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In vivo effects of chronic contamination with depleted uranium on CYP3A and associated nuclear receptors PXR and CAR in the rat

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Abstract

In addition to its natural presence at high concentrations in some areas, uranium has several civilian and military applications that could cause contamination of human populations, mainly through chronic ingestion. Reports describe the accumulation of this radionuclide in some organs (including the bone, kidney, and liver) after acute or chronic contamination and show that it produces chemical or radiological toxicity or both. The literature is essentially devoid of information about uranium-related cellular and molecular effects on metabolic functions such as xenobiotic detoxification. The present study thus evaluated rats chronically exposed to depleted uranium in their drinking water (1 mg/(rat day)) for 9 months. Our specific aim was to evaluate the hepatic and extrahepatic mRNA expression of CYP3A1/A2, CYP2B1, and CYP1A1 as well as of the nuclear receptors PXR, CAR, and RXR in these rats.

CYP3A1 mRNA expression was significantly higher in the brain (200%), liver (300%), and kidneys (900%) of exposed rats compared with control rats, while CYP3A2 mRNA levels were higher in the lungs (300%) and liver (200%), and CYP2B1 mRNA expression in the kidneys (300%). Expression of CYP1A1 mRNA did not change significantly during this study. PXR mRNA levels increased in the brain (200%), liver (150%), and kidneys (200%). Uranium caused CAR mRNA expression in the lungs to double. Expression of RXR mRNA did not change significantly in the course of this study, nor did the hepatic activity of CYP2C, CYP3A, CYP2A, or CYP2B.

Uranium probably affects the expression of drug-metabolizing CYP enzymes through the PXR and CAR nuclear receptors. These results suggest that the stimulating effect of uranium on these enzymes might lead to hepatic or extrahepatic toxicity (or both) during drug treatment and then affect the entire organism.

Keywords: Depleted uranium; CYP3A; PXR; CAR

Abbreviations: ALT, alanine amino-transferase; AST, aspartate amino-transferase; CAR, constitutive androstane receptor; CYP, cytochrome P450; DU, depleted uranium; PXR, pregnane X receptor; RXR, retinoid X receptor

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1. Introduction

In addition to its natural presence at high concentrations in some areas, uranium has several civilian and military applications that could cause contamination of human populations, mainly through chronic ingestion (Zamora et al., 1998; Kurttio et al., 2002). Reports describe its accumulation in some target tissues (bone, kidney) after acute or chronic contamination and show that it can produce chemical or radiological toxicity or both (Gilman et al., 1998a,b; Craft et al., 2004). No study however, showed the biological effects of low uranium levels in other organs, such as the liver, lung, intestine, and central nervous system. The literature appears essentially devoid of information about the cellular and molecular effects of uranium contamination on important metabolic functions in these organs, such as xenobiotic detoxification by drug-metabolizing enzymes. One study, however, reports histological and functional alterations of the biological detoxification system in the lung and liver following uranium contamination (Pasanen et al., 1995). These authors showed that uranium inhalation modulated cytochrome P450 (CYP) activity in both the short- and long-term. Their findings indicate that uranium toxicity may target key CYP enzymes in the liver that play a role in xenobiotic metabolism.

The CYP enzymes are a superfamily of hemoproteins involved in the metabolism of drugs, chemicals, and such endogenous substrates as steroids, fatty acids, prostaglandins, and vitamins (Nebert and Russell, 2002). They are present in many organs and tissues, but concentrate most abundantly in the liver (Debri et al., 1995). Most CYPs that biotransform xenobiotics and drugs belong to the CYP1A, CYP2B, and CYP3A families. Their gene expression is affected by chemical inducers, nutritional conditions, growth factors, and inflammation mediators (Morgan, 1989; Renton and Knickle, 1990; Wong et al., 1993; Kirby et al., 1994).

Detoxification and removal of many compounds, toxic metabolites, exogenous drugs, and hormones are critical for the homeostasis of the organism. These processes are mediated by CYPs and by the nuclear receptor signaling pathways that orchestrate the expression of the detoxifying enzymes. Two orphan nuclear receptors, the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR), mediate the effects of xenobiotics and therapeutic drugs on regulation of the *CYP3A* and *CYP2B* genes (Xie et al., 2004). Sequence analysis shows that PXR and CAR are closely related to each other, and both require heterodimerization with a retinoid X receptor (RXR) for high-affinity DNA binding (Blumberg and Evans, 1998).

Uranium-induced alterations of these metabolic processes may induce the onset of various pathologies, including hepatitis, cholestasis, and defects of drug metabolism. The objectives of this study were thus to evaluate the possible biological effects of chronic exposure to depleted uranium (DU) on major hepatic and extrahepatic CYPs involved in xenobiotic detoxification (CYP1A1, CYP2B1, and CYP3A1/2) and on two nuclear receptors (PXR and CAR) that are central regulators of detoxification enzymes.

2. Materials and methods

2.1. Chemicals and materials

Depleted uranyl nitrate hexahydrate (DU) was obtained from V.W.R. (Fontenay-sous-Bois, France). Hydroxypropyl-β-cyclodextrin (HPβCD) was a gift from Dr. Michel Riottot of Université d'Orsay, France. Chemicals and solvents of the highest purity available were purchased from Sigma Diagnostics (Isle d'Abeau Chenes, France).

2.2. Animals

Sprague–Dawley male rats (Charles River, France), weighing 250 g each, were divided into two groups of 10 rats each: a control group and an experimental group. The rats were housed in pairs, with a 12-h light/12-h dark cycle (light on: 08:00 h/20:00 h) and a temperature of $22 \pm 1\,^{\circ}C$. Water and food were delivered ad libitum. All experimental procedures were approved by the Animal Care Committee of the Institute of Radioprotection and Nuclear Safety and complied with French regulations for animal experimentation (Ministry of Agriculture Act No. 87-848, October 19, 1987, modified May 29, 2001).

2.3. Contamination

The rats in the experimental group were exposed to DU in their drinking water for 9 months, at a

dose of 40 mg/l (1 mg/(rat day)) (AREVA-COGEMA, France).

2.4. Plasma and organ sampling

Non-fasting animals were anesthetized by inhalation (TEM anesthesia, Limoges, France) of 95% air/5% isoflurane (Forène, Abbott France, Rungis) and killed by intracardiac puncture with a 2-ml insulin syringe to collect blood. After decapitation the brain (cortex) was removed, frozen immediately in liquid nitrogen and stored at −80 °C until processed for RNA studies. The abdomen was opened and the liver, kidney, lung, and small intestines were rapidly excised, weighed, and apportioned for preparing cellular fractions or storage at −80 °C for future use. Blood was centrifuged at 4000 × *g* at 4 °C for 10 min to collect the plasma. We used an automated Technicon RA-XT (Bayer Diagnostics) system to measure plasma creatinine, urea, alanine amino-transferase (ALT), aspartate amino-transferase (AST), gamma-glutamyl-transpeptidase (GGT), alkaline phosphatase (AP), bilirubin, iron, total cholesterol, and triglycerides (biological chemistry reagents, Bayer Diagnostics) in the control and DU-exposed rats.

2.5. RNA extraction and RT-PCR analysis

The mRNA levels of the CYPs, the nuclear receptors (PXR, CAR, and RXR), and the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) were measured by real-time PCR. Total RNA was prepared with the Rneasy total RNA isolation Kit (Qiagen, France) according to the manufacturer's instructions. The cDNA was produced from 1 μg of total RNA by reverse transcription with 200 U of Superscript reverse transcriptase (GIBCO) in a 20-μl reaction containing 1× superscript buffer (GIBCO), 1 mM 2-deoxynucleotide 5′-triphosphate, 20 ng random hexamer, 10 mM DTT, and 20 U Rnase inhibitor. After incubation for 50 min at 42 °C, the reaction was terminated by a denaturing enzyme for 10 min at 70 °C. RNA integrity was confirmed by denaturing agarose gel electrophoresis and ethidium bromide staining. PCR amplification of the CYPs, PXR, CAR, and RXR used Syber PCR master mix. Optimized PCR used the Abi Prism 7000 Sequence detection system (Qiagen). PCR fluorescent signals were normalized to the fluorescent signal obtained from the housekeeping gene HPRT for each sample. Sequences for the forward and reverse primers used in the present study are listed in Table 1.

2.6. Liver microsome preparation

Following anesthesia, the abdomen was surgically opened to permit removal and rapid chilling of the liver in ice-cold buffer (KH_2PO_4 50 mM, sucrose 300 mM, dithiothreitol 0.5 mM, EDTA 10 mM, NaCL 50 mM, pH 7.4). The liver was sliced and a 1 g portion was taken

Table 1
Nucleotide sequences of PCR primers

Gene	Primer sequence	PCR product (bp)	Reference
CYP1A1	Forward: 5′-CCT GGA GAC CTT CCG ACA TTC-3′ Reverse: 5′-GGG ATA TAG AAG CCA TTC AGA CTT G-3′	70	Su and Waxman (2004)
CYP2B1	Forward: 5′-GCT CAA GTA CCC CCA TGT CG-3′ Reverse: 5′-ATC AGT GTA TGG CAT TTT ACT GCG G-3′	109	Su and Waxman (2004)
CYP3A1	Forward: 5′-GAT GTT GAA ATC AAT GGT GTG T-3′ Reverse: 5′-TTC AGA GGT ATC TGT GTT TCC-3′	289	Rekka et al. (2002)
CYP3A2	Forward: 5′-AGT AGT GAC GAT TCC AAC ATA T-3′ Reverse: 5′-TCA GAG GTA TCT GTG TTT CCT-3′	252	Rekka et al. (2002)
CAR	Forward: 5′-ACC AGT TTG TGC AGT TCA GG-3′ Reverse: 5′-CTT GAG AAG GGA GAT CTG GT-3′	268	Rychlik et al. (2000)
RXR	Forward: 5′-CGC AAA GAC CTG ACC TAC ACC-3′ Reverse: 5′-TCC TCC TGC ACA GCT TCC C-3′	133	Primer express
PXR	Forward: 5′-GAC GGC AGC ATC TGG AAC TAC-3′ Reverse: 5′-TGA TGA CGC CCT TGA ACA TG-3′	112	Su and Waxman (2004)

and homogenized in buffer, as previously described by Souidi et al. (1998). The homogenate was centrifuged for 20 min at 20,000 × *g* and the supernatant centrifuged at 100,000 × *g* for 1 h. The pellet was resuspended in buffer and again centrifuged at 100,000 × *g* for 1 h. The microsomal pellet was homogenized in buffer, sampled, and stored at −80 °C until required.

2.7. Testosterone hydroxylase assay

Testosterone hydroxylase activity was determined with a technique adapted from Li et al. (2002) after minor modification. Briefly, for a total volume of 500 μl, each assay tube contained KH_2PO_4 75 mM (pH 7.4), EDTA 1 mM, DTT 0.5 mM, $MgCl_2$ 5 mM, NADPH 1 mM, 250 μg of liver microsomal protein, and testosterone 200 μM and was solubilized by 160 μl of buffer potassium phosphate containing 45% (w/v) HPβCD (for an enzyme assay 10 μl of this solution contained 100 nmol of testosterone and 4.5 mg HPβCD). The preincubation time, in which only NADPH was omitted, was 5 min at 37 °C, and the incubation time following initiation of the assay with NADPH was 15 min. The reaction was terminated by the addition of 2 ml methanol/chloroform (2:1, v/v) and 10 μl cortexolone (10 nmol) as an internal standard. Testosterone and its metabolites in the microsomal samples were extracted with 2 ml chloroform and 1.5 ml water. The tubes were again vortexed for 1 min, and 2 ml of the organic phase was collected and evaporated (nitrogen gas). The dry residue was reconstituted with 250 μl of 26% acetonitrile in water. An aliquot of 15 μl of the reconstituted solution was injected into the HPLC for analysis with a LiChrospher RP-18 (250 mm/5 μm particle size, flow rate of 1.3 ml/min, absorbance 247 nm). The mobile phase was acetonitrile:water (26:74). Activity of the testosterone hydroxylases (2α-testosterone-hydroxylase, 6β-testosterone-hydroxylase, 7α-testosterone-hydroxylase, and 16α-testosterone-hydroxylase) is expressed as picomoles per minute per whole liver.

2.8. Other assays

Microsomal protein content was determined as previously described, according to the Lowry method with bovine serum albumin as a standard (Lowry et al., 1951).

2.9. Statistical analysis

Results are reported as means ± S.E. Statistical analyses were performed with Student's *t*-test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Physiological and plasma parameters

Table 2 shows the physical and biochemical data for rats contaminated with DU for 9 months (1 mg/(rat day)) and control rats. The experimental contamination of this study did not affect food intake, weight gain, or the animals' general health status. The macroscopic appearance of the liver, kidney, lungs, intestines, and brain was normal and did not differ from that of the controls, thereby indicating that ingestion of DU at this quantity in drinking water did not induce major adverse effects in rats. There was no histological evidence of structural changes in the liver and kidney after uranium contamination (results not shown). DU increased plasma total cholesterol levels (>40%) but not plasma triglycerides. Concentrations of biochemical markers associated with liver function, including AST and ALT, were significantly lower in the uranium-contaminated animals.

Table 2
Physical and biochemical parameters in controls and uranium contaminated rats

	Control ($n = 10$)	Contaminated ($n = 10$)
Final body weight (g)	717 ± 20	740 ± 44
Liver (g)	23 ± 1	24 ± 2
Kidney (g)	4.5 ± 0.5	4.1 ± 0.3
Cholesterol (mmol/l)	2.89 ± 0.19	4.08 ± 0.54*
Triglyceride (mmol/l)	1.41 ± 0.12	1.80 ± 0.2
ALT (U/l)	73.50 ± 11.80	43.70 ± 6.57*
AST (U/l)	166.50 ± 23.15	98.20 ± 10.60**
AP (U/l)	158.60 ± 15.92	133.00 ± 10.25
GGT (U/l)	2.10 ± 0.55	3.20 ± 0.83
Bilirubine (μmol/l)	1.25 ± 0.29	1.16 ± 0.15
Creatinine (μmol/l)	48.60 ± 1.33	45.09 ± 4.35
Urea (mmol/l	5.74 ± 0.18	5.45 ± 0.27

Note: Results are expressed as means ± S.E.; *n*, number of rats per group.
* $p < 0.05$.
** $p < 0.02$.

Kidney function indicators (creatinine and urea) were unaffected.

3.2. Analysis of CYP mRNA levels

The effects of this 9-month contamination on the expression of CYP3A1, CYP3A2, CYP2B1, and CYP1A1 mRNA in rats are reported in Fig. 1. The mRNA level of the reference gene was unchanged in the liver, kidney, intestines, lungs, and brain after 9 months of contamination with DU. As Fig. 1 shows, CYP3A1 mRNA expression in contaminated compared with control animals increased in the brain (200%, $p < 0.05$), liver (300%, $p < 0.01$), and kidney (900%, $p < 0.05$). Similarly, the CYP3A2 mRNA level increased in the lungs (300%, $p < 0.01$) and liver (200%, $p < 0.05$) but did not change significantly in the kidney. CYP2B1 mRNA expression increased in the kidney (300%, $p < 0.01$), while CYP1A1 mRNA expression did not change significantly.

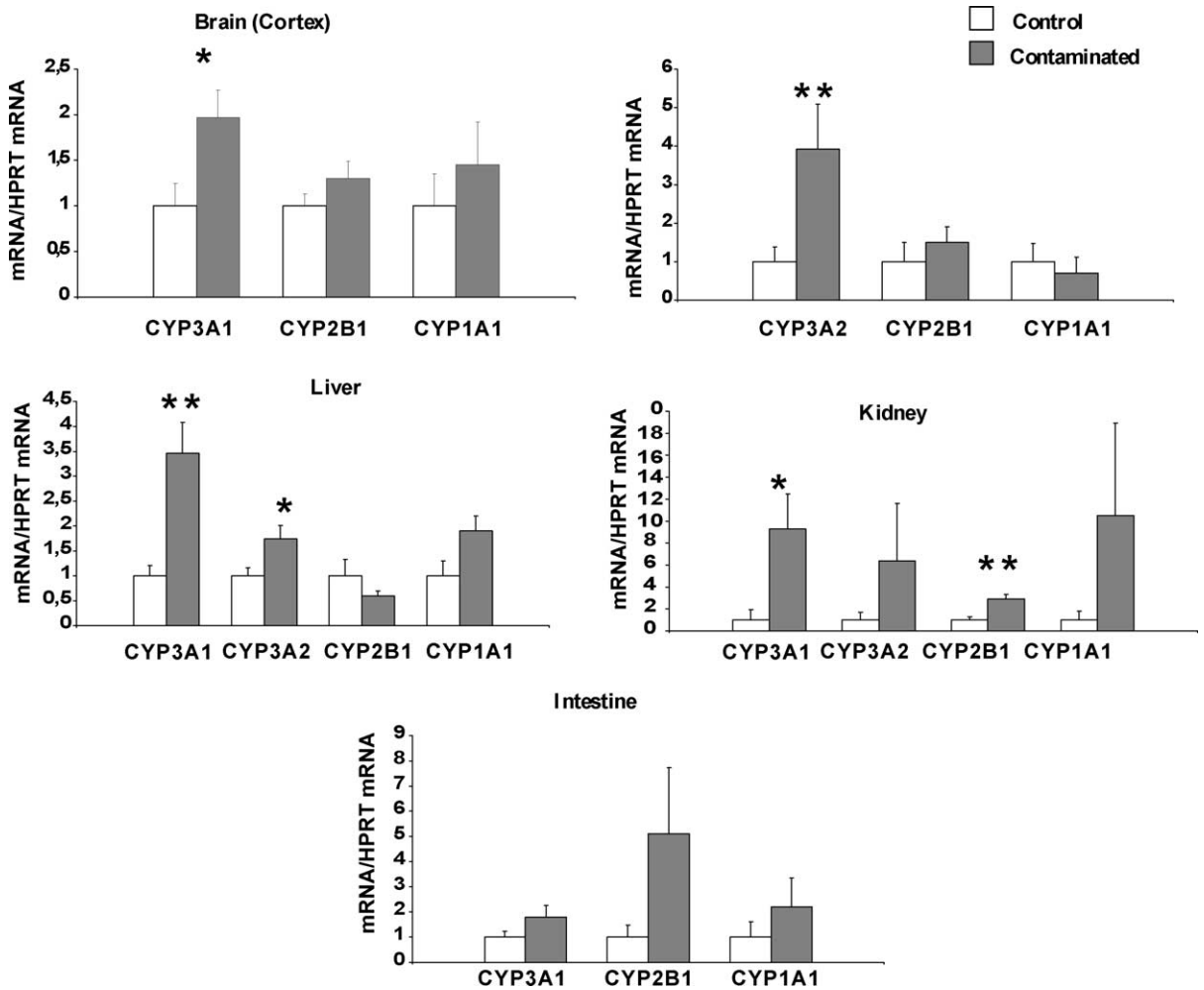

Fig. 1. Relative mRNA expression of CYP isoforms (1A1, 2B1, 3A1, 3A2) in the brain (cortex), lung, liver, kidney, and intestine of rats contaminated for 9 months with depleted uranium (40 mg/l) in their drinking water. The results are expressed as a ratio to the mRNA levels of the reference gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). Data are the means ± S.E. ($n = 6$); $^{*}p < 0.05$, $^{**}p < 0.01$ significantly different from control value.

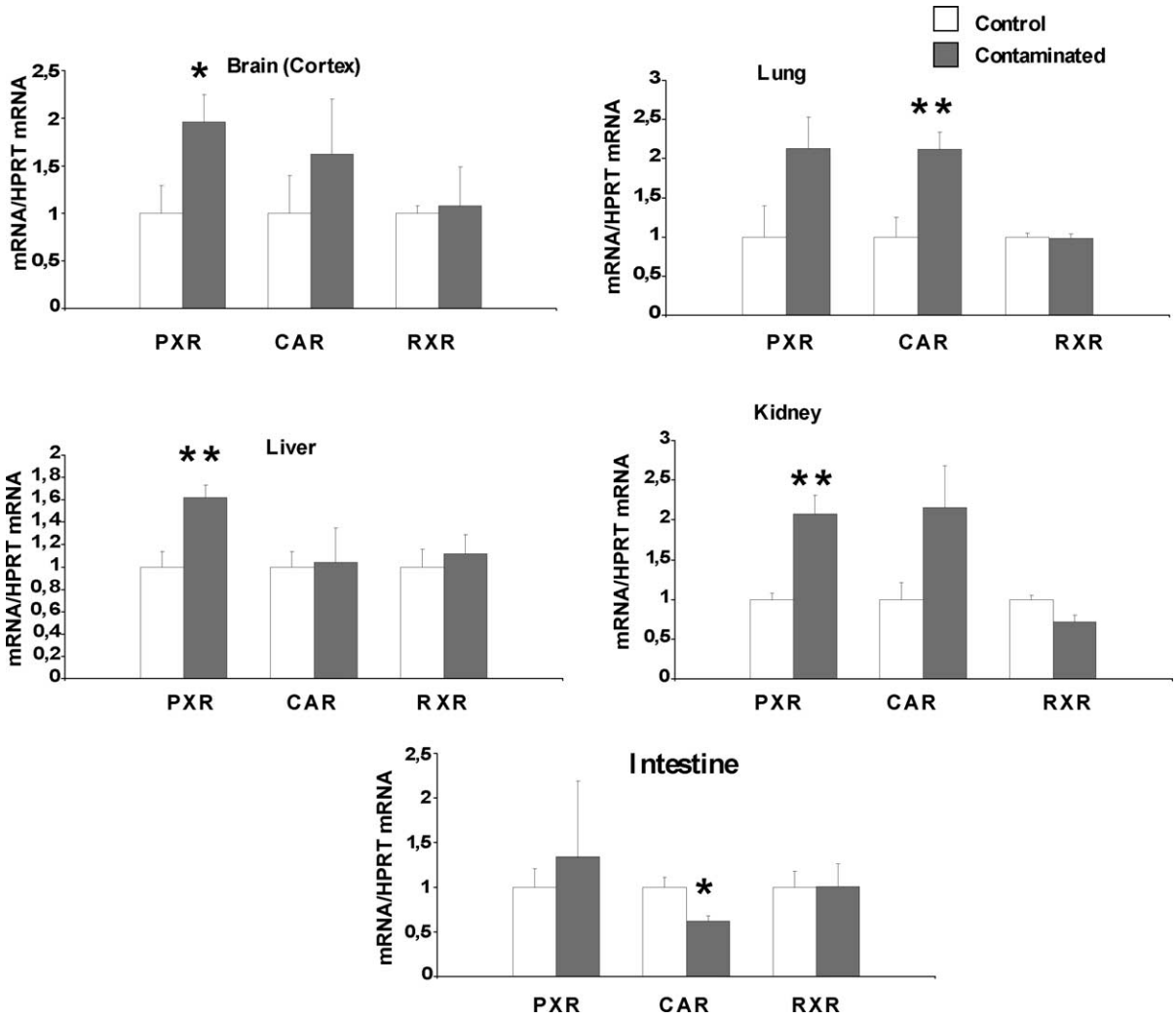

Fig. 2. Relative mRNA expression of PXR, CAR, and RXR in the brain (cortex), lung, liver, kidney, and intestine of rats contaminated for 9 months with depleted uranium (40 mg/l) in their drinking water. The results are expressed as a ratio to the mRNA levels of the reference gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). Data are the means ± S.E. ($n = 6$); $^{*}p < 0.05$, $^{**}p < 0.01$ significantly different from control value.

3.3. Analysis of PXR, CAR, and RXR mRNA levels

We evaluated the effects of DU contamination on the expression of mRNA of the nuclear receptors PXR, CAR, and RXR (Fig. 2) by quantifying it and reporting it as a ratio to HPRT. As Fig. 2 shows, PXR mRNA levels increased in the brain (200%, $p < 0.05$), liver (150%, $p < 0.01$), and kidney (200%, $p < 0.01$), compared with controls. Uranium contamination caused expression of CAR mRNA in the lungs to double ($p < 0.01$), but that of RXR did not change significantly.

3.4. Analysis of enzyme activity in the liver

Rat liver microsomes contain CYP enzymes that are known to metabolize testosterone (Chung et al., 1998). The CYP2C and CYP2B isozymes are responsible, respectively, for the 2α- and 16α-hydroxy testosterone metabolites. The 6β-hydroxy testosterone metabolite

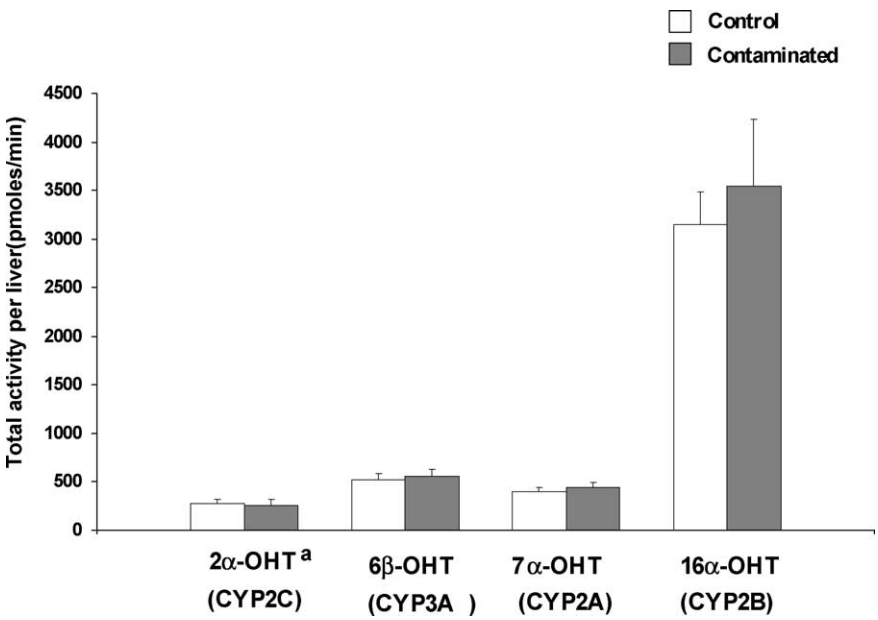

Fig. 3. In vitro metabolism of testosterone by hepatic microsome samples from male rats that were contaminated with uranium for 9 months. The associated isoforms of testosterone hydroxylation were: 2α (CYP2C), 6β (CYP3A), 7α (CYP2A) and in position 16α (CYP2B). Data are the means ± S.E. ($n = 6$); $^{*}p < 0.05$, $^{**}p < 0.01$ significantly different from control value. The abbreviation designates the regio- and stereochemistry of hydroxylated testosterone metabolite (i.e. 2α-OHT denotes 2α-hydroxytestosterone).

is the major product of CYP3A isozyme metabolic activity. CYP2A metabolizes testosterone, mainly to 7α-hydroxy testosterone. Testosterone metabolism is a useful indicator of these CYP enzyme activities. Here, analysis of the testosterone metabolites demonstrated that hepatic activity of CYP2C, CYP3A, CYP2A, and CYP2B did not change significantly after 9 months of chronic DU contamination (Fig. 3).

4. Discusssion

The growing use of DU in today's society requires that we study its biological effects. Its toxic effects have been reported in vitro (demonstration of its cellular toxicity (Miller et al., 2003, 2004) and in vivo, in animals (nephrotoxicity; Leggett, 1989). Effects on physiological systems such as reproduction and development have also been observed (Domingo, 2001). The rare studies in humans show biological effects on some physiological systems after chronic ingestion of uranium and thereby suggest its potential toxicity (Zamora et al., 1998; Kurttio et al., 2002).

To continue the investigation in this area, we studied the effects of DU on CYP enzymes in rats. This class of enzyme is particularly important in physiology; it participates in essential metabolic pathways of various substances, in particular, xenobiotics, including drugs (Hasler et al., 1999).

The rats in our experiment were contaminated at low doses by chronic ingestion during 9 months. The daily dose was 1 mg/(rat day) of DU, administered in the drinking water (40 mg/l). This concentration was chosen to correspond to values close to those found in some area in Finland (Auvinen et al., 2002) and Mexico (Hakonson-Hayes et al., 2002).

Different animal studies have shown that contamination with toxic doses of uranium induces harmful changes to the kidneys (Gilman et al., 1998a,b) and liver (Goel et al., 1979) as well as damaging general health and vital status (Gilman et al., 1998a,b). In the DU contamination conditions of our study, we observed no effects on their food intake, weight, or general health status. Histologic examination showed that the liver and kidneys of these animals, like those of the controls, appeared normal. These findings indi-

cate that DU ingestion at a dose of 1 mg/(rat day) for 9 months did not induce any visible toxic effect in the rats.

Blood tests showed no effect on renal function markers such as creatinine and urea and thus confirmed that the dose used in this study was not nephrotoxic. On the other hand, DU contamination did affect the plasma levels of some biochemical markers in our animals; total cholesterol increased while some liver function markers, including ALT and AST, decreased. The increased plasma levels of cholesterol have previously been described (Keller, 1953) with high doses of uranium. The effect of uranium on plasma cholesterol suggests that it affects the agents involved in cholesterol homeostasis and more particularly in the major organs of cholesterol metabolism, such as the liver and the intestines. An increase in plasma phospholipids was also observed and probably expressed changes in the organism's lipid metabolism. The authors suggest that uranium may thus be involved in the genesis of cardiovascular disease.

We also observed that some plasma markers of liver function, including ALT and AST, diminished after uranium contamination. This finding confirms the results of Ortega et al. (1989) and probably indicates a disruption of liver function. A diminution of plasma levels of these two proteins is described in humans during some viral liver diseases (Fabrizi et al., 2001) and in animals, in some conditions of hepatotoxicity (Solter et al., 2000). As a general rule, however, damage to liver function is characterized by an increase in plasma levels of ALT and AST, due to the cell cytolysis associated with liver diseases (Dufour et al., 2000), as Domingo et al. (1987) observed after high level of subcutaneous DU administration. These contradictory results are probably due to the quantity of uranium used and the duration of contamination.

In our study, we observed that uranium affected the CYPs involved in xenobiotic metabolism. We found that DU induces the expression of CYPs, including CYP3A1, CYP3A2, and CYP2B1, all important in metabolizing drugs as well as endogenous substances (You, 2004). The induction by uranium of these enzymes has been observed in different tissues, including the liver, kidneys, lungs, and brain (cortex). These results suggest that DU can induce the gene expression of some enzymes required for the phase I metabolism of xenobiotics. To our knowledge only one study has shown uranium to have any effect on the superfamily of CYPs and more particularly on those involved in the metabolism of xenobiotics and testosterone (Pasanen et al., 1995). In that study, the authors reported that inhalation of uranium inhibited the enzyme activity of some hepatic and pulmonary CYPs and suggested that these effects may damage the liver and lungs.

As a general rule, heavy metals, such as cadmium, arsenic, and zinc, are described as inhibitors of CYP activity in xenobiotic metabolism, through post-transcriptional regulation (Moore, 2004). Some heavy metals affect the metabolism of heme required for the catalytic function of the CYPs. Recent studies show that some of these metals may also act at a transcriptional level via transcription factors (Abu-Bakar et al., 2004; Tully et al., 2000): cadmium induces the expression of CYP2A mRNA and lead induces that of CYP1A mRNA. Our study suggests that DU can behave like other heavy metals and induce transcription of CYPs, particularly those in the CYP3A and CYP2B families.

Uranium in the liver did not affect the enzymatic activity of 2α-testosterone-hydroxylase (CYP2C), 6β-testosterone-hydroxylase (CYP3A), 7α-testosterone-hydroxylase (CYP2A), or 16α-testosterone-hydroxylase (CYP2B). One hypothesis is that in these conditions of long-term uranium contamination, the organism tries to compensate for the increase in CYP mRNA expression by post-transcriptional regulation to maintain a basal level of enzyme activity. This mechanism may play an important role in the adaptive response to uranium and in the protection against liver toxicity. A study of cadmium and its effects revealed this adaptive type of regulation. Cadmium induced a diminution in the enzyme activity of some CYPs involved in xenobiotic metabolism; at the same time it led to an increase in their mRNA levels (Elbekai and El-Kadi, 2004).

This study shows for the first time that uranium also affects expression of the nuclear receptors involved in gene control of CYP3A and CYP2B. That is, at the same time as expression of CYP3A1/2 mRNA increases in various tissues and CYP2B1 mRNA increases in the kidneys, PXR and CAR expression also increases. These two nuclear receptors are reported to have a primary role in the regulation of CYPs involved in xenobiotic metabolism (Sonoda et al., 2003). These results suggest that chronic uranium contamination

induced an increase in CYP3A and CYP2B expression by increasing PXR and CAR expression. This in turn suggests that uranium in the organism can interfere with the metabolism of xenobiotics and more particularly with the nuclear receptors, through a mechanism as yet unknown. The effects of uranium on CYP3A, CYP2B and on the PXR and CAR nuclear receptors thus raise public health questions.

In conclusion, our study suggests that DU induces expression of CYP3A and CYP2B, probably via an increase in the expression of PXR and CAR. These results indicate that chronic low-dose contamination by DU may produce public health consequences, such as alterations in drug metabolism during drug treatment and dysfunctions in steroid hormone metabolism.

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