TOXICOLOGICAL SCIENCES **89(1)**, 120–134 (2006)
doi:10.1093/toxsci/kfj017
Advance Access publication October 26, 2005

A Toxicogenomics Approach to Identify New Plausible Epigenetic Mechanisms of Ochratoxin A Carcinogenicity in Rat

M. Marin-Kuan,*,[1] S. Nestler,†,‡ C. Verguet,* C. Bezençon,* D. Piguet,* R. Mansourian,* J. Holzwarth,* M. Grigorov,* T. Delatour,* P. Mantle,‡ C. Cavin,* and B. Schilter*

*Nestlé Research Center, PO Box 44, Vers-chez-les-Blanc, CH-1000 Lausanne 26, Switzerland; †Nephro-Urology Unit, Institute of Child Health, University College London, 30 Guilford Street, London WC1E 1EH, UK; and ‡Department of Environmental Science and Technology, Imperial College London, London SW7 2AZ, UK

Received August 24, 2005; accepted October 7, 2005

Ochratoxin A (OTA) is a mycotoxin occurring naturally in a wide range of food commodities. In animals, it has been shown to cause a variety of adverse effects, nephrocarcinogenicity being the most prominent. Because of its high toxic potency and the continuous exposure of the human population, OTA has raised public health concerns. There is significant debate on how to use the rat carcinogenicity data to assess the potential risk to humans. In this context, the question of the mechanism of action of OTA appears of key importance and was studied through the application of a toxicogenomics approach. Male Fischer rats were fed OTA for up to 2 years. Renal tumors were discovered during the last 6 months of the study. The total tumor incidence reached 25% at the end of the study. Gene expression profile was analyzed in groups of animals taken in intervals from 7 days to 12 months. Tissue-specific responses were observed in kidney versus liver. For selected genes, microarray data were confirmed at both mRNA and protein levels. In kidney, several genes known as markers of kidney injury and cell regeneration were significantly modulated by OTA. The expression of genes known to be involved in DNA synthesis and repair, or genes induced as a result of DNA damage, was only marginally modulated. Very little or no effect was found amongst genes associated with apoptosis. Alterations of gene expression indicating effects on calcium homeostasis and a disruption of pathways regulated by the transcription factors hepatocyte nuclear factor 4 alpha (HNF4α) and nuclear factor-erythroid 2-related factor 2 (Nrf2) were observed in the kidney but not in the liver. Previous data have suggested that a reduction in HNF4α may be associated with nephrocarcinogenicity. Many Nrf2-regulated genes are involved in chemical detoxication and antioxidant defense. The depletion of these genes is likely to impair the defense potential of the cells, resulting in chronic elevation of oxidative stress in the kidney. The inhibition of defense mechanism appears as a highly plausible new mechanism, which could contribute to OTA carcinogenicity.

Key Words: **ochratoxin A; nephrocarcinogenicity.**

Ochratoxin A (OTA) is a naturally occurring mycotoxin produced by several species of *Aspergillus* and *Penicillium.* As a consequence of its widespread occurrence in a variety of food commodities such as cereals, green coffee, cocoa, dried fruits, and meat products (WHO, 2001), the human population is continuously exposed to OTA, although usually only in trace amounts.

OTA causes a range of adverse effects in monogastric animals, with renal toxicity being the most prominent (NTP, 1989; O'Brien and Dietrich, 2005; WHO, 2001). Other OTA-mediated toxic effects observed *in vitro* or *in vivo* include inhibition of protein synthesis (Dirheimer and Creppy, 1991), impairment of energetic metabolism (Gekle *et al*., 2005; WHO, 2001), induction of oxidative stress (Gautier *et al*., 2001a; Kamp *et al*., 2005; Petrik *et al*., 2003; WHO, 2001), and apoptosis (Gekle *et al*., 2005; O'Brien and Dietrich, 2005; Petrik *et al*., 2003; Sauvant *et al*., 2005).

In a 2-year carcinogenicity study, OTA administration by oral gavage caused dose-dependent incidence of renal tumors in male rats, while females were much less susceptible (NTP, 1989). Similar effects were observed in mice, although at much higher doses (Bendele *et al*., 1985). OTA has been suspected to be an etiological agent in the human disease Balkan endemic nephropathy (BEN) and in its association with an increased incidence of urinary tract tumors (Petkova-Bocharova *et al*., 1988; Plestina *et al*., 1990). However, causal involvement of the toxin in these two diseases lacks robust etiological evidence, and the actual health significance of current natural OTA exposure in the human is still unclear.

At present, there is still insufficient mechanistic understanding of OTA toxicity, particularly in respect to the occurrence of renal carcinomas. OTA has, for instance, been shown to alter several cell-signaling pathways known to be involved in carcinogenesis. They include modulation of the calcium-dependent signaling and the mobilization of two mitogen-activated protein kinases (MAPK), the extracellular signal-regulated kinase (ERK1/2) and the c-jun amino terminal kinase (JNK1/2) (Gekle *et al*., 2005; Sauvant *et al*., 2005).

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

Since the carcinogenic mechanism remains obscure, there is still significant debate about the best model for human risk assessment. Knowledge of the extent of a toxicant's genotoxicity is vital in evaluating its potency as a carcinogen. At present, the genotoxic status of OTA is still unclear (Manderville, 2005; Turesky, 2005), mainly due to contradictory experimental evidence. (1) Applying ^{32}P-postlabelling, some authors have found low levels of DNA lesions in organs of animals treated with OTA (Castegnaro *et al*., 1998; Faucet *et al*., 2004; Pfohl-Leszkowicz *et al*., 1993). These lesions have been interpreted as OTA-specific covalent DNA adducts, suggesting that OTA is a genotoxin *sensu stricto*. Such a compound would require dose-response modeling for quantitative risk assessment. It has, however, also been suggested that OTA-mediated DNA lesions detected by ^{32}P-postlabelling may have arisen through indirect mechanisms rather than direct DNA-binding (Gautier *et al*., 2001b). (2) In studies that used radiolabeled OTA, DNA-binding could not be detected (Gautier *et al*., 2001b; Gross-Steinmeyer *et al*., 2002; Mally *et al*., 2004), indicating that the compound may not be directly genotoxic. If this is the case, an alternative safety-based approach would need to take into account uncertainty factors in order to evaluate the human health risk of OTA exposure.

The aim of the present study was to exploit the power of toxicogenomics to seek evidence for potential involvement of epigenetic mechanisms in OTA carcinogenesis under exposure conditions producing a significant incidence of renal tumors without overt toxicity. Gene expression profiles were analyzed in kidney and liver of male Fischer (F-344) rats, given dietary OTA over various periods ranging from 7 days to 12 months. This duration spans the first half of the standard 2-year protocol for studying lifetime responses to carcinogens.

MATERIALS AND METHODS

Ochratoxins. Standardized OTA production was performed by growing *A. ochraceus* isolate D2306 (Harris and Mantle, 2001) in shaken solid substrate fermentations at 28°C for 2 weeks to yield a product containing 5–6 mg OTA/g. More specifically, 40 g of sterilized shredded wheat (Cereal Partners UK, Welwyn, UK) in 500 ml Erlenmeyer flasks was inoculated with a concentrated spore suspension in water (16 ml), and the flasks were shaken at 200 rpm and 10 cm eccentric throw. An aliquot of each fermentation was assayed for OTA-concentration by HPLC with diode array detection (Harris and Mantle, 2001). Batches of fermentation product contained ochratoxin B (OTB) equivalent to 5–10% of the amount of OTA. No other mycotoxins (e.g., penicillic acid, citrinin) were biosynthesized in this fermentation. Each week, a weighed amount of standardized fermentation product was homogenized into powdered standard commercial rat feed (Special Diets Services, UK) to a final concentration appropriate for the required OTA-intake per animal. Each animal was given 20 g of contaminated feed daily, which was always fully consumed.

Animal treatment. Male Fischer 344 (F-344) rats (B. & K. Universal Ltd., Hull, UK) in groups of five were administered OTA in diet given daily over 2 years. As from their initial weight of ~175 g, daily dietary intake was 300 μg OTA/kg bw, but was held at 100 μg/rat after animals reached 333 g. Over the period of the study, animals were housed in cages on absorbent paper under tightly controlled conditions (21 ± 1°C, 55 ± 10% relative humidity, air-exchange, 12-h light-dark cycle). Animal growth and welfare were monitored by regular weighing and daily surveillance. For the gene expression study, five time points were selected to represent early (7 and 21 days) and later (4, 7, and 12 months) responses. At each time point, four control and treated animals were randomly chosen for tissue harvest. Kidneys and liver from these rats were immediately snap-frozen in liquid nitrogen. All handling and procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

RNA isolation. Homogenates from liver and kidney tissue samples were prepared with the FastPrep system (Q.BIOgene, Germany). Total RNA was isolated using the Clontech Atlas Pure Total RNA Labeling System (BD Biosciences, Switzerland) following the manufacturer's protocol. Total RNAs were treated with DNase I to remove genomic DNA contamination. RNA concentration was measured with the RiboGreen RNA quantification Kit (Molecular Probes, Eugene, OR) according to the manufacturer's instruction. Assessment of RNA quality and measurement of the 28S to 18S ratio were performed by dynamic gel electrophoresis using the Agilent Bioanalyser (Agilent Biotechnologies, Germany).

cRNA preparation. Five μg of total RNA from kidney and liver samples of treated and control rats was used for the synthesis of double-stranded cDNA (Superscript Choice System; Invitrogen Life Technologies, Carlsbad, CA) in the presence of a T7-(dT) DNA oligonucleotide primer. After synthesis, cDNA was purified by phenol/chloroform/isoamyl alcohol extraction and subsequent ethanol precipitation. The purified cDNA was transcribed *in vitro* using a MEGAscript kit (Ambion Diagnostics, Austin, TX) in the presence of biotinylated ribonucleotides to form biotin-labeled cRNA (ENZO Life Sciences, Farmingdale, NY). Labeled cRNA was purified using a nucleospin matrix (Macherey-Nagel, Germany), quantified by RiboGreen, and its quality was assessed with Agilent Bioanalyzer before subsequent fragmentation.

Hybridisation and labeling. Twenty μg of each fragmented cRNA was hybridized to a GeneChip cartridge, rotated at 60 rpm for 16 h at 40°C. Sample was analyzed independently using the Affymetrix platform RG-U34A expression probe arrays (Affymetrix Inc., Santa Clara, CA) containing 8,799 probe sets. For each time point, at least three independent controls and treated animals were analyzed individually on a GeneChip cartridge. Quality control steps were performed during the microarray procedure and were collected according to the laboratory information management system (L.I.M.S). After hybridization, GeneChips were washed 10 times in 6× SSPE-T at 25°C, followed by four washes in 0.5× SSPE-T at 50°C. GeneChips were stained with 10 μg/ml streptavidin-P-phycoerythrin, SAPE (Molecular Probes Inc., Eugene, OR), in a GeneChip Fluidics Workstation 400 (Affymetrix Inc., Santa Clara, CA). The signal was amplified using biotinylated goat antistreptavidin antibody (Vector Laboratories, Burlingame, CA), followed from a second staining with SAPE. The array was washed and scanned at 560 nm using a confocal laser scanner (GeneArray Scanner 2500, Hewlett Packard).

Data analysis and clustering algorithms. Signals from scanned images were quantified using the Affymetrix Gene Expression Analysis software (MAS 5.0). Normalization of gene expression data was performed. Differences in gene expression occurring in treated rats compared to controls were calculated by applying analysis of variance (ANOVA). This analysis was performed on log-transformed signal values for each gene individually (By-Gene test). Three factors were used for ANOVA: treatment, time, and the interaction between treatment and time. ANOVA between the treatments was performed applying the Global Error Assessment method (GEA) as previously described (Mansourian *et al.*, 2004; Mutch *et al.*, 2004). This approach increases the statistical power of the data. Complete data sets generated in this study are available at the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), access number GSE2852.

Gene clustering dendrograms were obtained with the software Spotfire (Spotfire Inc.) following the UPGMA clustering and Tanimoto similarity measure (Matter, 1997). Only genes with a significant $p \leq 0.001$ in at least two time points were included in dendrograms of differential expression of genes in

kidney and liver. The vertical axis of each dendrogram shows individually selected probes, whereas the horizontal axis represents the clustering level at the time points studied (7 days, 21 days, 4 months, 7 months, and 12 months).

Single value decomposition. The variability of gene modulation induced by OTA during the time was also investigated by a matrix factorization technique equivalent to Principal Component Analysis (PCA), the singular value decomposition (SVD) method (Alter *et al.*, 2003). SVD method provides a low-dimensional projection of the original dataset by its decomposition in three quantities—the matrices containing the left- and right-singular vectors of the original data matrix, and the diagonal matrix containing the singular values. Clusters formed by the SVD method can be interpreted as the relative frequencies with which a given gene is modulated at every one of the original single time points which, when combined, form the temporal modes of gene modulation. Average from each SVD-generated cluster was performed in order to evaluate the profile of the gene expression during the time.

Real-time polymerase chain reaction (PCR). Several key genes, each of which represents a category of genes found to be modulated by OTA, were selected to validate the microarray data. RNA preparation, quantification, and quality control were conducted as described above. RNA was converted to cDNA using the two-step TaqMan Gold RT-PCR system (Applied Biosystem, Foster City, CA) containing the TaqMan Reverse Transcription Reagents and the TaqMan PCR Core kit for relative quantification experiments. Reverse transcription was performed in triplicate, according to the manufacturer's instructions (Applied Biosystems, Foster City, CA), using the random hexamers primer and 5 μg of pooled RNA samples. cDNA synthesis was performed in a Thermal Cycler® 9600 (Applied Biosystems) under the following conditions: 10 min at 25°C, 30 min at 48°C, and a final inactivation step of 5 min at 95°C. Samples were amplified in a MicroAmp 96-well reaction plate with the ABI Prism® 7000 Sequence Detection System using 1 μl of cDNA template and the TaqMan PCR kit (Applied Biosystems). The thermocycler conditions included two incubations of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles, each consisting of a denaturing step for 15 sec at 95°C and a second annealing and extension step for 1 min at 60°C. Quantification of amplified PCR products was performed using ABI Prism® 7000 Sequence Detection System software Version 1.1, normalized to the rat 18S gene as internal control. All rat PCR probes and primers used in this study were obtained from the Assays on Demand (AoD) Service of Applied Biosystems.

Protein expression analysis by Western blot. Correlation between mRNA and protein expression was confirmed by Western blot. Briefly, 50 mg of liver and kidney tissue samples were directly homogenized using RIPA (150 mM PBS containing 1% (vol/vol) Igepal CA630, 0.5% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS, and 5 μg/μl protease inhibitor mixture), pH 7.4 (SantaCruz Biotechnology, Santa Cruz, CA) following the manufacturer's instructions. Protein concentration was determined with a BioRad Protein assay kit (Bio-Rad, Richmond, CA). Using the MiniCell XCellSureLock system (Invitrogene), 15 μg of protein was loaded in the NuPAGE® Bis-Tris Pre-Cast Gel 4–12% System (Invitrogene Ltd, Paisley, UK). Protein was transferred to a nitrocellulose transfer membrane (Invitrogene) for 1 h at 30 V. Transferred membranes were probed with antibodies specific for the proteins Gstp, Gclc (kindly offered by Dr. Leslie McLellan, Dundee University, Scotland, UK), Oatp1 (kindly offered by Prof. B. Hagenbuch, University of Kansas Medical Center, Kansas City, KS), HNF4α (Abcam, Cambridge, UK), Oct2 and Oat-k1 (Alpha Diagnostic, San Antonio, TX). Each blot was subsequently probed with horseradish peroxidase linked to the specific secondary antibody. Chemiluminescence of the immunoblots was detected using ECL solution (Pierce, Rockford, IL). Membranes were wrapped in polythene film and exposed to Kodak films.

RESULTS

OTA treatment was generally well tolerated throughout the 2-year carcinogenicity study from which the tissue samples for the gene expression study originated. The body weight of treated animals did not differ significantly from that of control animals. All tumors that were found before the 2-year end point of the carcinogenicity study were from animals that had been euthanized because of loss of condition. The first renal tumor was found 75 weeks after OTA treatment commenced (Fig. 1). The total incidence of animals with a renal tumor reached 25% ($n = 64$) at the end of the study. At least 75% of these animals had unilateral renal carcinoma as determined by tumor size, proliferating and disorganized histopathology, and abundance of metastasis (Mantle *et al*., in press).

Gene Selection and Clustering

Applying the stringency criteria for selection of significantly differentiated genes, statistical analysis revealed that the expression of 470 genes in the kidney and 233 genes in the liver were differentially modulated ($p \leq 0.001$ in at least two time points) by the OTA treatment. Of these, only 45 genes were modulated in both kidney and liver.

Hierarchical clustering analysis was performed for kidney and liver mRNA expression profiles (Figs. 2A and 2B). The vertical axis of the dendrograms represents each transcript, and the x-axis represents the various time points, and at the intersection of these two values, the expression intensity as compared to control (fold-expression) can be seen. The organization in both the horizontal and vertical axes represents the similarity of the expression values as detailed by the tree connecting each category. The distance of the connecting lines in the tree represents the extent of similarity. Gene expression clusters were generated accordingly; interestingly, the 7-day and 12-month profiles exhibited the most similar time points (in terms of the expression values of the affymetrix probes at these time points) in both organs. In the kidney, these two time points taken together are then most similar to the 7-month time point; these three are most similar to the 21-day and, finally, the 4-month time point. In the liver, these two time points taken as a cluster are most similar to the 12-month, 7-day, and finally, the 7-month time point. In both organs, down regulation was

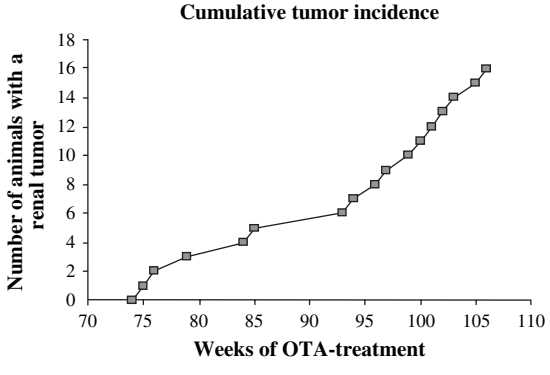

FIG. 1. Cumulative renal tumor incidence during the last quarter of live in rats given OTA-contaminated diet continuously for up to 2 years.

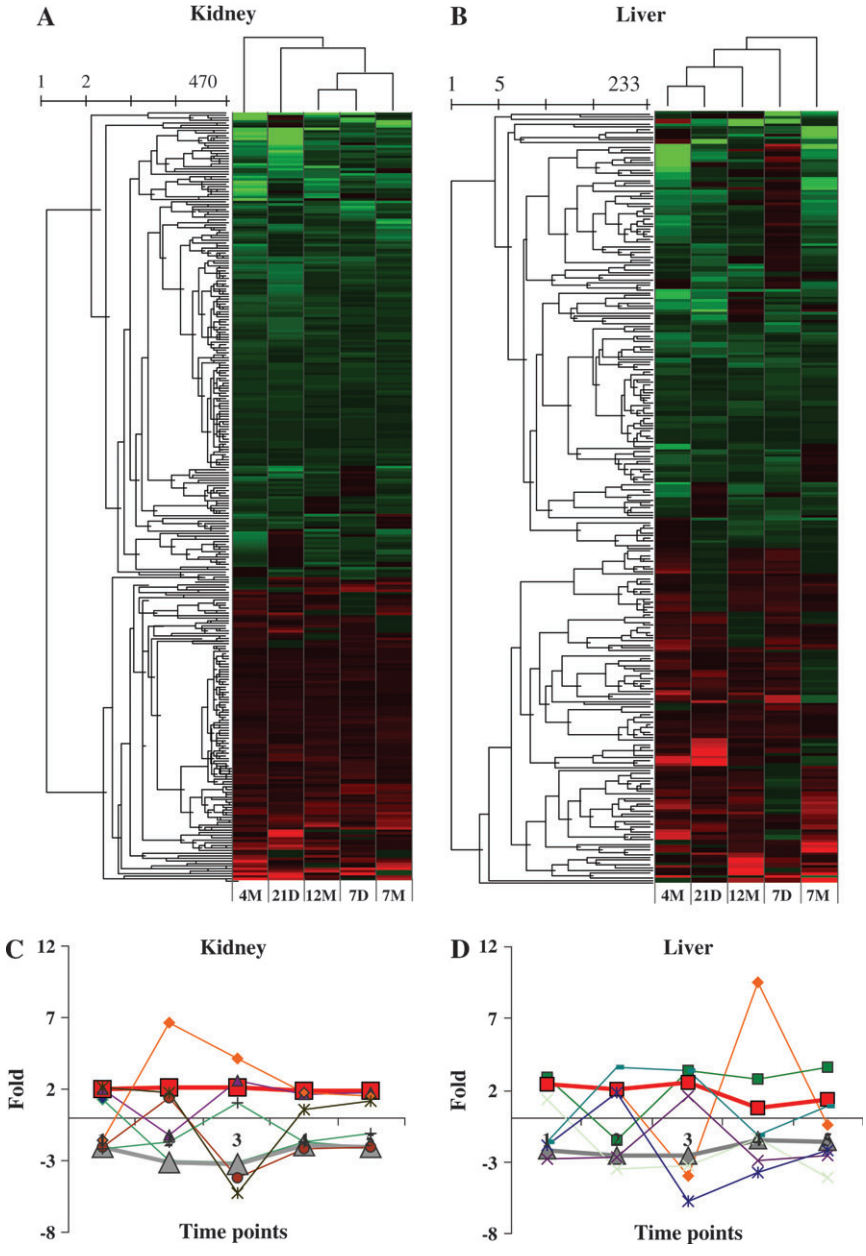

FIG. 2. Hierarchical clustering representing the genes significantly modulated ($p \leq 0.001$) in at least two time points in the kidney (A) and the liver (B). The y-axis of the dendrograms represents the genes and the corresponding expression level displayed in green for down-regulation, red for up-regulation, and black for insignificant change in gene expression. Time points of analysis are plotted on the x-axis at 7 days (7D), 21 days (21D), 4 months (4M), and 12 months (12M), and their corresponding time clustering is shown at the top of each dendrogram. Mean values of the fold-expression change for the eight clusters, as determined by the single value decomposition (SVD) method, are plotted as a function of time points for kidney (C) and liver (D). The two main clusters identified in each organ are highlighted in bold (red squares: up-regulation; grey triangles: down-regulation). They illustrate the gene expression pattern typical for the majority of significantly modulated genes.

the predominant effect, with 60% and 56% of genes down-regulated in kidney and liver, respectively.

Further analysis of the gene expression profile in kidney and liver was performed by application of a single value decomposition (SVD) method along the time coordinate. The mean fold-expression values were plotted as a function of time (Figs. 2C and 2D). In both organs, eight clusters with differentiated time-dependent expression profile were identified. The modulation of OTA-induced gene expression was generally less than two-fold. The two main clusters include only genes that, over the five time points, are either continuously up-regulated (31% in kidney, 25% in liver) or

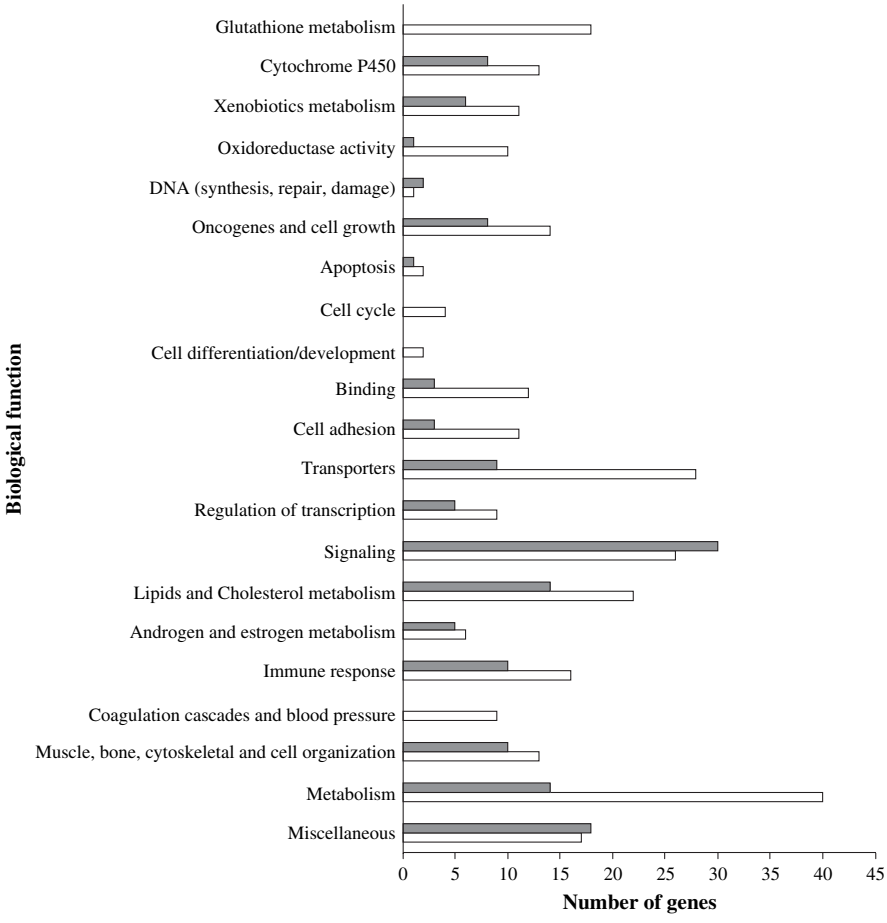

FIG. 3. Biological function clustering. Genes exhibiting at least 1.5-fold up- or down-regulation in kidney (white columns) and liver (black columns), plotted as a function of the total number of genes in each category.

continuously down-regulated (47% in kidney, 27% in liver). No other clear and easily interpretable time-dependent trends, such as an early and/or late response, could be observed.

Grouping of genes modulated by OTA was performed according to their biological functions. For this analysis, a gene ontology approach was applied to genes exhibiting at least 1.5-fold expression values. The number of genes attributed to each functional class is depicted in Figure 3, showing a wide diversity of highly tissue-selective biological responses. In the kidney, genes involved in xenobiotic metabolism and oxidative stress response were generally down-regulated by OTA, whereas this group of genes was much less modulated in the liver. Interestingly, many of these genes share the antioxidant regulatory element (ARE) as a regulatory motif (Table 1). Other gene classes, such as several enzymes involved in fatty acid metabolism and cytochrome P450, were also selectively down-regulated in the kidney of OTA-treated animals. Many of these genes possess a common promoter targeted by the transcription factor hepatocyte nuclear factor alpha (HNF4α). Several genes, which belong to different functional classes and were down-regulated following OTA-treatment, were identified as being under transcriptional control of HNF4α (Table 2). Of interest was the finding that the mRNA specific for HNF4α itself was down-regulated.

Expression of many transporter genes was down-regulated in the kidney (Table 3). Several genes known as markers of kidney injury, cell regeneration, and oncogenesis were significantly modulated by OTA. For example, the kidney injury molecule (*KIM-1,* 4.4-fold) and the survival markers *c-myc* (3.7-fold), *Akt-1* (1.7-fold), Polo-like kinase (*Plk,* 1.5-fold), and *Cdkn1a* alias *p21* (2.3-fold) were up-regulated. The regucalcin (*REG*), also known as the senescence marker protein 30 (*Smp30*), was down-regulated more than 10-fold by OTA.

Only small changes occurred in expression of genes known to be involved in DNA synthesis and repair or in genes induced as a result of DNA damage. Few genes associated with DNA repair were slightly down-regulated. Similarly, very little or no effects were found on expression of apoptosis-related genes. Three genes involved in the regulation of protein synthesis were modulated by OTA. The prostaglandin F2 receptor negative regulator (*Ptgfrn*) and the eukaryotic translation initiation factor 4E binding protein 1 (*Eif4ebp1*) were up-regulated

TABLE 1
Genes Modulated by OTA under Transcriptional Control of Nrf2

Accession no.	Gene title	Symbol	Fold[a]	Fold 7D	Fold 21D	Fold 4M	Fold 7M	Fold 12M
Xenobiotic metabolism								
X65296	carboxylesterase 3	Ces3	**−3.4***	−1.5	−9.8*	−4.2*	−3.3*	−2.0*
M33747	UDP-glucuronosyltransferase 2 family, member 5	*Ugt2b5*	**−2.7***	−2.5	−1.3	−5.2*	−3.1	−3.0
X81395	carboxylesterase 1	*Ces1*	**−1.9***	−2.0	−2.2	−2.7	−1.8	−1.3
S81433	Heme-oxygenase 2/5′ region	*HMOX2*	**−1.6***	−2.4	−3.0*	1.1	−1.0	−1.6
AF045464	aflatoxin B1 aldehyde reductase	*Akr7a3/ Afar*	**−1.5***	1.0	−2.0*	−2.1*	−1.2	−1.4
J02722cds	heme oxygenase 1	*Hmox1*	**2.1***	1.4	4.4	1.6*	1.3	3.2*
Carbon metabolism								
AI008020	malic enzyme 1	*Me1*	**−1.8***	−1.3	−2.8*	−3.2*	1.0	−1.7
AJ005046	fructose bisphosphatase 2	*Fbp2*	**2.6***	1.8	1.8	2.9	4.1	3.2
Glutathionerelated genes								
J05181	glutamate-cysteine ligase catalytic subunit	*Gclc*	**−4.8***	−3.4*	−8.0*	−6.0*	−2.7*	−5.8*
S65555	glutamate cysteine ligase, modifier subunit	*Gclm*	**−3.1***	−3.5*	−3.4*	−4.1*	−1.7	−3.5*
L38615	glutathione synthetase	*Gss*	**−1.9***	−1.1	−2.5*	−2.7*	−1.8*	−1.8
AI138143	glutathione S-transferase, theta 2	*Gstt2*	**−1.8***	−1.7	−1.8*	−2.6*	−1.7*	−1.6
X03518	gamma-glutamyl transpeptidase	*Ggtp*	**−1.8***	1.0	−1.8	−2.6*	−1.8	−2.1*
X02904	glutathione S-transferase, pi 2	*Gstp2*	**−1.7***	−1.4	−1.5	−2.0*	−1.8	−2.1*
S72506	glutathione-S-transferase, alpha type2	*Gsta2*	**3.8***	−1.6	2.9	21.3*	4.4	4.3
U76252	gamma-glutamyltransferase-like activity 1	*Ggtla1*	**1.7***	2.8*	2.2	1.5	1.1	1.2
Amino acid metabolism								
M93297	ornithine aminotransferase	*Oat*	**−2.7***	−1.6	−6.2*	−2.4*	−2.8*	−2.1*
Others								
X06107	insulin-like growth factor 1	*Igf1*	**−1.9***	−2.8	−1.5	−3.8	2.9	−4.9*
L48060	prolactin receptor	*Prlr*	**−1.7***	−1.1	−3.4*	−1.6	−1.4	−1.8
X71127	complement component 1, q subcomponent, beta polypeptide	*C1qb*	**2.0***	1.6	3.6*	1.7	2.0*	1.8

[a]Average fold-change in gene expression from the five time points (7D and 21D days and 4M, 7M, and 12M months).
*Statistical significance represented by the alpha risk <0.001.

(3.2- and 1.5-fold, respectively), while the Elongation factor 1-alpha 1 (*eEF1A-1*) was down-regulated (−3.3-fold).

Confirmation of Microarray Expression Data

In order to confirm the microarray data, selected gene products assigned to various functional classes were analyzed by quantitative real-time PCR (TaqMan-PCR). As with the microarray data, TaqMan RT-PCR data is presented as fold-expression value of OTA-treated samples as compared to controls. As shown in Figure 4, expression values obtained with RT-PCR are highly comparable to the data generated by microarray technology.

Protein expression of six genes was measured from samples taken at the early and late time-points (21 days and 12 months) by Western blot analysis. A good correlation was observed between microarray data and measured protein expression (Fig. 5).

DISCUSSION

OTA is a common trace contaminant of some foodstuffs. It produces various toxicological effects, the most relevant being nephrotoxicity and nephrocarcinogenicity in rat (NTP, 1989). There is significant debate on the use of rat carcinogenicity data to assess the potential carcinogenic risk to humans. A very relevant question with respect to OTA-mediated carcinogenicity and risk assessment is whether the toxin acts through genotoxic or epigenetic mechanisms. For human safety assessment, a nongenotoxic mechanism of OTA would trigger the use of a health-based guidance value established by the application of uncertainty factors to the pivotal No Observed Adverse Effect Level obtained in animal studies (WHO, 2001).

The present work was aimed to generate data relevant to understanding the mechanism of OTA action and should help to select the most appropriate risk assessment procedure. This study was intended better to reflect the pattern of human exposure, and therefore, for the first time, oral feeding was chosen as the route of administration. This allowed ingestion of OTA in the most natural way for the experimental animals, homogenized into feed and consumed as desired, mainly in the dark part of the diurnal cycle. The dose was chosen to produce tumors, although avoiding overt nephrotoxicity, which could interfere with the carcinogenic process. A previous acute study had shown a marked difference between the toxicity of single

TABLE 2
Genes Modulated by OTA under Transcriptional Control of HNF4α

Accession no.	Gene title	Gene symbol	Fold[a]	Fold 7D	Fold 21D	Fold 4M	Fold 7M	Fold 12M
Glucose metabolism								
X05684	pyruvate kinase, liver and RBC	*Pklr*	**−1.6***	−1.8	−2.1*	−1.8	−1.6	−1.1
Androgen and estrogen metabolism								
L19998	sulfotransferase family 1A, phenol-preferring, member 1	*Sult1a1*	**1.9***	1.3	2.0*	2.2*	2.0*	1.9*
Lipids and steroid metabolism								
M73714	*aldehyde dehydrogenase family 3, subfamily A2*	*Aldh3a2*	**1.9***	1.6	−1.2	4.4*	−1.0	4.6*
D90109	fatty acid Coenzyme A ligase	*Facl2*	**1.6***	2.2*	1.1	1.7	1.5	1.8*
Xenobiotic metabolism								
J02657	cytochrome P450, subfamily IIC	*Cyp2c*	**10.0***	−8.2*	−7.5*	−64.9*	−1.8	−13.5*
J02861	cytochrome P450 2c13	*Cyp2c13*	**−2.4***	−2.5*	−1.1	−2.6*	−4.8*	−2.2*
M18335	cytochrome P450, 2c39	*Cyp2c39*	**−1.5***	−1.3	−1.2	1.1	−3.0*	−2.0*
Lipids and retinal transport								
M10934	retinol binding protein	*RBP4*	**−2.2***	1.3	−4.9*	−4.6*	−1.7*	−1.8*
S69874	fatty acid binding protein 5, epidermal	*Fabp5*	**−2.1***	−1.6	−2.7*	−1.8*	−2.1*	−2.5*
M27440	apolipoprotein B	*Apob*	**−1.6***	−1.4	−1.8*	−2.7	−1.7*	1.1*
D38380	transferrin	*Tf*	**2.5***	3.2*	1.9*	1.4	3.0*	3.9*
Transcription factors								
X57133	hepatocyte nuclear factor 4, alpha	*Hnf4α*	**−2.4***	−1.9	−4.3*	−1.3	−3.8*	−2.0*
Coagulation cascades and blood pressure								
M12112	angiotensinogen	*Agt*	**−3.5***	1.1	−10.5*	−5.8*	−2.2*	−4.4*
M81397	coagulation factor 2	*F2*	**−1.5***	−1.6*	−2.3*	−1.7*	−1.1	−1.1
Transporters (See Table 3)								

[a]Average fold-change in gene expression from the five time points (7D and 21D days and 4M, 7M, and 12M months).
*Statistical significance represented by the alpha risk <0.001.

daily doses by gavage and the same amount homogenized into feed (Miljkovic *et al*., 2003). In the initial phase of the study (until rats reached 333 g), the chosen dose used (300 μg/kg bw) was twice that estimated as the highest average daily intake in the NTP study (NTP, 1989), in which the toxin was administered by oral gavage. After rats reached 333 g, the dose was held to 100 μg/rat, aimed to gradually reduce the two-fold difference in intake between the NTP and the present regime as the body weight further increased. This protocol was considered to better mimic the human intake pattern (Mantle *et al*., in press). Dosing adult rats according to body weight (as in the NTP study) would have resulted in an increasing exposure to OTA (due to weight gain from fat accumulation), which would not have reflected exposure of human adults. At a given occurrence level of OTA, human intake is likely to be relatively stable because of the limited expected age-dependent variations in body weight and food intake. The various selected time points represented early (7 and 21 days) and late (up to 12 months) exposure stages. The treatment was well tolerated, and cumulative incidence of animals with a renal tumor of 25% had occurred by the 2-year end point of the experiment. This incidence of carcinomas was significantly lower than that observed in the NTP study at the highest dose (Mantle *et al*., in press). However, the development of renal cancer in this study makes it a suitable source for studying carcinogenic processes. The difference of cancer incidence between the two studies highlights the importance of the route of administration for tumor outcomes.

OTA-modulated gene expression profiles were obtained in both kidney, the toxin's main target organ, and liver. Overall, the effects observed were relatively modest. Real-time PCR data confirmed the results. The effect on abundance of certain mRNAs correlated with the change in expression of respective proteins, suggesting that such OTA-induced changes could be of biological significance. The various clustering approaches showed the distinctive difference between kidney and liver responses, consistent with the known organ selectivity of OTA carcinogenicity in the male rat. It is also possible that the apparently higher susceptibility of kidney is due to a dose-response effect from the elevated content and the prolonged residence of OTA in that organ. Indeed, it is well documented

TABLE 3
Transporter-Related Genes Modulated by OTA

Accession no.	Gene title	Gene symbol	Fold[a]	Fold 7D	Fold 21D	Fold 4M	Fold 7M	Fold 12M	Renal proximal tubule	Function	Regulation	Ref.
U15176	ATPase, Na^+/K^+	*Atp1a4*	**−4.3***	−3.3	−4.3	−6.4*	−4.6	−3.6	basolateral	Intracellular sodium and potassium balance	PKC, ERK1/2	Khundmiri *et al.*, 2005
AB004559	solute carrier family 22, member 6	*Slc22a6/rOAT1*	−2.7*	−2.7*	−4.4*	−3.6*	−1.8	−1.9	basolateral	Endogenous and exogenous anions ***Ochratoxin A***	PKC, ERK1/2	Koepsell and Endou, 2004 Tsuda *et al.*, 1999
D79981	solute carrier family 21, member 4	*Slc21a4/OAT-K1*	−2.5*	−2.7	−5.6*	−3.0	−1.2	−1.8	apical	Organic anions, xenobiotics, ***Ochratoxin A***		Takeuchi *et al.*, 2001
L19031	solute carrier family 21, member 1	*Slc21a1/Oatp1*	**−2.5***	−1.6	−3.8*	−2.9	−3.9*	−1.3	apical	Organic anions **Ochratoxin A**	PKC, testosterone	Terlouw *et al.*, 2003 Hagenbuch and Meier, 2003
AB005547	aquaporin 8	*Aqp8*	**−2.5***	−4.6*	−1.0	−2.1	−2.1	−4.4*	apical/ basolateral	H_2O, Urine concentration	Vasopressin, adenylate cyclase	Verkman *et al.*, 1996
D13871	solute carrier family 2, member 5	*Slc2a5/GLUT5*	−2.2*	−1.5	−2.6*	−2.7*	−2.4*	−2.2*	apical	fructose transport		Swissprot
D86086	ATP-binding cassette	*Abcc2, Mrp-2*	**−2.1***	−2.1	−1.9	−2.5*	−1.9	−2.2	apical	Organic anions, glutathione conjugated		Lee and Kim, 2004
AB013455	solute carrier family 34, member 1	*Slc34a1, NaPi-2*	**−1.8***	−1.5	−3.0*	−1.9	−1.6*	−1.3*	apical	Na/Pi cotransport	Dietary Pi intake and parathyroid hormone	Magagnin *et al.*, 1993
D83044	solute carrier family 22, member 2	*Slc22a2/Oct2*	−1.7*	−1.0	−3.0*	−2.0	−1.8*	−1.4*	basolateral	Endogenous and exogenous cations	>Male	Koepsell and Endou, 2004 Lee and Kim, 2004
M80804	solute carrier family 3, member 1	*Slc3a1/NBAT*	**−1.7***	−1.5	−2.4*	−2.0*	−1.6	−1.2	apical	Amino acid transport		Wells and Hediger, 1992
X67948	aquaporin	*Aqp1*	**−1.7***	−1.8	−2.5*	−1.5	−1.5	−1.4	apical/ basolateral	H_2O, Urine concentration	Vasopressin, adenylate cyclase	Ma *et al.*, 1998
U76379	solute carrier family 22, member 1	*Slc22a1/OCT1A*	−1.5*	−1.9*	−1.3	−1.7*	−1.5	−1.1	basolateral	Organic cations, anions, weak bases	PKC/PKA, TK cGMP	Koepsell and Endou, 2004 Lee and Kim, 2004
U28504	solute carrier family 17, member 1	*Slc17a1/ NaPi-1*	−1.5*	−1.3	−2.3*	−1.8	−2.3*	1.5	apical	Na/Pi co-transport		Swissprot
M81855	P-glycoprotein/ multidrug resistance 1	*Mdr1/Pgy1*	**4.6***	−1.0*	6.9	21.8*	6.5*	2.2	apical	Removal of xenobiotics		Lee and Kim, 2004

TABLE 3—*Continued*

Accession no.	Gene title	Gene symbol	Fold[a]	Fold 7D	Fold 21D	Fold 4M	Fold 7M	Fold 12M	Renal proximal tubule	Function	Regulation	Ref.
L28135	solute carrier family 2, member 2	*Slc2a2/GLUT2*	3.0*	2.0*	6.7*	3.9*	1.6	3.2*	apical	fructose		Rat Genome Database (RGD)
J04024	ATPase, Ca^{++}	*Atp2a2*	**1.7***	2.2	1.6	1.9	1.6	1.5	membrane	calcium		RGD
U78977	ATPase, class II	*Atp9a*	**1.6***	1.8	1.7	1.2	1.5	1.6	membrane	cations		Swissprot
AA800120	solute carrier family 25, member 20	*Slc25a20/Cact*	1.5*	1.6	1.7	1.7	1.2	1.4	mitochondria	Fatty acylcarnitine carrier		Swissprot

Note. Underlined genes correspond to the genes under the regulation of HNF4α.
[a]Average fold-change in gene expression from the five time points (7D and 21D days and 4M, 7M, and 12M months).
*Statistical significance represented by the alpha risk <0.001.

that OTA is actively transported in kidney cells (Gekle *et al*., 2005; O'Brien and Dietrich, 2005; WHO, 2001).

Hierarchical clustering analysis resulted in the identification of relatively complex and unexpected time-related responses. For example, a cluster was noted between the early response (at 7 days) and the late response (at 12 months). Currently, this finding is difficult to interpret. Time-related differences may occur as a result of toxicokinetics, treatment duration, and/or age-specific factors. The experimental design and the statistical approach applied in the present study did not allow thorough addressing of these factors.

General Markers of Nephrotoxicity and Carcinogenicity

Up-regulation of the prototypical renal toxicity marker kidney injury molecule (*KIM-1*) was observed in treated animals. *KIM-1* is a type-1 membrane protein highly expressed both in early stage renal tubular injury and in regenerating proximal tubule epithelial cells (Ichimura *et al*., 1998). Early induction of *KIM-1* expression with a maximum at 21 days (21-fold) shows that the OTA-dose in the present study produced some nephrotoxicity, even if not clinically apparent. From a mechanistic perspective, this finding may be relevant. It is acknowledged that toxicity might induce tissue regeneration and cell proliferation, which may then promote tumor development. OTA-induced proliferation has been observed *in vivo* (NTP, 1989) and *in vitro* (Hong *et al*., 2000; Kamp *et al*., 2005). However, there was no histological evidence of renal cell proliferation at any time point analyzed in this study, apart from the consistent presence of karyomegalic nuclei (data not shown). In the present study, genes implicated in cell survival and proliferation were induced by OTA, including *c-myc* (Amati *et al*., 1998), *Akt1* (Vanhaesebroeck and Alessi, 2000), and *Plk* (Holtrich *et al*., 1994). In addition, a strong up-regulation of P-glycoprotein (*P-gp*) was observed. P-gp belongs to the ATP-binding cassette (ABC) multidrug transporter superfamily overexpressed in tumor cells (Thomas and Coley, 2003).

Inhibition of protein synthesis is thought to be a major mode of OTA toxicity, although the exact molecular mechanism involved is not known (Creppy *et al*., 1983). However, only few differentially expressed genes relate directly to a known mechanism of protein synthesis inhibition. The prostaglandin F2 receptor negative regulator (*Ptgfrn;* Orlicky, 1996) and the eukaryotic translation initiation factor 4E binding protein 1 (*Eif4ebp1;* Gingras *et al*., 1999) were up-regulated. Both are documented to be involved in negative regulation of protein synthesis. In contrast, elongation factor 1-alpha 1 (*eEF1A-1*) was down-regulated. This protein is known to promote the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis (Petroulakis and Wang, 2002). Although our data imply inhibition of protein synthesis, comprehensive data on protein expression and modification would be required for a more definite conclusion. For example,

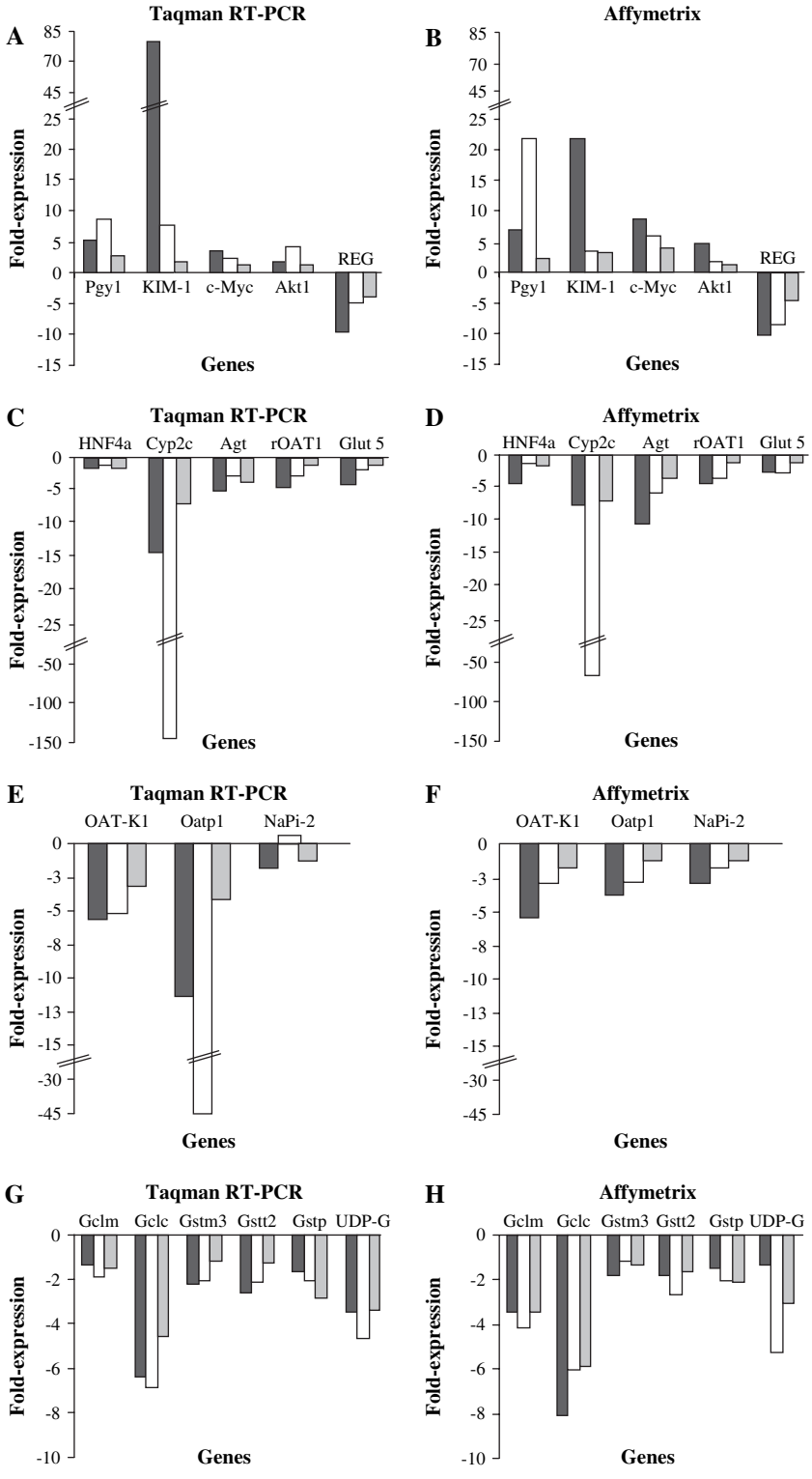

FIG. 4. Quantitation of gene expression changes for selected kidney genes as measured by TaqMan and Affymetrix methods at three time points: 21 days (black), 4 months (white), and 12 months (grey). The relative quantities of TaqMan RT-PCR products were normalized to the 18S rat housekeeping gene. TaqMan RT-PCR data are presented as the fold expression values as compared to those of control samples from technical replicates from pooled kidney mRNA ($n = 3$). (A–B) Nephrotoxicity markers. (C–D) HNF4α-regulated genes. (E–F) Transport-related genes. (G–H) Nrf2-regulated genes.

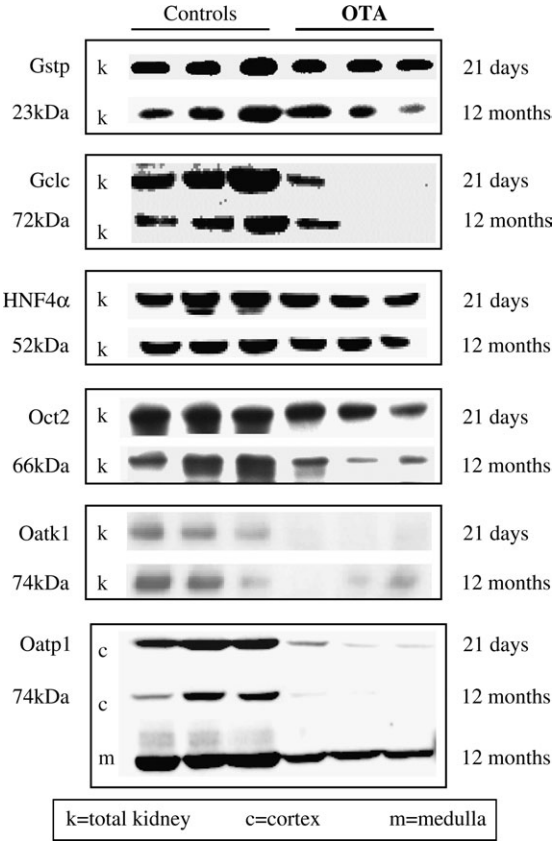

FIG. 5. Correlation between changes in gene and protein expression. Kidney protein lysates from control and OTA-treated animals were analyzed by Western blots and visualized by chemiluminescence. Transferred membranes were probed with antibodies specific to the following proteins: Gstp, Gclc, HNF4α, Oct2, Oat-k1, Oatp1 (cortex only), and Oatp1 (medulla only). Three controls and three samples from OTA-treated animals were analyzed at 21 days and 12 months.

it is known that the activity of the *Eif4ebp1* protein is dependent on its phosphorylation status (Gingras *et al*., 1999).

One of the most striking effects obtained in the present study was a strong down-regulation of regucalcin, also known as the senescence marker protein30 (*Smp30*). The maximum effect was observed after 21 days of treatment. In kidney tubule cells, regucalcin is known to play a role in the regulation of intracellular Ca^{2+} homeostasis. The reduction of regucalcin is compatible with documented effects of OTA on intracellular calcium and on calcium signaling homeostasis (Sauvant *et al*., 2005). In rat renal cortex, regucalcin was shown to produce a suppressive effect on DNA synthesis (Xue *et al*., 2000; Morooka and Yamaguchi, 2002). Interestingly, recent studies have suggested that regucalcin may possess tumor suppression activity in hepatoma cells (Tsurusaki and Yamaguchi, 2003, 2004). Inhibition of regucalcin expression was also observed with other nephrotoxicant and nephrocarcinogenic compounds like cisplatin (Huang *et al*., 2001; Misawa and Yamaguchi, 2001). Taken together, these data confirm a potential key role of calcium homeostasis in the mechanism of OTA action, which deserves further investigation.

OTA was found to significantly reduce the expression of a number of genes encoding transport proteins, such as those involved in organic anion transport. Similar trends were observed in an acute and high-dose experiment (Luhe *et al*., 2003). Importantly, these results support previous physiological data *in vitro* and *in vivo* showing that chronic treatment with OTA resulted in an impairment of the secretion of organic anions in the proximal tubules (Gekle and Silbernagl, 1994; Gekle *et al*., 2005; Sauvant *et al*., 2005). In this context it is interesting to note that angiotensinogen mRNA expression was down-regulated in our study. This may explain the effects of OTA on renal hemodynamics, which is thought to be mediated by angiotensin II (Gekle *et al*., 2005).

The transport proteins Oat1, Oat-k1, and Oatp1 are well documented to selectively transport OTA (Gekle and Silbernagl, 1994; Gekle *et al*., 2005; O'Brien and Dietrich, 2005; Zepnik *et al*., 2003). In our study they were markedly down-regulated at RNA and protein levels, indicating that OTA is likely to affect its own toxicokinetics. In addition, the down-regulation of transporters involved in the excretion of xenobiotic metabolites (e.g., multidrug resistance-associated protein 2, mrp2) may possibly result in interactions between OTA and other toxic chemicals.

Recently, it has been proposed that gene expression profiling can identify distinctive fingerprints or signatures to discriminate between compounds that act either as directly genotoxic or are genotoxic through indirect mechanisms (Dickinson *et al*., 2004; Hu *et al*., 2004). Although this method of classification still requires validation, a trend has been observed in which direct-acting genotoxins induce toxicity-associated pathways followed by early modulation of specific genes related to DNA-damage. Interestingly, the genes reported as potentially specific for direct-acting genotoxins were not modulated in our study, which may strongly suggest an indirect genotoxic mechanism for OTA.

Disruption of Biological Pathways

Analysis of DNA-binding sites revealed that many genes down-regulated by OTA share the motif 5′-AGGTCA-3′ as a DNA-binding domain. This sequence is the regulatory motif through which the transcription factor HNF4α acts (Ellrott *et al*., 2002). This strongly suggests that OTA may disrupt HNF4α-dependent regulatory pathways. The exact mechanism involved in the inhibition of HNF4α-regulated gene expression is not clear, although it was found that down-regulation of HNF4α expression was observed at both mRNA and protein levels.

From the perspective of cancer development, the biological significance of down-regulation of HNF4α-regulated genes is difficult to define. Many of the HNF4α target genes play important roles in development, differentiation, and homeostasis

(Sladek, 1993), and the depletion of HNF4α-pathway was previously associated with cancer development (Sel *et al*., 1996). HNF4α-expression and activity was analyzed in human renal carcinomas (Sel *et al*., 1996). The data indicated that in tumor samples both the expression and activity of HNF4α were reduced as compared to normal tissue, suggesting that this pathway might be an important molecular mechanism in renal carcinogenesis. However, the present analyses occurred well before any renal tumors were evident in the OTA-treated rats.

In the kidney, OTA treatment resulted in an average 10-fold down-regulation of the expression of the male-specific, HNF4α-regulated cytochrome P450 CYP 2C11. CYP 2C11 is involved in phase I metabolism of endogenous compounds (i.e., steroids) and several xenobiotics (Riddick *et al*., 2004). A potential role of CYP 2C11 in bioactivation of OTA was suggested (Pfohl-Leszkowicz *et al*., 1998), but data indicating a strong down-regulation of this gene do not support this hypothesis.

OTA has been reported to induce oxidative stress. For example, an increased formation of malondialdehyde (MDA) was observed in the kidney of rats exposed to 120 μg/kg bw/day of OTA for 60 days (Petrik *et al*., 2003). Other studies confirmed lipid peroxidation as a result of OTA exposure (Omar *et al*., 1990; Sauvant *et al*., 2005). Contrary to this, in another study, treatment of male rats with OTA (up to 2.0 mg/kg bw administered by oral gavage) did not increase the formation of biomarkers of oxidative damage such as the lipid peroxidation marker MDA in plasma, kidney, and liver, or the DNA damage marker 8-oxo-7,8-dihydro-2′deoxyguanosine in kidney DNA (Gautier *et al*., 2001b). However, a significant increase in the expression of the marker of oxidative stress response HSP32 protein was observed in kidney but not in liver. Surprisingly, in the present study, many genes normally induced by oxidative stress were down-regulated during OTA treatment. This response was only observed in kidney, and the mechanism of this down-regulation is still unknown. Interestingly, these genes share the antioxidant regulatory element (ARE) as a regulatory motif in their promoter region. The ARE-motif is recognized by the nuclear factor-erythroid 2-related factor 2 (Nrf2), a member of the ''Cap-n-Collar'' family of basic-region leucine zipper transcription factors (Mathers *et al*., 2004). Nrf2 is involved in both the basal expression and the induction of genes encoding mainly for detoxication, cytoprotective, and antioxidant enzymes (Lee and Johnson, 2004; Nguyen *et al*., 2004). These proteins are responsible for cellular redox status and for cellular defense against oxidative damage. We are currently studying inhibition of Nrf2-activation by OTA as a molecular mechanism involved in OTA toxicity.

Although the expression of most of the renal Nrf2-regulated genes seemed to be inhibited by OTA treatment, a limited number of them appeared to be induced (e.g., Gsta2). It is important to note that, for many genes, the 5′ promoter region may contain multiple binding sites specific for several transcription factors, and therefore the observed effects on mRNA expression may be the combined effects on different transcription factors. Previous investigations have provided evidence that phase II enzymes may not be regulated exclusively by Nrf2-mediated mechanisms (Kang *et al*., 2003). For example, recent studies indicated that Nrf2 might not significantly contribute to the expression of Gsta1/2 in the mouse small intestine (McMahon *et al*., 2001). Our results show that some Nrf2-regulated genes are not down- but up-regulated, suggesting that OTA may also alter other signaling pathways that have not yet been identified.

The toxicological consequences of the OTA-mediated down-regulation of many Nrf2-regulated genes are likely to be biologically significant. It is well documented that chemicals inducing an oxidative stress response through activation of Nrf2 are associated with chemoprotective properties (Chen and Kong, 2004; Zhang and Gordon, 2004). With OTA, the inhibition of expression of genes involved in the biosynthesis of glutathione (including the rate-limiting Gclc) is likely to result in a reduction of the cellular glutathione content, as already documented in OTA-treated cell cultures (Schaaf *et al*., 2002). Because of its major importance in the cellular redox-balance, a reduction of intracellular glutathione content should compromise the cellular defense against oxidative damage. 4-Hydoxynonenal (4-HNE) is a reactive metabolite resulting from lipid peroxidation that forms DNA adducts (Hartley *et al*., 1995). Because of the affinity to conjugate 4-HNE with glutathione, Gstp is thought to play a role in the detoxication of this lipid peroxidation product (Hartley *et al*., 1995). It can be hypothesized that the OTA-mediated down-regulation of Gstp may impair the detoxication of 4-HNE and thus result in increased oxidative damage.

Many Nrf2-regulated gene products down-regulated by OTA are involved in xenobiotic detoxication processes. They include several glutathione S-transferases and UDP-glucuronyltransferases. Their reduced expression may possibly result in a higher concentration of xenobiotics, normally detoxified by these enzymes, which could act synergistically with OTA, as suggested for OTA and other mycotoxins (Creppy *et al*., 2004).

Finally, an attempt to link HNF4α and Nrf2-pathways pointed to protein kinase C (PKC) as a common regulatory feature. PKC is the major kinase involved in ARE-mediated gene induction by Nrf2 (Bloom and Jaiswal, 2003; Numazawa *et al*., 2003) and in activation of the HNF4α−pathway (Hashimoto *et al*., 2005; Roy *et al*., 2001). Notably, in our study several transport-related genes, reported to be regulated through PKC-dependent processes, were also down-regulated by OTA (see Table 2). These data strongly suggest that disruption of PKC-mediated processes may play a role in the toxicity of OTA (Fig. 6). Further investigation is necessary to confirm this hypothesis.

Summary and Conclusion

In the present study significantly different gene expression profiles were observed in kidney and liver of male Fischer rats

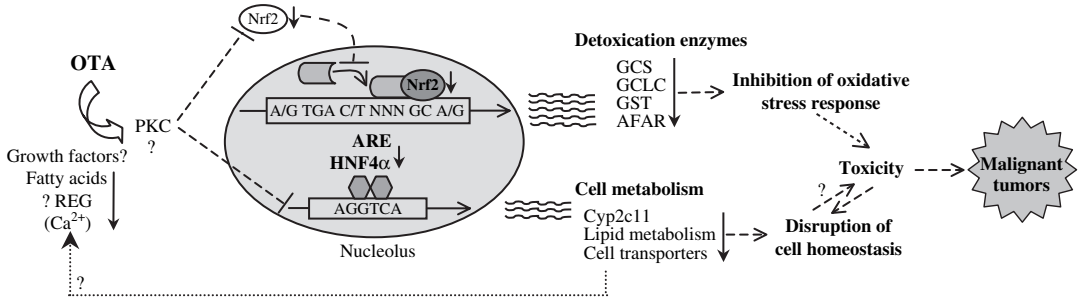

FIG. 6. Role of PKC in the response of kidney cells to OTA. Putative scheme of plausible mechanism of OTA toxicity, assembled from the gene expression data. Effect of OTA on potential signaling molecules (growth factors, fatty acids, and/or Ca^{2+}) could disrupt PKC-regulated pathways downstream. Down-regulation of genes under transcriptional control of Nrf2 may lead to a reduced oxidative stress response. In addition, OTA-induced down-regulation of genes under HNF4α-control may affect key metabolic processes. This could make kidney cells more vulnerable to OTA-induced toxicity leading to tumor development. Dashed lines: hypothetical effects (⊥ inhibition). Solid lines: OTA-effects demonstrated in this study. Down-regulation is denoted by ↓.

following chronic dietary exposure to OTA in a concentration sufficient to cause renal carcinoma late in life. Gene expression data indicated occurrence of renal toxicity, which may involve oxidative damage and eventually lead to cell proliferation and cancer. Gene expression analysis pointed toward predominant involvement of epigenetic mechanisms in OTA carcinogenicity. These mechanisms may include alteration of calcium homeostasis and disruption of pathways regulated by the transcription factors HNF4α and Nrf2. The depletion of Nrf2-regulated enzymes is likely to impair the cellular defense potential, resulting in chronic elevation of oxidative stress in the kidney. Sources of oxidants can be either endogenous (normal physiological processes) or exogenous (xenobiotics). Low concentration of antioxidants can result in cell proliferation and, ultimately, cancer development (Klaunig and Kamendulis, 2004). Therefore, inhibition of cellular defense appears to be a highly plausible novel mechanism, which could contribute to OTA-mediated carcinogenicity. However, it has to be acknowledged that the present data do not provide any absolute demonstration of a direct link between the gene expression effects observed and the late development of tumors. Further investigations are necessary to further establish the relevance of the biological effects to the carcinogenic process. In addition, it has to be noted that the interpretation of gene expression data in this study has focused on significantly modulated genes. It is, of course, feasible that subtle changes in the expression of other genes, together with cell signaling and post-translational modification processes, which were not further analyzed in this study, are also implicated in OTA carcinogenesis and could help to accomplish the comprehension of the OTA mechanism of action.

ACKNOWLEDGMENTS

We would like to thank the Central Biomedical Services at Imperial College London for their technical help, Prof. B. Hagenbuch, Dr. Leslie McLellan, and Dr. S. Lariani for their advice and contributions to our studies. The work of the authors was supported by the EU-Grant # QLK1-CT-2001–011614. Conflict of interest: none declared.

REFERENCES

Alter, O., Brown, P. O., and Botstein, D. (2003). Generalized singular value decomposition for comparative analysis of genome-scale expression datasets of two different organisms. *Proc. Natl. Acad. Sci. U.S.A.* **100,** 3351–3356.

Amati, B., Alevizopoulos, K., and Vlach, J. (1998). Myc and the cell cycle. *Front. Biosci.* **3,** d250–d268.

Bendele, A. M., Carlton, W. W., Krogh, P., and Lillehoj, E. B. (1985). Ochratoxin A carcinogenesis in the (C57BL/6J×C3H)F1 mouse. *J. Natl. Cancer Inst.* **75,** 733–742.

Bloom, D. A., and Jaiswal, A. K. (2003). Phosphorylation of Nrf2 at Ser40 by protein kinase C in response to antioxidants leads to the release of Nrf2 from INrf2, but is not required for Nrf2 stabilization/accumulation in the nucleus and transcriptional activation of antioxidant response element-mediated NAD(P)H:quinone oxidoreductase-1 gene expression. *J. Biol. Chem.* **278,** 44675–44682.

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.

Chen, C., and Kong, A. N. (2004). Dietary chemopreventive compounds and ARE/EpRE signaling. *Free Radic. Biol. Med.* **36,** 1505–1516.

Creppy, E. E., Stormer, F. C., Kern, D., Roschenthaler, R., and Dirheimer, G. (1983). Effects of ochratoxin A metabolites on yeast phenylalanyl-tRNA synthetase and on the growth and *in vivo* protein synthesis of hepatoma cells. *Chem. Biol. Interact.* **47,** 239–247.

Creppy, E. E., Chiarappa, P., Baudrimont, I., Borracci, P., Moukha, S., and Carratu, M. R. (2004). Synergistic effects of fumonisin B1 and ochratoxin A: Are *in vitro* cytotoxicity data predictive of *in vivo* acute toxicity? *Toxicology* **201,** 115–123.

Dickinson, D. A., Warnes, G. R., Quievryn, G., Messer, J., Zhitkovich, A., Rubitski, E., and Aubrecht, J. (2004). Differentiation of DNA reactive and non-reactive genotoxic mechanisms using gene expression profile analysis. *Mutat. Res.* **549,** 29–41.

Dirheimer, G., and Creppy, E. E. (1991). Mechanism of action of ochratoxin A. *IARC Sci. Publ.* **115,** 171–186.

Ellrott, K., Yang, C., Sladek, F. M., and Jiang, T. (2002). Identifying transcription factor binding sites through Markov chain optimization. *Bioinformatics.* **18(Suppl. 2)**, S100–S109.

Faucet, V., Pfohl-Leszkowicz, A., Dai, J., Castegnaro, M., and Manderville, R. 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.

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

Gautier, J., Richoz, J., Welti, D. H., Markovic, J., Gremaud, E., Guengerich, F. P., and Turesky, R. J. (2001b). Metabolism of ochratoxin A: Absence of formation of genotoxic derivatives by human and rat enzymes. *Chem. Res. Toxicol.* **14,** 34–45.

Gekle, M., Sauvant, C., and Schwerdt, G. (2005). Ochratoxin A at nanomolar concentrations: A signal modulator in renal cells. *Mol. Nutr. Food Res.* **49,** 118–130.

Gekle, M., and Silbernagl, S. (1994). The role of the proximal tubule in ochratoxin A nephrotoxicity *in vivo:* Toxodynamic and toxokinetic aspects. *Ren. Physiol. Biochem.* **17,** 40–49.

Gingras, A. C., Gygi, S. P., Raught, B., Polakiewicz, R. D., Abraham, R. T., Hoekstra, M. F., Aebersold, R., and Sonenberg, N. (1999). Regulation of 4E-BP1 phosphorylation: A novel two-step mechanism. *Genes Dev.* **13,** 1422–1437.

Gross-Steinmeyer, K., Weymann, J., Hege, H. G., and Metzler, M. (2002). Metabolism and lack of DNA reactivity of the mycotoxin ochratoxin A in cultured rat and human primary hepatocytes. *J. Agric. Food Chem.* **50,** 938–945.

Hagenbuch, B., and Meier, P. J. (2003). The superfamily of organic anion transporting polypeptides. *Biochim. Biophys. Acta* **1609,** 1–18.

Harris, J. P., and Mantle, P. G. (2001). Biosynthesis of ochratoxins by *Aspergillus ochraceus. Phytochemistry* **58,** 709–716.

Hartley, D. P., Ruth, J. A., and Petersen, D. R. (1995). The hepatocellular metabolism of 4-hydroxynonenal by alcohol dehydrogenase, aldehyde dehydrogenase, and glutathione S-transferase. *Arch. Biochem. Biophys.* **316,** 197–205.

Hashimoto, N., Kido, Y., Uchida, T., Matsuda, T., Suzuki, K., Inoue, H., Matsumoto, M., Ogawa, W., Maeda, S., Fujihara, H., *et al.* (2005). PKClambda regulates glucose-induced insulin secretion through modulation of gene expression in pancreatic beta cells. *J. Clin. Invest* **115,** 138–145.

Holtrich, U., Wolf, G., Brauninger, A., Karn, T., Bohme, B., Rubsamen-Waigmann, H., and Strebhardt, K. (1994). Induction and down-regulation of PLK, a human serine/threonine kinase expressed in proliferating cells and tumors. *Proc. Natl. Acad. Sci. U.S.A* **91,** 1736–1740.

Hong, J. T., Park, K. L., Han, S. Y., Park, K. S., Kim, H. S., Oh, S. D., Lee, R. D., and Jang, S. J. (2000). Effects of ochratoxin A on cytotoxicity and cell differentiation in cultured rat embryonic cells. *J. Toxicol. Environ. Health A* **61,** 609–621.

Hu, T., Gibson, D. P., Carr, G. J., Torontali, S. M., Tiesman, J. P., Chaney, J. G., and Aardema, M. J. (2004). Identification of a gene expression profile that discriminates indirect-acting genotoxins from direct-acting genotoxins. *Mutat. Res.* **549,** 5–27.

Huang, Q., Dunn, R. T., Jayadev, S., DiSorbo, O., Pack, F. D., Farr, S. B., Stoll, R. E., and Blanchard, K. T. (2001). Assessment of cisplatin-induced nephrotoxicity by microarray technology. *Toxicol. Sci.* **63,** 196–207.

Ichimura, T., Bonventre, J. V., Bailly, V., Wei, H., Hession, C. A., Cate, R. L., and Sanicola, M. (1998). Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury. *J. Biol. Chem.* **273,** 4135–4142.

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.

Kang, K. W., Park, E. Y., and Kim, S. G. (2003). Activation of CCAAT/enhancer-binding protein beta by 2′-amino-3′-methoxyflavone (PD98059) leads to the induction of glutathione S-transferase A2. *Carcinogenesis* **24,** 475–482.

Khundmiri, S. J., Dean, W. L., McLeish, K. R., and Lederer, E. D. (2005). Parathyroid hormone-mediated regulation of Na^+-K^+-ATPase requires ERK-dependent translocation of protein kinase Calpha. *J. Biol. Chem.* **280,** 8705–8713.

Klaunig, J. E., and Kamendulis, L. M. (2004). The role of oxidative stress in carcinogenesis. *Annu. Rev. Pharmacol. Toxicol.* **44,** 239–267.

Koepsell, H., and Endou, H. (2004). The SLC22 drug transporter family. *Pflugers Arch.* **447,** 666–676.

Lee, J. M., and Johnson, J. A. (2004). An important role of Nrf2-ARE pathway in the cellular defense mechanism. *J. Biochem. Mol. Biol.* **37,** 139–143.

Lee, W., and Kim, R. B. (2004). Transporters and renal drug elimination. *Annu. Rev. Pharmacol. Toxicol.* **44,** 137–166.

Luhe, A., Hildebrand, H., Bach, U., Dingermann, T., and Ahr, H. J. (2003). A new approach to studying ochratoxin A (OTA)-induced nephrotoxicity: Expression profiling *in vivo* and *in vitro* employing cDNA microarrays. *Toxicol. Sci.* **73,** 315–328.

Ma, T., Yang, B., Gillespie, A., Carlson, E. J., Epstein, C. J., and Verkman, A. S. (1998). Severely impaired urinary concentrating ability in transgenic mice lacking aquaporin-1 water channels. *J. Biol. Chem.* **273,** 4296–4299.

Magagnin, S., Werner, A., Markovich, D., Sorribas, V., Stange, G., Biber, J., and Murer, H. (1993). Expression cloning of human and rat renal cortex Na/Pi cotransport. *Proc. Natl. Acad. Sci. U.S.A* **90,** 5979–5983.

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

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.

Mantle, P., Kulinskaya, E., and Nestler, S., (2005). Renal tumourigenesis in male rats in response to chronic dietary ochratoxin A. *Food Add. Contam.* (in press).

Mansourian, R., Mutch, D. M., Antille, N., Aubert, J., Fogel, P., Le Goff, J. M., Moulin, J., Petrov, A., Rytz, A., Voegel, J. J., *et al.* (2004). The Global Error Assessment (GEA) model for the selection of differentially expressed genes in microarray data. *Bioinformatics* **20,** 2726–2737.

Mathers, J., Fraser, J. A., McMahon, M., Saunders, R. D., Hayes, J. D., and McLellan, L. I. (2004). Antioxidant and cytoprotective responses to redox stress. *Biochem. Soc. Symp.* **71,** 157–176.

Matter, H. (1997). Selecting optimally diverse compounds from structure databases: A validation study of two-dimensional and three-dimensional molecular descriptors. *J. Med. Chem.* **40,** 1219–1229.

McMahon, M., Itoh, K., Yamamoto, M., Chanas, S. A., Henderson, C. J., McLellan, L. I., Wolf, C. R., Cavin, C., and Hayes, J. D. (2001). The Cap'n'Collar basic leucine zipper transcription factor Nrf2 (NF-E2 p45-related factor 2) controls both constitutive and inducible expression of intestinal detoxification and glutathione biosynthetic enzymes. *Cancer Res.* **61,** 3299–3307.

Miljkovic, A., Pfohl-Leszkowicz, A., Dobrota, M., and Mantle, P. G. (2003). Comparative responses to mode of oral administration and dose of ochratoxin A or nephrotoxic extract of *Penicillium polonicum* in rats, *Exp. Toxicol. Pathol.* **54,** 305–312.

Misawa, H., and Yamaguchi, M. (2001). Involvement of nuclear factor-1 (NF1) binding motif in the regucalcin gene expression of rat kidney cortex: The expression is suppressed by cisplatin administration. *Mol. Cell Biochem.* **219,** 29–37.

Morooka, Y., and Yamaguchi, M. (2002). Endogenous regucalcin suppresses the enhancement of protein phosphatase activity in the cytosol and nucleus of kidney cortex in calcium-administered rats. *J. Cell Biochem.* **85,** 553–560.

Mutch, D. M., Anderle, P., Fiaux, M., Mansourian, R., Vidal, K., Wahli, W., Williamson, G., and Roberts, M. A. (2004). Regional variations in ABC transporter expression along the mouse intestinal tract. *Physiol. Genomics* **17,** 11–20.

Nguyen, T., Yang, C. S., and Pickett, C. B. (2004). The pathways and molecular mechanisms regulating Nrf2 activation in response to chemical stress. *Free Radic. Biol. Med.* **37,** 433–441.

NTP (1989). Toxicology and Carcinogenesis Studies of Ochratoxin A (CAS No. 303–47–9) in F344/N Rats (Gavage Studies). *Natl. Toxicol. Program Tech. Rep. Ser.* **358,** 1–142.

Numazawa, S., Ishikawa, M., Yoshida, A., Tanaka, S., and Yoshida, T. (2003). Atypical protein kinase C mediates activation of NF-E2-related factor 2 in response to oxidative stress. *Am. J. Physiol Cell Physiol.* **285,** C334–C342.

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

Orlicky, D. J. (1996). Negative regulatory activity of a prostaglandin F2 alpha receptor associated protein (FPRP). *Prostaglandins Leukot. Essent. Fatty Acids* **54,** 247–259.

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.

Petkova-Bocharova, T., Chernozemsky, I. N., and Castegnaro, M. (1988). Ochratoxin A in human blood in relation to Balkan endemic nephropathy and urinary system tumours in Bulgaria. *Food Addit. Contam.* **5,** 299–301.

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.

Petroulakis, E., and Wang, E. (2002). Nerve growth factor specifically stimulates translation of eukaryotic elongation factor 1A-1 (eEF1A-1) mRNA by recruitment to polyribosomes in PC12 cells. *J. Biol. Chem.* **277,** 18718–18727.

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., Pinelli, E., Bartsch, H., Mohr, U., and Castegnaro, M. (1998). Sex- and strain-specific expression of cytochrome P450s in ochratoxin A-induced genotoxicity and carcinogenicity in rats. *Mol. Carcinog.* **23,** 76–85.

Plestina, R., Ceovic, S., Gatenbeck, S., Habazin-Novak, V., Hult, K., Hokby, E., Krogh, P., and Radic, B. (1990). Human exposure to ochratoxin A in areas of Yugoslavia with endemic nephropathy. *J. Environ. Pathol. Toxicol. Oncol.* **10,** 145–148.

Riddick, D. S., Lee, C., Bhathena, A., Timsit, Y. E., Cheng, P. Y., Morgan, E. T., Prough, R. A., Ripp, S. L., Miller, K. K., Jahan, A., *et al.* (2004). Transcriptional suppression of cytochrome P450 genes by endogenous and exogenous chemicals. *Drug Metab. Dispos.* **32,** 367–375.

Roy, N., Guruprasad, M. R., Kondaiah, P., Mann, E. A., Giannella, R. A., and Visweswariah, S. S. (2001). Protein kinase C regulates transcription of the human guanylate cyclase C gene. *Eur. J. Biochem.* **268,** 2160–2171.

Sauvant, C., Holzinger, H., and Gekle, M. (2005). The nephrotoxin ochratoxin A induces key parameters of chronic interstitial nephropathy in renal proximal tubular cells. *Cell. Physiol. Biochem.* **15,** 125–134.

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.

Sel, S., Ebert, T., Ryffel, G. U., and Drewes, T. (1996). Human renal cell carcinogenesis is accompanied by a coordinate loss of the tissue specific transcription factors HNF4 alpha and HNF1 alpha. *Cancer Lett.* **101,** 205–210.

Sladek, F. M. (1993). Orphan receptor HNF-4 and liver-specific gene expression. *Receptor* **3,** 223–232.

Takeuchi, A., Masuda, S., Saito, H., Abe, T., and Inui, K. (2001). Multispecific substrate recognition of kidney-specific organic anion transporters OAT-K1 and OAT-K2. *J. Pharmacol. Exp. Ther.* **299,** 261–267.

Terlouw, S. A., Masereeuw, R., and Russel, F. G. (2003). Modulatory effects of hormones, drugs, and toxic events on renal organic anion transport. *Biochem. Pharmacol.* **65,** 1393–1405.

Thomas, H., and Coley, H. M. (2003). Overcoming multidrug resistance in cancer: An update on the clinical strategy of inhibiting p-glycoprotein. *Cancer Control* **10,** 159–165.

Tsuda, M., Sekine, T., Takeda, M., Cha, S. H., Kanai, Y., Kimura, M., and Endou, H. (1999). Transport of ochratoxin A by renal multispecific organic anion transporter 1. *J. Pharmacol. Exp. Ther.* **289,** 1301–1305.

Tsurusaki, Y., and Yamaguchi, M. (2003). Overexpression of regucalcin modulates tumor-related gene expression in cloned rat hepatoma H4-II-E cells. *J. Cell. Biochem.* **90,** 619–626.

Tsurusaki, Y., and Yamaguchi, M. (2004). Role of regucalcin in liver nuclear function: Binding of regucalcin to nuclear protein or DNA and modulation of tumor-related gene expression. *Int. J. Mol. Med.* **14,** 277–281.

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

Vanhaesebroeck, B., and Alessi, D. R. (2000). The PI3K-PDK1 connection: More than just a road to PKB. *Biochem. J.* **346,** 561–576.

Verkman, A. S., van Hoek, A. N., Ma, T., Frigeri, A., Skach, W. R., Mitra, A., Tamarappoo, B. K., and Farinas, J. (1996). Water transport across mammalian cell membranes. *Am. J. Physiol.* **270,** C12–C30.

Wells, R. G., and Hediger, M. A. (1992). Cloning of a rat kidney cDNA that stimulates dibasic and neutral amino acid transport and has sequence similarity to glucosidases. *Proc. Natl. Acad. Sci. U.S.A* **89,** 5596–5600.

WHO (2001). Safety Evaluation of certain mycotoxins in food. Fifty-sixth meetingt of the Joint FAO/WHO Expert Committee on Food Additives. WHO Food Additives Series **47,** i-706.

Xue, J. H., Takahashi, H., and Yamaguchi, M. (2000). Stimulatory effect of regucalcin on mitochondrial ATP-dependent calcium uptake activity in rat kidney cortex. *J. Cell Biochem.* **80,** 285–292.

Zepnik, H., Volkel, W., and Dekant, W. (2003). Toxicokinetics of the mycotoxin ochratoxin A in F 344 rats after oral administration. *Toxicol. Appl. Pharmacol.* **192,** 36–44.

Zhang, Y., and Gordon, G. B. (2004). A strategy for cancer prevention: Stimulation of the Nrf2-ARE signaling pathway. *Mol. Cancer Ther.* **3,** 885–893.