.

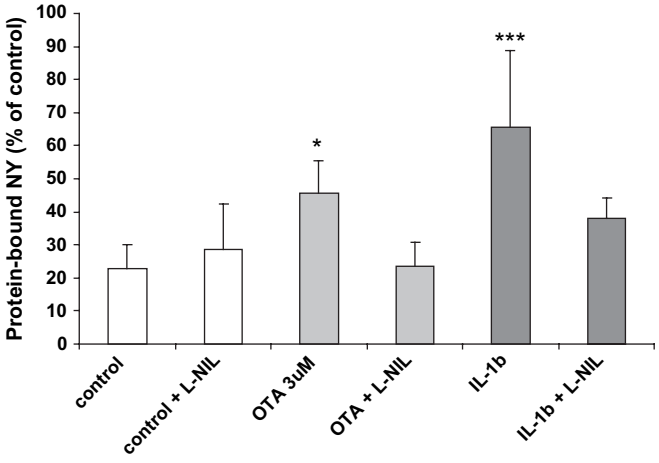

FIG. 5. Effect of L-NIL on the level of protein-bound NY in rat primary hepatocytes exposed 48 h to either OTA (1.5–3μM) or 10 ng/ml IL-1β. NY measurements were carried out by LC-MS/MS, and L-NIL was supplemented at 250μM. Values are expressed as mean ± SD, $n = 4$. Significantly different ($*p < 0.05$, $***p < 0.001$) from control values using the Newman-Keuls multiple comparison test.

(3uM), the NY level (23.6 ± 7.2 pg/μg of tyrosine) was not significantly different from the control value, indicating a prevention of protein nitration by the iNOS inhibitor (Fig. 5).

Further experiments were performed to assess the effect of L-NIL on the formation of DNA abasic sites. As shown in Figure 6, induction of DNA abasic sites by OTA (3μM) was confirmed after 24 or 48h of cell treatment. The measurement of abasic sites in DNA of rat primary hepatocytes treated simultaneously with OTA (3.0μM) and L-NIL (250μM) for 24 h did not reveal any preventive effect of L-NIL.

Nuclear Factor-Erythroid 2 p45-Related Factor 2 Activators Prevent NY Formation

OTA is known to inhibit the activity of the transcription factor nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) resulting in a reduction in cellular antioxidant gene expression (Cavin *et al*., 2007). This was correlated with a concomitant increase in oxidative DNA damage measured by an increase in abasic site formation. Pretreatment of cells with Nrf2 inducers was shown to prevent all the reported Nrf2-dependent effects of OTA. Since oxidative stress and nitrosative stress are closely related, the impact of Nrf2 activators on NO-mediated damage was investigated.

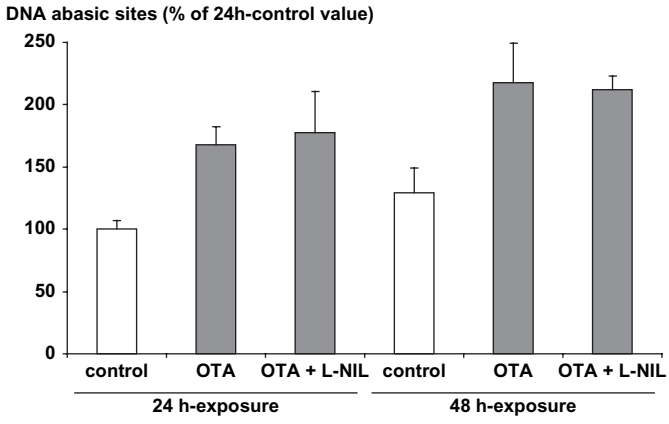

FIG. 6. Slot blot analysis of the effect of L-NIL (250μM) on the OTA-mediated increase of DNA abasic sites after 24 or 48 h treatment with OTA (3μM) in rat primary hepatocytes. Values are expressed as mean ± SD, $n = 4$.

To confirm the production of oxidative stress/damage in our experimental conditions, protein adducts of the lipid peroxidation product 4-hydroxynonenal (4-HNE) were analyzed in control and OTA-treated NRK kidney and hepatocyte cell cultures. In both systems, 48 h of OTA treatment led to a dose-dependent increase in 4-HNE as measured by Western blot (data not shown). Pretreatment of the cells for 24 h with two different Nrf2 activators (the coffee diterpenes C+K, and Cou) prevented the OTA-dependent formation of 4-HNE adducts (Fig. 7A). These results were further confirmed by MALDI-TOFMS analyses (Fig. 7B). The spectrum of the immunoaffinity-purified rat hepatocyte homogenates exhibited a major peak corresponding to a 34.7-kDa protein. The response of the protein was very intense when cells were treated with OTA, and approximately 20% (10% respectively) of the signal was remaining when C+K (4 μg/ml) or Cou (250μM), respectively, was used as a pretreatment (Fig. 7B). This protein could be correlated with an intense band observed at approximately 38 kDa in the anti-HNE Western blot. The relative response of this band was very similar to the response of the peak at 34.7 kDa in the MALDI-TOFMS spectrum toward C+K and Cou.

The impact of preventing oxidative stress on OTA-mediated protein nitration was then studied. Pretreatment of the cultures with C+K for a 24-h period prior to a cotreatment with C+K and OTA prevented the increased formation of NY observed with OTA alone (Fig. 8).

Cytotoxicological Impact of OTA-Mediated Stimulation of Oxidative and Nitrosative Stresses.

To study whether the OTA-dependent increase in oxidative and nitrosative stress was sufficient to impact the viability of the cells, the cytotoxicity of OTA in presence or absence of pretreatment with Nrf2 activators was assessed by measuring the release of LDH in the extracellular medium. In both NRK kidney and hepatocyte cell culture models, OTA treatment over 24 h triggered a dose-dependent release of LDH (Fig. 9A). However, within the dose range tested (up to 9μM), the cytotoxicity observed was relatively low as compared to overt toxicity produced by high concentrations of cadmium chloride (4μM). In both cell culture systems, pretreatment with Nrf2 activators (C+K or Cou for 24 h) followed by treatment with OTA (1.5–6μM) and either C+K (4 μg/ml) or Cou (250μM) for 24 h prevented the OTA-dependent release of LDH (Fig. 9B).

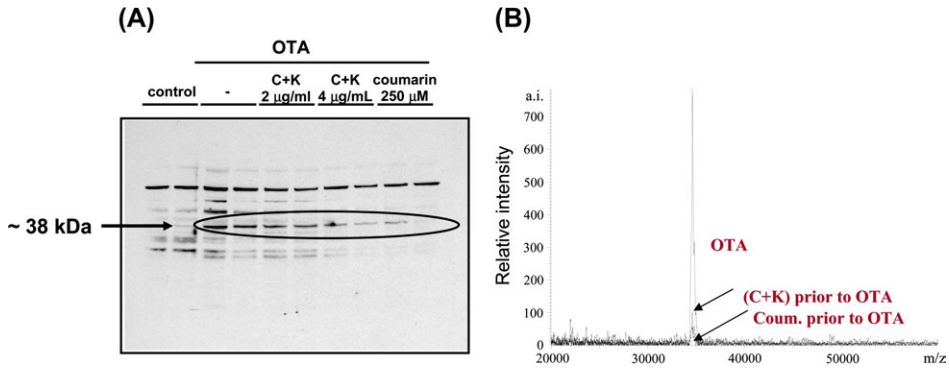

FIG. 7. (A) Western blot analysis of the inhibitory effects of C+K (2-4 µg/ml) and Cou (250µM) on the increased formation of HNE-protein adducts by OTA (3µM) in rat hepatocytes. (B) MALDI-TOFMS spectrum of the immunoaffinity (anti-HNE)-purified rat hepatocytes homogenates following 48 h treatment with OTA (3µM). Effect of C+K (4 µg/ml) and Cou (250µM) on the relative intensity of the major peak observed at 34.7 KDa.

DISCUSSION

Exposure to OTA is a worldwide phenomenon, as evidenced by its detection in sera from human individuals of many countries (EFSA, 2006; WHO, 2001). Because of lack of data, the actual health significance of low levels of OTA exposure in humans cannot be evaluated based on epidemiology (EFSA, 2006; WHO, 2007). Instead, risk assessment has to rely on studies conducted in laboratory animals where it causes various toxic effects, the most relevant being nephrotoxicity and nephrocarcinogenicity in rats (EFSA, 2006; WHO, 2007). It is widely acknowledged that the risk assessment of dietary OTA based on animal carcinogenicity data would significantly benefit from the elucidation of the mechanism of action involved. Several authors and expert groups have concluded that OTA is unlikely to act through a direct genotoxic mechanisms (EFSA, 2006; WHO, 2007) since various laboratories have failed to detect any OTA-DNA adducts in treated animals (Delatour *et al.*, 2008; Mally *et al.*, 2004, 2005; Turesky, 2005). However, using ^{32}P-postlabeling method, some authors have found low levels of DNA lesions interpreted as DNA adducts (Castegnaro *et al.*, 1998; Pfohl-Leszkowicz *et al.*, 1993, 2007). Altogether, these data strongly indicate that OTA is genotoxic. However, further work is necessary to describe precisely the molecular mechanisms involved.

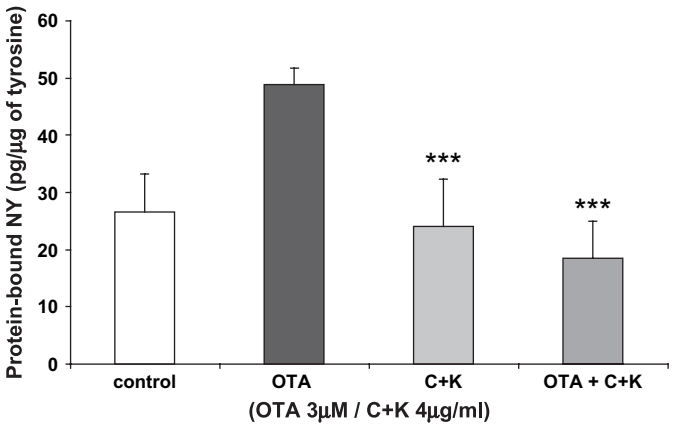

FIG. 8. Effect of C+K on the OTA-mediated increase in protein-bound NY in rat hepatocytes. LC-MS/MS analysis was performed with protein extract from cells treated with OTA (3µM) or C+K (4 µg/ml) and cells pretreated for 24 h with C+K before cotreatment with OTA and C+K for 24 h. Values are expressed as mean ± SD, $n = 4$. Significantly different (***$p < 0.001$) from OTA (3µM) using the Newman-Keuls multiple comparison test.

OTA was shown to induce iNOS expression in macrophages and brain cell cultures (Ferrante *et al.*, 2008; Zurich *et al.*, 2005), suggesting the possibility of an increased production of NO. Other authors have observed in OTA-treated cells a mutation profile similar to those induced by reactive species derived from NO (Palma *et al.*, 2007; Zhuang *et al.*, 2000). Taken together, this information raised the hypothesis that OTA may stimulate the production of NO resulting in nitrosative stress and DNA damage. In the present investigation, OTA was shown to activate the transcription factor NF-κB and induce iNOS protein expression. This response was observed in both NRK kidney cells and primary hepatocytes, indicating that in contrast to the *in vivo* situation, the renal target selectivity was not observed in cell culture *in vitro*. This suggests that as previously highlighted (see EFSA, 2006; O'Brien and Dietrich, 2005; Schwerdt *et al.*, 1996), the high renal selectivity of OTA effects observed *in vivo* is compatible with an increased concentration of this mycotoxin in kidney cells as a result of active transport. However, if intracellular concentration reaches sufficiently high level, any other cell type would be expected to respond in a similar way.

The iNOS induction was associated with an increase in intracellular nitrosative stress. Results of protein-bound NY demonstrated that OTA can generate an overnitration of tyrosine residues in rat kidney cells and hepatocytes. Interestingly, Western blot analysis suggested that overnitration did not concern all proteins but seem to be selective to some of them. The reasons and toxicological significance of such selectivity in protein nitration are not known and deserve further investigations.

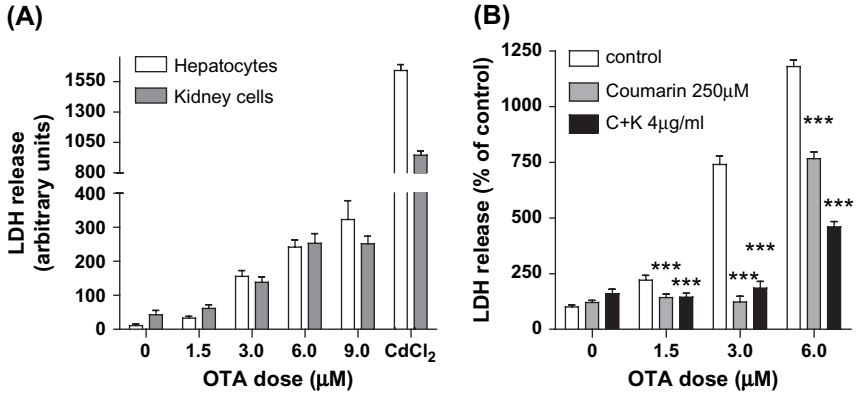

FIG. 9. (A) Dose-dependent cytotoxicity of OTA (1.5–6.0μM) in rat primary hepatocytes (white) and NRK kidney cells (gray) over 24-h exposure. Cytotoxicity was measured as leakage of LDH in the extracellular medium (± SD). (B) Dose-response effect of C+K and Cou against the cytotoxicity induced by OTA in rat hepatocytes. Cells were pretreated for 24 h with C+K (4 μg/ml) or Cou (250μM) followed by treatment with OTA (0–6μM) for 24 h. Results are expressed as the percentage of the mean value derived from control cultures (± SD). Significantly different (***$p < 0.001$) from control values using the Newman-Keuls multiple comparison test.

The experiments performed with a specific inhibitor of iNOS activity (L-NIL) indicated that the increased iNOS expression was likely to play a key role in the process of tyrosine nitration in the presence of OTA. However, oxidative stress is involved in the reaction with NO resulting in the production of the peroxinitrite anion at an approximate rate constant of 1.9×10^{10}/M/s (Kissner *et al*., 1997), which is about threefold faster than the rate at which superoxide dismutase can scavenge radical anion superoxide (Fielden *et al*., 1974). Recent data showed that treatment with OTA reduced the superoxide dismutase (SOD) activity in LLC-PK1 (Proximale tubule porcine kidney cell line) kidney tubular cells (Boesch-Saadatmandi *et al*., 2008a). In addition, NO is known to react with glutathione, forming stable nitrosothiols (Chiueh and Rauhala, 1999; Heikal, *et al*., 2009). OTA was shown by different laboratories to deplete cellular glutathione (Cavin *et al*., 2007; Schaaf *et al*., 2002). Altogether these data suggest that the increase in protein nitration induced by OTA treatment may likely result from the concomitant actions of three different mechanisms, an increase production of NO due to the increase expression of iNOS, a reduced detoxification of NO due to a depletion of glutathione, and an increase in superoxide anions due to an inhibition of SOD activity.

With regard to DNA damage, the adverse consequences of peroxinitrite formation by OTA and fast depurination of 8-nitroguanine were supported by our initial data showing a significant increase in the formation of DNA abasic sites in cell cultures. However, unlike previously observed with protein nitration, L-NIL could not prevent the OTA-mediated increase in DNA abasic sites formation. These DNA damage are the consequence of both spontaneous depurination (i.e., 8-nitroguanine) and base excision repair subsequent to excessive structural modifications of nucleobases (such as resulting from oxidative stress). In our case, the measurement of abasic sites was conducted to indirectly assess the 8-nitroguanine formed in DNA. However, the contribution of the depurination of 8-nitroguanine in the formation of abasic sites as compared to other oxidative stress–mediated modifications is unknown. In the case of OTA, the data indicate that oxidative damage \contributes clearly more than nitrosative stress to the formation of abasic sites. To definitely prove, OTA-mediated DNA nitration would require the direct measurement of 8-nitroguanine. Since 8-nitroguanine is rapidly depurinated from DNA, such analysis is still very challenging.

It is widely acknowledged that OTA produces oxidative stress resulting in increased DNA damage (Arbillaga *et al*., 2007; Cavin *et al*., 2007; Kamp *et al*., 2005; Mally *et al*., 2005) and lipid peroxidation (Baudrimont *et al*., 1994; Omar *et al*., 1990; Rahimtula *et al*., 1988), both *in vivo* and *in vitro*. The mechanisms involved have not been elucidated yet. Potential oxidoreduction mechanisms directly involving OTA and Fe^{3+} have been proposed (Omar *et al*., 1990). Other authors have reported the generation of quinone/hydroquinone (OTQ/OTHQ) couple from OTA oxidation, which may undergo redox cycling generating reactive oxygen species and, thus, oxidative lesions (Faucet-Marquis *et al*., 2006; Manderville and Pfohl-Leszkowicz, 2008). The actual relevance of these reactions for the physiological *in vivo* situation has still to be confirmed (Mally and Dekant, 2009). In addition, a depletion of cellular antioxidant defenses through an inhibition of Nrf2 activity was described as a likely mechanism of OTA-mediated increase in oxidative stress (Cavin *et al*., 2007; Marin-Kuan *et al*., 2006, 2008). Nrf2 is a transcription factor regulating the expression of many genes, including genes encoding for detoxification, cytoprotective, and antioxidant enzymes (Kensler *et al*., 2007). Recently, an OTA-mediated down-regulation of genes involved in oxidative stress response was further confirmed in rat and porcine kidney cell lines (Arbillaga *et al*., 2007; Boesch-Saadatmandi *et al*., 2008a,b). The application of Nrf2 activators was also shown to prevent the

depletion of Nrf2 gene expression caused by OTA, resulting in a protection against the formation of DNA abasic sites (Cavin *et al*., 2007).

In the present work, we confirmed that Nrf2 activators could counteract oxidative stress by showing a protection against OTA-induced lipid peroxidation. Moreover, it was also shown that Nrf2 activators could prevent OTA-mediated protein nitration, suggesting a protection against nitrosative stress. This protection may result from the restoration of efficient intracellular antioxidant defenses by Nrf2 activators, especially the prevention of glutathione depletion. However, preliminary data in our laboratory have indicated that the Nrf2 activator C+K could also inhibit the OTA-dependent increase in iNOS expression, suggesting that more complex mechanisms involving possible cross talks between Nrf2 and NF-κB may also play a role. This hypothesis is supported by data indicating that C+K could inhibit LPS-dependent NF-κB activation by preventing IκB degradation (Kim *et al*., 2004).

It is interesting to notice that the biological effects observed with OTA, including Nrf2-mediated intracellular defenses depletion, increased oxidative stress, and stimulation of nitrosative stress were observed in a very narrow range of OTA concentrations (1.5–3μM), which are slightly cytotoxic as determined by LDH release. All effects were prevented by Nrf2 activators, suggesting possible interconnections. This supports the hypothesis that active doses of OTA produce toxicity, together with other effects such as, for example, DNA damage (Cavin *et al*., 2007) and mobilization of protein kinase activation cascade (Marin-Kuan *et al*., 2007). Toxicity may be of key importance with respect to OTA carcinogenicity. It is well recognized that toxicity induce tissue damage triggering cell regeneration and proliferation, which may then lead to cell transformation and tumor development. The hypothesis of a role of toxicity in OTA carcinogenicity was supported by *in vivo* data using gene-profiling techniques (Marin-Kuan *et al*., 2006). In addition, OTA-induced tumors were only observed at nephrotoxic doses and frank nephrotoxicity was occurring before the onset of carcinogenicity (EFSA, 2006; WHO, 2001). Taken together, these data further support that as suggested earlier (Rached *et al*., 2007; Stemmer *et al*., 2007), tissue regeneration and cell proliferation are likely to play a role in OTA carcinogenicity.

For most of the effects described above, dose-responses were not simple and a plateau was observed at 6μM OTA. There is currently no data explaining the plateau. One plausible hypothesis is that at 6μM, macromolecular damages exceed a threshold triggering repair mechanisms. Such a threshold was documented for DNA repair in OTA-treated rat hepatocytes (Dörrenhaus and Föllmann, 1997). In addition, it is known that exceeding a defined level of cellular oxidative damage may trigger apoptosis to eliminate abnormal cells. Apoptosis has been shown to be induced by OTA both *in vitro* and *in vivo* (O'Brien and Dietrich, 2005). Therefore, the induction of apoptosis resulting in the elimination of the cells which have exceeded the threshold of macromolecular damage may be an alternative explanation for the plateau observed with OTA-mediated DNA and protein damages.

In summary, we showed that OTA generates nitrosative stress in kidney and liver cells through an increased iNOS expression. This effect is likely to be mediated by an activation of NF-κB. The iNOS induction was correlated with an increase in protein nitration and DNA abasic sites formation. Although protein nitration was clearly shown to be dependent upon iNOS induction, this could not be demonstrated for DNA abasic site formation. This suggests a high contribution of oxidative stress as compared to nitrosative stress for the formation of abasic sites and DNA damage. However, the analytical methodology used did not allow to completely rule out the possibility of OTA-induced formation of 8-nitroguanine. In conclusion, the present data indicate reactive oxygen species but not NO as a key source of OTA-induced DNA damage and provide additional evidence for a role of oxidative stress and Nrf2-mediated antioxidant defense depletion as mechanisms of OTA toxicity and carcinogenicity. In contrast, the actual role of nitrosative stress still remains to be established using improved analytical tools.

ACKNOWLEDGMENTS

The authors would like to address special thanks to Sylviane Junod and are very grateful to her for excellent technical assistance.

REFERENCES

Arbillaga, L., Azqueta, A., van Delft, J. H., and Lopez de Cerain, A. (2007). In vitro gene expression data supporting a DNA non-reactive genotoxic mechanism for ochratoxin A. *Toxicol. Appl. Pharmacol.* **220,** 216–224.

Baudrimont, I., Betbeder, A. M., Gharbi, A., Pfohl-Leszkowicz, A., Dirheimer, G., and Creppy, E. E. (1994). Effect of superoxide dismutase and catalase on the nephrotoxicity induced by subchronical administration of ochratoxin A in rats. *Toxicology* **89,** 101–111.

Bertholet, R. (1987). Nestec S. A. Preparation of a mixture of cafestol and kahweol, United States Patent 4748258, patent number 4692534.

Boesch-Saadatmandi, C., Loboda, A., Jozkowicz, A., Huebbe, P., Blank, R., Wolffram, S., Dulak, J., and Rimbach, G. (2008a). Effect of ochratoxin A on redox-regulated transcription factors, antioxidant enzymes and glutathione-S-transferase in cultured kidney tubulus cells. *Food Chem. Toxicol.* **46,** 2665–2671.

Boesch-Saadatmandi, C., Wagner, A. E., Graeser, A. C., Hundhausen, C., Wolffram, S., and Rimbach, G. (2008b). Ochratoxin A impairs Nrf2-dependent gene expression in porcine kidney tubulus cells. *J. Anim. Physiol. Anim. Nutr.* (*Berl*) (Abstract), (June 2008) 10.1111/j.1439-0396.2008.00838.

Castegnaro, M., Canadas, D., Vrabcheva, T., Petkova-Bocharova, T., Chernozemsky, I. N., and Pfohl-Leszkowicz, A. (2006). Balkan endemic nephropathy: Role of ochratoxin A through biomarkers. *Mol. Nutr. Food Res.* **50,** 519–529.

Castegnaro, M., Mohr, U., Pfohl-Leszkowicz, A., Esteve, J., Steinmann, J., Tillmann, T., Michelon, J., and Bartsch, H. (1998). Sex- and strain-specific induction of renal tumors by ochratoxin A in rats correlates with DNA adduction. *Int. J. Cancer* **77,** 70–75.

Cavin, C., Delatour, T., Marin-Kuan, M., Holzhauser, D., Higgins, L., Bezencon, C., Guignard, G., Junod, S., Richoz-Payot, J., Gremaud, E., *et al.* (2007). Reduction in antioxidant defenses may contribute to ochratoxin A toxicity and carcinogenicity. *Toxicol. Sci.* **96,** 30–39.

Chiueh, C. C., and Rauhala, P. (1999). The redox pathway of S-nitrosoglutathione, glutathione and nitric oxide in cell to neuron communications. *Free Radic. Res.* **31**(6), 641–650.

Clark, H. A., and Snedeker, S. M. (2006). Ochratoxin a: Its cancer risk and potential for exposure. *J. Toxicol. Environ. Health B Crit. Rev.* **9,** 265–296.

Delatour, T., Guy, P. A., Stadler, R. H., and Turesky, R. J. (2002a). 3-Nitrotyrosine butyl ester: A novel derivative to assess tyrosine nitration in rat plasma by liquid chromatography-tandem mass spectrometry detection. *Anal. Biochem.* **302,** 10–18.

Delatour, T., Mally, A., Dekant, W., Marin-Kuan, M., Schilter, B., and Cavin, C. (2009). Answer to Prof. Pfohl-Leszkowicz's letter to the editor. *Mol. Nutr. Food Res.* **53,** 1–2.

Delatour, T., Mally, A., Richoz, J., Ozden, S., Dekant, W., Ihmels, H., Otto, D., Gasparutto, D., Marin-Kuan, M., Schilter, B., *et al.* (2008). Absence of 2′-deoxyguanosine-carbon 8-bound ochratoxin A adduct in rat kidney DNA monitored by isotope dilution LC-MS/MS. *Mol. Nutr. Food Res.* **52,** 472–482.

Delatour, T., Richoz, J., Vuichoud, J., and Stadler, R. H. (2002b). Artifactual nitration controlled measurement of protein-bound 3-nitro-L-tyrosine in biological fluids and tissues by isotope dilution liquid chromatography electrospray ionization tandem mass spectrometry. *Chem. Res. Toxicol.* **15,** 1209–1217.

Dörrenhaus, A., and Föllmann, W. (1997). Effects of ochratoxin A on DNA repair in cultures of rat hepatocytes and porcine urinary bladder epithelial cells. *Arch. Toxicol.* **71,** 709–713.

European Food Safety Authority (EFSA, 2006). (2006). Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to ochratoxin A in food. *EFSA J.* **365,** 1– 56; Q-2005-154.

Faucet, V., Pfohl-Leskowicz, A., Dai, J., Castegnaro, M., and Manderville, M. A. (2004). Evidence for covalent DNA adduction by ochratoxin A following chronic exposure to rat and subacute exposure to pig. *Chem. Res. Toxicol.* **17,** 1289–1296.

Faucet-Marquis, V., Pont, F., Stomer, C., Rizk, T., Castegnaro, M., and Pfohl-Leskowicz, A. (2006). Evidence of a new dechlorinated ochratoxin A derivative formed in opossum kidney cell cultures after pretreatment by modulators of glutathione pathways: Correlation with DNA-adduct formation. *Mol. Nutr. Food Res.* **50,** 530–542.

Fenaille, F., Morgan, F., Parisod, V., Tabet, J. C., and Guy, P. A. (2003). Solid-state glycation of beta-lactoglobulin monitored by electrospray ionisation mass spectrometry and gel electrophoresis techniques. *Rapid Commun. Mass Spectrom.* **17,** 1483–1492.

Fenaille, F., Tabet, J. C., and Guy, P. A. (2002). Immunoaffinity purification and characterization of 4-hydroxy-2-nonenal- and malondialdehyde-modified peptides by electrospray ionization tandem mass spectrometry. *Anal. Chem.* **74,** 6298–6304.

Ferrante, M. C., Raso, G. M., Bilancione, M., Esposito, E., Iacono, A., and Meli, R. (2008). Differential modification of inflammatory enzymes in J774A.1 macrophages by ochratoxin A alone or in combination with lipopolysaccharide. *Toxicol. Lett.* **181,** 40–46.

Fielden, E. M., Roberts, P. B., Bray, R. C., Lowe, D. J., Mautner, G. N., Rotilio, G., and Calabrese, L. (1974). Mechanism of action of superoxide dismutase from pulse radiolysis and electron paramagnetic resonance. Evidence that only half the active sites function in catalysis. *Biochem. J.* **139,** 49–60.

Fink-Gremmels, J. (2005). Conclusions from the workshops on ochratoxin A in food: Recent developments and significance, organized by ILSI Europe in Baden (Austria), 29 June–1 July 2005. *Food Addit. Contam.* **22**(Suppl. 1), 1–5.

Gautier, J. C., Holzhaeuser, D., Markovic, J., Gremaud, E., Schilter, B., and Turesky, R. J. (2001). Oxidative damage and stress response from ochratoxin a exposure in rats. *Free Radic. Biol. Med.* **30,** 1089–1098.

Gentil, A., Cabral-Neto, J. B., Mariage-Samson, R., Margot, A., Imbach, J. L., Rayner, B., and Sarasin, A. (1992). Mutagenicity of a unique apurinic/apyrimidinic site in mammalian cells. *J. Mol. Biol.* **227,** 981–984.

Grollman, A. P., and Jelakovic, B. (2007). Role of environmental toxins in endemic (Balkan) nephropathy. *J. Am. Soc. Nephrol.* **18,** 2817–2823.

Grollman, A. P., Shibutani, S., Moriya, M., Miller, F., Wu, L., Moll, U., Suzuki, N., Fernandes, A., Rosenquist, T., Medverec, Z., *et al.* (2007). Aristocholic acid and the etiology of endemic (Balkan) nephropathy. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 12129–12134.

Hecker, M., Preiss, C., and Schini-Kerth, V. B. (1997). Induction by staurostorine of nitric oxide synthase expression in vascular smooth muscle cells. Role of NF-kappa B, CREB and C/EBP. *Br. J. Pharmacol.* **120,** 1067–1074.

Heikal, L., Martin, G. P., and Dailey, L. A. (2009). Characterisation of the decomposition behaviour of S-nitrosoglutathione and a new class of analogues: S-Nitrosophytochelatins. *Nitric Oxide* **20**(3), 157–165.

Jörgensen, K., Rasmussen, G., and Thorup, I. (1996). Ochratoxin A in Danish cereals 1986–1992 and daily intake by the Danish population. *Food Addit. Contam.* **13**(1), 95–104.

Kamp, H. G., Eisenbrand, G., Schlatter, J., Wurth, K., and Janzowski, C. (2005). Ochratoxin A: Induction of (oxidative) DNA damage, cytotoxicity and apoptosis in mammalian cell lines and primary cells. *Toxicology* **206,** 413–425.

Kensler, T. W., Wakabayashi, N., and Biswal, S. (2007). Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu. Rev. Pharmacol. Toxicol.* **47,** 89–116.

Kim, J. Y., Jung, K. S., and Jeong, H. G. (2004). Suppressive effects of the kahweol and cafestol on cyclooxygenase-2 expression in macrophages. *FEBS Lett.* **569,** 321–326.

Kissner, R., Nauser, T., Bugnon, P., Lye, P. G., and Koppenol, W. H. (1997). Formation and properties of peroxynitrite as studied by laser flash photolysis, high-pressure stopped-flow technique, and pulse radiolysis. *Chem. Res. Toxicol.* **10,** 1285–1292.

Mally, A., and Dekant, W. (2009). Mycotoxins and the kidney: Modes of action for renal tumor formation by ochratoxin A in rodents. *Mol. Nutr. Food. Res.* **53**(4), 467–478.

Mally, A., Pepe, G., Ravoori, S., Fiore, M., Gupta, R. C., Dekant, W., and Mosesso, P. (2005). Ochratoxin A causes DNA damage and cytogenetic effects but no DNA adducts in rats. *Chem. Res. Toxicol.* **18,** 1253–1261.

Mally, A., Zepnik, H., Wanek, P., Eder, E., Dingley, K., Ihmels, H., Volkel, W., and Dekant, W. (2004). Ochratoxin A: Lack of formation of covalent DNA adducts. *Chem. Res. Toxicol.* **17,** 234–242.

Manderville, R. A. (2005). A case for the genotoxicity of ochratoxin a by bioactivation and covalent DNA adduction. *Chem. Res. Toxicol.* **18,** 1091–1097.

Manderville, R. A., and Pfohl-Leszkowicz, A. (2008). Bioactivation and DNA adduction as a rationale for ochratoxin A carcinogenesis. *World Mycotoxin J.* **1,** 357–367.

Marin-Kuan, M., Cavin, C., Delatour, T., and Schilter, B. (2008). Ochratoxin A carcinogenicity involves a complex network of epigenetic mechanisms. *Toxicon* **52**(2), 195–202.

Marin-Kuan, M., Nestler, S., Verguet, C., Bezencon, C., Piguet, D., Delatour, T., Mantle, P., Cavin, C., and Schilter, B. (2007). MAPK-ERK activation in kidney of male rats chronically fed ochratoxin A at a dose causing a significant incidence of renal carcinoma. *Toxicol. Appl. Pharmacol.* **224,** 174–181.

Marin-Kuan, M., Nestler, S., Verguet, C., Bezencon, C., Piguet, D., Mansourian, R., Holzwarth, J., Grigorov, M., Delatour, T., Mantle, P., *et al.* (2006). A toxicogenomics approach to identify new plausible epigenetic mechanisms of ochratoxin a carcinogenicity in rat. *Toxicol. Sci.* **89,** 120–134.

Nakamura, J., Walker, V. E., Upton, P. B., Chiang, S.-Y., Kow, Y. W., and Swenberg, J. A. (1998). Highly sensitive apurinic/apyrimidinic site assay can detect spontaneous and chemically induced depurination under physiological conditions. *Cancer Res.* **58,** 222–225.

O'Brien, E., and Dietrich, D. R. (2005). Ochratoxin A: The continuing enigma. *Crit. Rev. Toxicol.* **35,** 33–60.

Oldreive, C., and Rice-Evans, C. (2001). The mechanisms for nitration and nitrotyrosine formation in vitro and in vivo: Impact of diet. *Free Radic. Res.* **35,** 215–231.

Omar, R. F., Hasinoff, B. B., Mejilla, F., and Rahimtula, A. D. (1990). Mechanism of ochratoxin A stimulated lipid peroxidation. *Biochem. Pharmacol.* **40,** 1183–1191.

Palma, N., Cinelli, S., Sapora, O., Wilson, S. H., and Dogliotti, E. (2007). Ochratoxin A-induced mutagenesis in mammalian cells is consistent with the production of oxidative stress. *Chem. Res. Toxicol.* **20,** 1031–1037.

Petrik, J., Zanic-Grubisic, T., Barisic, K., Pepeljnjak, S., Radic, B., Ferencic, Z., and Cepelak, I. (2003). Apoptosis and oxidative stress induced by ochratoxin A in rat kidney. *Arch. Toxicol.* **77,** 685–693.

Pfohl-Leszkowicz, A., Gabryelski, W., and Manderville, R. A. (2009). Formation of 2′-deoxyguanosine-carbon 8-bound ochratoxin A adduct in rat kidney DNA. *Mol. Nutr. Food Res.* **53,** 154–155.

Pfohl-Leszkowicz, A., Grosse, Y., Kane, A., Creppy, E. E., and Dirheimer, G. (1993). Differential DNA adduct formation and disappearance in three mouse tissues after treatment with the mycotoxin ochratoxin A. *Mutat. Res.* **289,** 265–273.

Pfohl-Leszkowicz, A., and Manderville, R. A. (2007). Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. *Mol. Nutr. Food Res.* **51,** 61–99.

Pfohl-Leszkowicz, A., Tozlovanu, M., Manderville, R., Peraica, M., Castegnaro, M., and Stefanovic, V. (2007). New molecular and field evidences for the implication of mycotoxins but not aristocholic acid in human nephropathy and urinary tract tumor. *Mol. Nutr. Food Res.* **51,** 1131–1146.

Pitt, J. I. (1987). Penicillium viridicatum, Penicillium verrucosum, and production of ochratoxin A. *Appl. Environ. Microbiol.* **53,** 266–269.

Rached, E., Hard, G. C., Blumbach, K., Weber, K., Draheim, R., Lutz, W. K., Ozden, S., Steger, U., Dekant, W., and Mally, A. (2007). Ochratoxin A: 13-week oral toxicity and cell proliferation in male F344/n rats. *Toxicol. Sci.* **97,** 288–298.

Radi, R. (2004). Nitric oxide, oxidants, and protein tyrosine nitration. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 4003–4008.

Rahimtula, A. D., Bereziat, J. C., Bussacchini-Griot, V., and Bartsch, H. (1988). Lipid peroxidation as a possible cause of ochratoxin A toxicity. *Biochem. Pharmacol.* **37,** 4469–4477.

Schaaf, G. J., Nijmeijer, S. M., Maas, R. F., Roestenberg, P., de Groene, E. M., and Fink-Gremmels, J. (2002). The role of oxidative stress in the ochratoxin A-mediated toxicity in proximal tubular cells. *Biochim. Biophys. Acta* **1588,** 149–158.

Schwerdt, G., Bauer, K., Gekle, M., and Silbernagl, S. (1996). Accumulation of ochratoxin A in rat kidney in vivo and in cultivated renal epithelial cells in vitro. *Toxicology* **114,** 177–185.

Sidhu, J. S., Farin, F. M., and Omiecinski, C. J. (1993). Influence of extracellular matrix overlay on phenobarbital-mediated induction of CYP2B1, 2B2, and 3A1 genes in primary adult rat hepatocyte culture. *Arch. Biochem. Biophys.* **301,** 103–113.

Spencer, J. P., Wong, J., Jenner, A., Aruoma, O. I., Cross, C. E., and Halliwell, B. (1996). Base modification and strand breakage in isolated calf thymus DNA and in DNA from human skin epidermal keratinocytes exposed to peroxynitrite or 3-morpholinosydnonimine. *Chem. Res. Toxicol.* **9,** 1152–1158.

Stemmer, K., Ellinger-Ziegelbauer, H., Ahr, H. J., and Dietrich, D. R. (2007). Carcinogen-specific gene expression profiles in short-term treated Eker and wild-type rats indicative of pathways involved in renal tumorigenesis. *Cancer Res.* **67,** 4052–4068.

Taylor, B. S., Alarcon, L. H., and Billiar, T. R. (1998). Inducible nitric oxide synthase in the liver: Regulation and function. *Biochemistry (Mosc.)* **63,** 766–781.

Turesky, R. J. (2005). Perspective: Ochratoxin a is not a genotoxic carcinogen. *Chem. Res. Toxicol.* **18,** 1082–1090.

World Health Organization (WHO). (2001). *Ochratoxin A, Safety Evaluation of Certain Mycotoxins in Food WHO Food Additives, Series 47*, Vol. 74. pp. 281–415. WHO, Genèva, Switzerland.

World Health Organization (WHO). (2007). Joint FAO/WHO Expert Committee on Food Additives (JECFA). Evaluation of certain food additives and contaminants, Ochratoxin A. *WHO Tech. Rep. Ser.* **947,** 169–180.

Yermilov, V., Rubio, J., and Ohshima, H. (1995). Formation of 8-nitroguanine in DNA treated with peroxynitrite in vitro and its rapid removal from DNA by depurination. *FEBS Lett.* **376,** 207–210.

Zhuang, J. C., Wright, T. L., deRojas-Walker, T., Tannenbaum, S. R., and Wogan, G. N. (2000). Nitric oxide-induced mutations in the HPRT gene of human lymphoblastoid TK6 cells and in Salmonella typhimurium. *Environ. Mol. Mutagen.* **35,** 39–47.

Zimmerli, B., and Dick, R. (1996). Ochratoxin A in table wine and grape-juice: Occurrence and risk assessment. *Food Addit. Contam.* **13**(6), 655–668.

Zurich, M. G., Lengacher, S., Braissant, O., Monnet-Tschudi, F., Pellerin, L., and Honegger, P. (2005). Unusual astrocyte reactivity caused by the food mycotoxin ochratoxin A in aggregating rat brain cell cultures. *Neuroscience* **134,** 771–782.