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ANALYSIS FOR DNA ADDUCTS, OCHRATOXIN A CONTENT AND ENZYME EXPRESSION IN KIDNEYS OF PIGS EXPOSED TO MILD EXPERIMENTAL CHRONIC OCHRATOXICOSIS

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Summary. *Further study of pig renal tissue obtained by unilateral nephrectomy after six months on feed artificially contaminated with ochratoxin A (OTA) has revealed significant new information. Whereas there was no evidence of DNA adducts in either control animal, as tested by ^{32}P post-labelling, there was a differential pattern of occurrence in male and female treated pigs. DNA adducts occurred across cortex and medulla in the male, but most clearly in the cortico-medullary region. Adducts were only detected in the female in the cortex. Microsomal preparation from these kidneys consistently catalysed the formation of DNA adducts in vitro in the presence of OTA*; *none was formed without OTA. The ochratoxin A content of a kidney of a treated pig (about 15 μg) contrasted with the 1 μg found in that of control pigs, the latter reflecting significant though slight contamination of some commercial feed with ochratoxin A in Bulgaria but which did not appear to have any adverse effects in this pig experiment. Small amounts of fluorescent compounds more polar than OTA were detected in kidney extracts. Activities of the enzymes ECOD, PROD and EROD in microsome preparations from renal cortex and medulla were variously different in male and female animals, reflecting different expressions of cytochromes P_{450}. Western blot analysis also indicated that OTA modulated cyclooxygenase (COX) pathways differently in males and females in ways that might theoretically be disadvantageous for the male.*

Key words: *Ochratoxin A, DNA adducts, ^{32}P post-labelling, protein synthesis, renal enzyme activity, pig, nephrotoxicity*

Introduction

Experimental ochratoxicosis in pigs has sought to define the role of ochratoxin A in natural occurrence of porcine nephropathy in pig production, notably and classically in Denmark (1), but also in Bulgaria (2). Its primary role as causal agent in the former is well recognised, if also occasionally in conjunction with other natural mycotoxins in feed. Its role in Bulgaria is less clear (2). Thus experimental chronic exposure to a modest intake of ochratoxin A over one year was made there recently (3) to assess whether the mycotoxin alone could elicit the natural morbidity seen in some commercial pig production. One year is a common maximum life duration in commercial pig production. Choice of feed contamination at 800 micrograms of ochratoxin A /kg feed (800 ppb) was a value several times higher than average natural contamination found in previous studies in Bulgaria (2). The chronic dosing study showed rather slight effects on liveweight gain and on kidney weight, morphology and histopathology, a much less marked morbidity than in spontaneous natural porcine nephropathy in Bulgaria.

Recently, opportunity has arisen to extend the parameters studied initially, using renal tissue archived deep-frozen after unilateral nephrectomy at the six months stage of experimental feeding on an ochratoxin-contaminated diet. The present paper reports these findings which therefore constitute a sequel to the published description (3).

Materials and methods

Pigs

From pigs given *ad libitum* a diet containing ochratoxin A at 800 ppb continuously from about 10 weeks of age, and corresponding controls (3), four animals (one male and one female each from treated and control groups) were selected at random after 6 months for unilateral nephrectomy. Immediately after excision the left kidney was weighed and a segment cut across cortex and medulla for histological fixation. Other parallel segments were immediately frozen in dry ice and subsequently stored at -20C, pending chemical and biochemical analysis.

When opportunity arose to analyse for DNA adducts a kidney segment was divided into three regions (cortex, cortico-medullary junction and medulla) before analysis. For expression of cytochrome P_{450} enzymes and

arachidonic acid enzymes, a segment was divided at the cortico-medullary junction into only two parts, mainly cortex and mainly medulla. Measurement of OTA content was made on three slices of undivided tissue (each 300-400 mg) traversing both cortex and medulla.

Chemicals for ^{32}P- postlabelling assay for DNA-adducts:

Proteinase K, spleen phosphodiesterase, RNase A and T1, and micrococcal nuclease were purchased from Sigma (L'Isle d'Abeau, France); T4 polynucleotide kinase and [γ ^{32}P-ATP], 444 Tbq /mmol (6000 Ci/mmol) were from Amersham (Les Ullis, France); nuclease P1 from Boehringer (Manheim, Germany); rotiphenol from Rothsichel (Lauterbourg, France); cellulose MN 301 was from Macherey Nagel (Düren, Germany); polyethyleneimine (PEI) was from Corcat (Virginia Chemicals, Portsmouth, VA, USA). The PEI/cellulose TLC plates were prepared in the Toulouse laboratory.

Analytical methods

DNA adduct analysis was as previously described (4). Microsome extraction and western blot analysis was as described in (5). EROD, PROD and ECOD activities were measured as described in (6).

Ochratoxin A analysis was as follows; 0.2 g kidney tissue was homogenized at 4°C in 4 ml of a solution of $MgCl_2$ (0,1M) in 0.05 M HCl (pH 1.2). The homogenate was then extracted twice by shaking with 4 ml chloroform for 10 minutes. The chloroform extracts were pooled and the supernatant discarded. The pooled chloroform phases were extracted twice by shaking with 8 ml of 0.1 M $NaHCO_3$ solution for 5 minutes. After each extraction, the mixture was centrifuged and the bicarbonate (upper) phase collected. The two bicarbonate extracts were pooled and adjusted to pH 1.5 with HCl. The acidified aqueous extract was extracted again with chloroform, first with 16 ml and then 8 ml, for 10 minutes. The two chloroform extracts were pooled and evaporated to dryness under nitrogen. After addition of 200 µl of methanol, the sample was analysed by HPLC–spectrofluorimetry using a Gilson 811B dynamic chromatography pump, a Spectra Physics 2000 fluorescence spectrophotometer and ICS autosampler. A spherisorb column (5 µm C18, 0.46 × 25 cm), preceded by a C18 pre-column, both from ICS was used. The system was run isocratically with methanol/acetonitrile/sodium acetate 0.005 M/acetic acid glacial (300/300/400/14). For the analysis of OTA, the excitation and emission wavelengths were, respectively, 335 and 465 nm. A mixture of OT beta, OT alpha, 4-OH-OTA, open-ring OTA (OP-OTA) and OTB was also chromatographed in the same conditions.

Results

Autoradiographs showing DNA adduct patterns in pig kidney are shown in Fig. 1. No DNA adducts could be observed in either control animals (male and female). In pigs fed with OTA, DNA adducts occurred in all parts of the male kidney but were only observed in the cortex of the female pig (Table 1). Qualitatively, it appeared that two spots were common to four autoradiographs and that perhaps the closest pattern similarity was between female cortex and male cortico-medullary regions. Some adducts were only observed in the male.

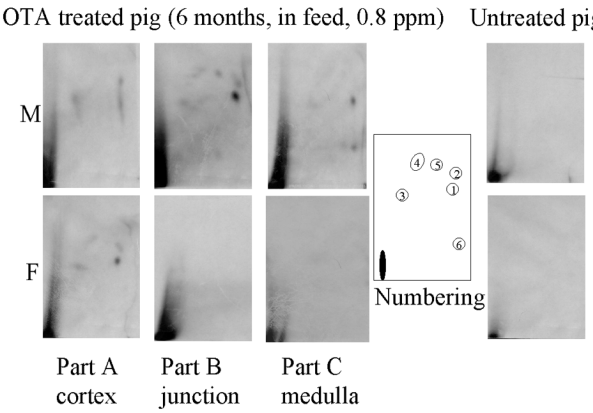

Fig. 1. Autoradiographs from ^{32}P postlabelling for DNA adducts in parts of kidney of pigs given feed with or without added ochratoxin A

The HPLC profiles for OTA analysis are shown in Figs. 2 & 3. In addition to the OTA extracted from kidneys (98.3 and 103.8 ng/g for OTA-treated male and female, respectively; 12.1 and 9.6 ng/g for controls) small amounts of several other more polar compounds were detected by their emitted fluorescence after UV excitation optimum for OTA (Figs 2 & 3 and Table 2). Specifically, the group of three fluorescent compounds eluting during 9 to 11 minutes may be the same in each OTA-treated pig, as also the compound eluting at 6 min. Occurrence of these compounds in Figs. 2 & 3, expressed quantitatively relative to OTA, is in Table 2, incidence of the principal ones being in the range 1-6% of OTA. Even in control animals, ingesting natural OTA

Table 1. Incidence of DNA adducts in renal cortex (Part A), cortico-medullary region (Part B), and medulla (Part C) of pigs fed 0.8ppm OTA, 6 months

Spot tissues		1	2	3	4	5	6	Total adducts per 10^9 nucleotides
Male	Part A	16.8 ± 4	4.5 ± 0.2	7.4 ± 2	ND	ND	ND	28.5 ± 0.5
	Part B	29.3 ± 5	11 ± 2	13 ± 4	1 ± 0.5	4 ± 0.9	ND	58 ± 10
	Part C	27 ± 5	ND	4.3 ± 0.4	ND	ND	10 ± 2	41 ± 3
Female	Part A	18 ± 2	4.6 ± 1.5	7.2 ± 2	4.5 ± 1	ND	ND	34 ± 5
	Part B	ND	ND	ND	ND	ND	ND	0
	Part C	ND	ND	ND	ND	ND	ND	0

contamination in feed, traces of similar compounds were evident. Seven different compounds were detected in kidney of the male pig, whereas only five were found in the female. Except for the compound called Z, the amounts of these compounds are higher in male kidney. Some compounds had retention times very close to those of OT beta, OT alpha, OP-OTA and 4-OH-OTA.

Activities of ECOD (reflecting mainly CYP 2A, but also 1B, 2B & 2E), PROD (reflecting CYP 2B) and EROD (reflecting CYP 1A & 1B) were analysed in microsomes from the cortex and medulla of kidneys (Table 3). Untreated female cortex exhibited higher PROD and ECOD activities than male cortex, whereas male cortex exhibited higher EROD activity.

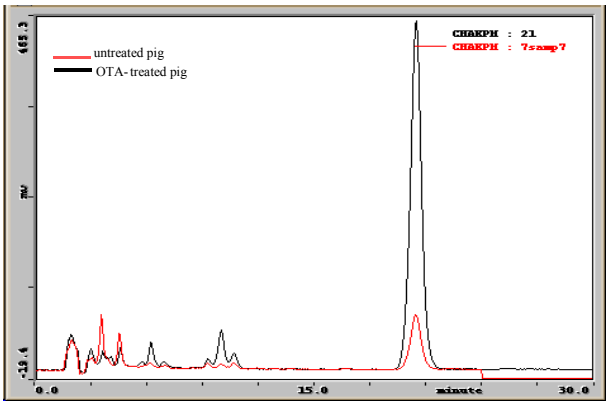

Fig. 2. HPLC profile of OTA and associated fluorescent compounds isolated from kidney of male pigs with and without added OTA

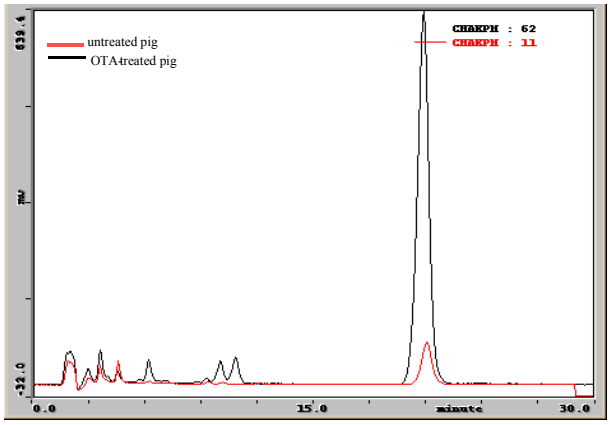

Fig. 3. HPLC profile of OTA and associated fluorescent compounds isolated from kidney of female pigs with and without OTA

Amongst the results of western blot analysis, measuring expression of protein synthesis (Fig. 4), some complementary patterns were evident concerning COX_1 and COX_2 both in cortex and medulla. OTA treatment was associated with reduced expression of COX_1 in the male, but an increase in the female to which its homeostatic and protective effects could be applied. Conversely, OTA increased COX_2 in the male, possibly adding its more malign activity to the already reduced homeostasis conferred by reduced COX_1. Microsomal preparations from mainly cortex and mainly medulla regions, incubated *in vitro* with DNA and OTA, gave the DNA adduct patterns displayed also in Fig. 4. Considerable qualitative homogeneity was evident particularly concerning two radioactive spots in autoradiographs. Both cortex and medulla in both sexes was an effective source of microsome activity; no radioactive nucleotides were found without OTA in 'control' incubations (Fig. 4).

Table 2. Estimated incidence of polar fluorescent compounds relative to the concentration of OTA

Retention time in minutes	4.53 *OTβ*	5.78 *OTα*	6.25 *X*	7.03 *OPOTA*	9.38 Y	10 *4OHOTA*	10.78 *Z*	11.24 *OTB*
Male	3.91%	0.64%	3.13%	1%	1.32%	6.25%	2.50%	ND
Female	1.8%	ND	2.5%	ND	0.29%	2.86%	3.57%	ND

Table 3. PROD, EROD, ECOD activities in kidney of pigs with or without OTA 0.8ppm, 6 months

	Sample	Activity (pmol/min/mg of microsomal proteins)		
		PROD	ECOD	EROD
Cortex	Untreated male	1.15 ± 0.2	70 ± 5	30.3 ± 0.5
	OTA treated Male	2.3 ± 0.2 a*	90 ± 5 a*	69 ± 0.5 a*
	Untreated Female	2.65 ± 0.2 c*	239 ± 7 c**	12.5 ± 0.5 c**
	OTA treated Female	2.4 ± 0.2	124 ± 5 b **; d *	74 ± 1 b**
Medulla	Untreated male	4.85 ± 0.4	55 ± 2	63.4 ± 0.5
	OTA treated Male	2.7 ± 0.3 a*	80 ± 5 a*	82 ± 1 a*
	Untreated Female	2.6 ± 0.2 c*	140 ± 5 c**	54.1 ± 0.5 c*
	OTA treated Female	7 ± 0.2 b**; d**	72 ± 5 b**	53 ± 0.5 d**

a:statistical difference between untreated *versus* treated male;
b:statistical difference between untreated *versus* treated female;
c:statistical difference between untreated male *versus* untreated female;
d: statistical difference between treated male *versus* treated female
* $p<0.05$; ** $p<0.01$

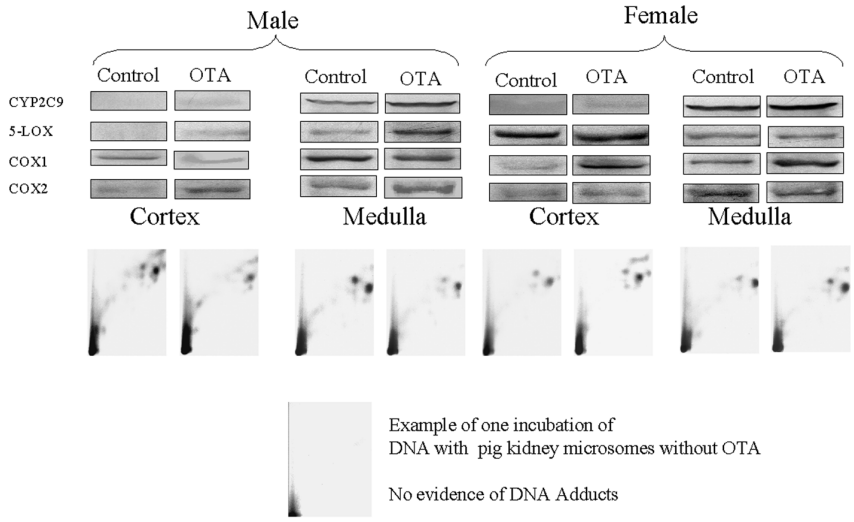

Fig. 4. Western blots and ^{32}P post-labelling autoradiographs from comparison of cortical and medullary microsome activities in male and female pigs with and without added OTA in feed for 6 months.

Discussion

Relative concentrations of ochratoxin A measured in kidney of treated and control pigs corresponded broadly with those of serum concentrations measured earlier (3). As described in 3, there was already evidence of some indigenous ochratoxin A in commercial pig feed, implied at about 40 ppb, revising the actual OTA concentration to about 840 ppb in the treated diet. This is confirmed by the analysis of kidney tissue. Thus it can be calculated that an amount of OTA equivalent to only about 2% of the daily intake resided in the kidneys. Comparison with standard retention times for OT beta, OT alpha, OTB and OP-OTA indicated that OTB was not present. However, the compound eluting at about 7 minutes may be OP-OTA, and that at 4.6 min particularly in the male control is likely to be OT beta. The compound eluting at 5.8 minutes could be OT alpha, and that at 10 minutes could be 4-OH-OTA. This clearly leaves three unrecognized polar compounds associated with the OTA treatment.

The new data is in the context of 6 months of continuous exposure to diets contaminated with OTA, either naturally slightly or experimentally increased approximately 20-fold. Initially this stage was part of a planned long-term study that unfortunately had to terminate at one year for economic reasons. The endpoint at one year showed only small adverse response expressed as slightly hypertrophied kidneys with a faintly mottled surface, though probably not recognizable as a cause of concern during meat inspection in routine slaughterhouse processing (3). At 6 months, the 20-fold difference in OTA contamination between treated and untreated feeds had been closely reflected in the serum OTA concentration (3). However, in the present analyses only a ten-fold difference was found in kidney tissues, compensated somewhat on a whole organ basis [about 14-16 µg OTA in a treated kidney and 1.0-1.3 µg OTA in a control], if the increased weight of the excised kidney of treated pigs is taken into account.

The pigs had been castrated at 12-15 kg body weight, before the experiment commenced; thus subsequent expression of gender will have been independent of gonadal influences. Nevertheless, different expressions of the proteins COX_1 and COX_2 were consistent with the male probably being more influenced by OTA circulating through kidney. Indeed, COX_1 plays a crucial role in the preservation of renal function and is constitutively expressed in all tissues, whereas COX_2 is less constitutively apparent but can readily be induced (7). This induction has been related to tumour cell growth and progressive renal injury (8-11). Gender did not seem to be a factor in the microscopic histopathological changes reported for the pig kidneys at one year (3) so it is difficult to predict how different expressions of COX_1 and COX_2 might affect pigs with entire sexuality. Correspondingly, since expression of COX_1 and COX_2 is not regulated by gonadal hormones, the differential occurrence of DNA adducts seemed also to be a constitutive feature of the fundamental genetic status before castration. In previous papers we correlated animal susceptibility to OTA-induced cancer to particular metabolic profile (12-13). In the present study we note that male pig kidney exhibited more toxifying CYP, in addition to COX_2, than female kidney.

The limited amounts of DNA from these archived tissues precluded co-chromatography of post-labelling products to attempt matching of radioactive regions in analyses from whole kidney tissue with those of *in vitro* preparations. Nevertheless, it is tantalizing to consider whether the adducts-like spots in Fig. 1, correspond to any in Fig. 4. Of course, it is not possible to predict whether, or to what extent and under what circumstances, DNA adducts revealed in pigs given the 800 ppb OTA-treated feed potentially could affect animal health. Nor is there any evidence that adducts observed in autoradiographs actually involved OTA directly and constituted evidence of mutagenicity. The incidence of adducts was also of an order similar to that of other studies on rats, implying that these DNA modifications

are not notably cumulative during long-term exposure to the mycotoxin. Nevertheless, it is well known that in the carcinogenic process, the most important event is the accuracy of DNA repair, more than the level of DNA modification. For some carcinogens, it has been demonstrated that a minor modification (e.g. alkylation of thymine on position 4 badly repaired) is highly mutagenic. Thus the original objective of a lifetime study of mild chronic ochratoxicosis in the pig (3) is seen to have been even more desirable concerning evaluating the toxicity of ochratoxin A.

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ANALIZA DNA ADUKTA SADRŽAJA OCHRATOXIN-A I EKSPRESIJE ENZIMA U BUBREZIMA PRASADI IZLOŽENIH BLAGOJ EKSPERIMENTALNOJ HRONIČNOJ OCHRATOXICOSI

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Kratak sadržaj: *Dalja studija tkiva bubrega dobivenih unilateralnom nefrektomijom prasadi koji su hranjeni hranom veštački kontaminiranom ochratoxin-om A (OTA), dala je značajne nove informacije. Nije bilo dokaza DNA adukta kod kontrolnih životinja testiranih obeležavanjem pomoću ^{32}P, već je zabeleženo različito ponašanje muških i ženskih tretiranih životinja. DNA adukti su se javljali u korteksu i meduli mužjaka, ali najjasnije u kortiko-medularnoj regiji. Kod ženki su uglavnom nalaženi u korteksu. Mikrozomalna preparacija ovih bubrega, permanentno je katalizovala stvaranje DNA adukta in vitro u prisustvu OTA, dok se isti nije formirao u odsustvu OTA. Sadržaj ochratoxin-a A u bubregu tretiranih prasadi (oko 15 μg) značajno se razlikovao od 1 μg nadjenog u kontroli, što je bila posledica kontaminacije neke komercijalne hrane ochratoxin-om A u Bugarskoj, što nije dovelo do neželjenih efekata u ovim eksperimentima. Male količine fluorescentnih sastojaka polarnijih od OTA su otkrivene u ekstraktima bubrega. Aktivnosti enzima ECOD, PROD i EROD u preparacijama mikrozoma iz korteksa i medule bubrega u različitom stepenu su se razlikovale kod ženki i mužjaka, što je odražavalo različitu ekspresiju cytochrom-a P_{450}. Western blot analiza je takodje pokazala da OTA različito moduliše cyclooxigenase-ni put (COX) u mužjaka i ženke na način koji bi možda teorijski bio nepovoljniji za mužjake.*

Ključne reči: *Ochratoxin A, DNA adukti, obeležavanje ^{32}P, sinteza proteina, aktivnost renalnih enzima, prase, nefrotoksičnost*