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Effects of serum from patients with chronic renal failure on rat hepatic cytochrome P450

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1 In humans, chronic renal failure (CRF) is associated with decreased hepatic drug metabolism, particularly that mediated by the cytochrome P450 (P450). The mechanisms remain poorly understood. The present study aimed to investigate the effects of the serum of patients with CRF on liver P450, and to evaluate whether renal replacement therapies (dialysis or transplantation) impede the inhibition of CRF serum on P450.

2 Rat hepatocytes were incubated for 24 h with serum from patients with severe CRF and from controls to measure (1) P450 level, (2) protein expression and mRNA levels of P450 isoforms and (3) metabolic activities of CYP3A and CYP1A. Similar experiments were performed with serum of patients once on chronic hemodialysis and after kidney transplantation.

3 In rat hepatocytes incubated for 24 h with serum from patients with CRF, P450 level and protein expression, as well as mRNA levels of P450 isoforms (CYP1A2, 2C6, 2C11, 2D1/2D2, 3A2 and 4A1/4A3), were decreased by more than 45% ($P<0.001$) compared to control serum, while the levels of CYP2E1 were not modified. CYP3A and CYP1A activities were decreased by 51 and 59% ($P<0.001$), respectively. The inhibitory effect of serum obtained from patients before first dialysis was similar after 1 or 6 months on chronic hemodialysis but was lost after successful kidney transplantation. In CRF serum, the fraction containing proteins between 10 and 15 kDa decreases P450.

4 Human uremic serum contains mediator(s) that decreases rat hepatic P450 activity and expression secondary to reduced gene expression. The inhibitory effect of serum persists even after initiation of dialysis, but disappears after normalization of renal function following kidney transplantation.
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Abbreviations: AST, aspartate amino *S*-transferase; CRF, chronic renal failure; EROD, 7-ethoxyresorufin-*O*-deethylase; ESRD, end stage renal disease; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; P450, cytochrome P450; PTH, parathyroid hormone; RT–PCR, reverse transcriptase–polymerase chain reaction; s.e.m., standard error (of estimated mean value)

Introduction

Despite dosage adjustment for the decrease in renal function, patients with chronic renal failure (CRF) present an elevated number of drug adverse effects (Matzke & Frye, 1997). This is related, in part, to the fact that CRF not only affects the renal clearance of drugs but also the metabolism of xenobiotics by inhibiting key enzymatic systems in the liver and the intestine. Indeed, several pharmacokinetics studies conducted in patients with CRF demonstrate that the nonrenal clearance of multiple drugs is reduced (Touchette & Slaughter, 1991; Talbert, 1994; Matzke & Frye, 1997; Dreisbach & Lertora, 2003; Pichette & Leblond, 2003).

Several animal studies have shown major repercussions of CRF on drug metabolism enzymes. In the liver, selected isoforms of the cytochrome P450 (P450) are reduced in CRF secondary to a decrease in gene expression (Leber & Schutterle, 1972; Van Peer & Belpaire, 1977; Leber *et al*., 1978; Patterson & Cohn, 1984; Uchida *et al*., 1995; Leblond *et al*., 2001). Similar results have been shown in the intestine (Leblond *et al*., 2002). This downregulation of P450 is associated with important reductions in the *in vitro* and *in vivo* metabolism of xenobiotics (Leblond *et al*., 2000; 2001). The main hypothesis for the liver P450 downregulation is the presence of endogenous inhibitors in the uremic blood. Recently, we have shown that the serum of rats with CRF contains mediator(s) able to downregulate the P450 of normal rat hepatocytes secondary to reduced gene expression (Guevin *et al*., 2002).

The mechanism underlying the decrease in drug metabolism in CRF patients remains to be defined. However, this decrease appears to be mainly secondary to alteration in drug metabolism by the liver, particularly by the P450 (Touchette & Slaughter, 1991). Indeed, some authors have reported a decrease in CYP2D6 activity in CRF patients (Kevorkian *et al*., 1996; Rostami-Hodjegan *et al*., 1999). More recently, hepatic

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CYP2C9 and CYP3A activities have been characterized in patients with end stage renal disease (ESRD) (Dowling *et al*., 2003; Dreisbach *et al*., 2003). The results of these studies revealed that CYP3A and CYP2C9 activities are reduced. The presence of a circulating factor in uremia that inhibits liver metabolism has also been implicated in CRF patients (Taburet *et al*., 1996). However, no study has directly evaluated the effects of serum from patients with CRF on liver P450 activity and expression.

The objectives of this study were to determine the effects of serum from patients with severe CRF on liver P450 and to define the mechanisms leading to its downregulation. For this purpose, we incubated normal rat hepatocytes with serum from CRF and control patients in order to measure (1) total P450 levels, (2) protein expression of P450 isoforms involved in drug metabolism as well as some of their specific metabolic activities and (3) the mRNA encoding these specific isoforms. Similar experiments were performed with the serum of patients once on chronic hemodialysis and after successful kidney transplantation. Finally, CRF sera were fractionated by size exclusion chromatography to evaluate the molecular weight of the factors implicated in the decrease of P450.

Table 1 Characteristics of the CRF patients

	Etiology of CRF	*Age*	*Gender*	*GFR*
Patient 1	Diabetes	65	M	11.7
Patient 2	Ischemic	66	M	8.6
Patient 3[a]	Ischemic	75	M	7.6
Patient 4	Obstructive	74	M	9.5
Patient 5[a]	Glomerulonephritis	68	M	7.7
Patient 6	Ischemic	30	F	1.6
Patient 7	Diabetes	73	F	6.9
Patient 8	FSGS	35	F	4.8
Patient 9	Hypertension	75	F	6.6
Patient 10	Diabetes	70	F	3.9
Patient 11	Diabetes	64	M	8.8
Patient 12	Hypertension	59	M	5.0
Patient 13	Diabetes	49	M	5.4
Patient 14	Glomerulonephritis	58	F	5.2
Patient 15	Diabetes	58	F	11.5
Patient 16	Diabetes	39	F	9.0

GFR: residual glomerular filtration rate ($ml\,min^{-1}\,1.73\,m^{-2}$). FSGS: focal and segmental glomerulosclerosis. Measurements were made before the first hemodialysis session or before the initiation of peritoneal dialysis.
[a]Peritoneal dialysis.

Methods

Experimental model

Male Sprague–Dawley rats (Charles River, Saint-Charles, PQ, Canada), weighing 200–300 g were housed in the Research Centre animal care facility and maintained on Purina rat pellets and water *ad libitum*. An acclimatization period of at least 3 days was allowed to the animals before any experimental work was undertaken. All the experiments were conducted according to the Canadian Council on Animal Care guidelines for care and use of laboratory animals.

Rat hepatocyte isolation and culture

Hepatocytes were isolated from normal rats according to the two-step liver perfusion method of Seglen (1976), with modifications as published previously (Guevin *et al*., 2002). Aliquots of 1.5×10^6 hepatocytes were transferred into each well of a six-well plastic culture plate coated with type I rat tail collagen. A preincubation of 2 h was performed at 37°C in William E medium, supplemented with 10% fetal calf serum, and 1 mM insulin in an atmosphere of 95% O_2 and 5% CO_2.

Effect of CRF serum on rat hepatocyte P450

Blood samples were obtained, after informed consent, from patients (over 18 years of age) with severe CRF prior to their first treatment of chronic hemodialysis. After coagulation, serum was recovered by centrifugation ($600 \times g$ for 10 min) and stored at −80°C. To be included in the study, patients needed to demonstrate progressive renal failure of more than a year (excluding patients with acute and rapidly progressive renal failure). Other exclusion criteria were factors known to modulate P450: presence of acute (e.g. bacterial or viral infections) or chronic inflammatory process; specific drugs such as systemic corticosteroids and antiepileptic drugs; smoking and chronic alcoholism. Characteristics of the patients (numbers 1–10) are summarized in Table 1. Control sera were obtained from healthy volunteers, on the basis of the same exclusion criteria.

After preincubation, the medium was changed for 2 ml of William E medium containing 10% of serum from patients with CRF or from controls. The serum of one patient was used for one experiment ($n = 10$). Thereafter, the rat hepatocytes were incubated for another 24 h. Hepatocytes were then harvested by scraping in phosphate-buffered saline (one well). For mRNA analysis, cells of a two more wells were harvested in RLT buffer. Samples were stored at −80°C till analysis. Three different rat hepatocyte preparations were used for this experiment.

Dose–response experiment

To assess whether downregulation of P450 produced by serum mediator(s) was dose-dependent, dose–response curves were obtained by measuring the ability of a wide range of serum concentrations (1–20%) to depress the P450 of normal rat hepatocytes. The serum of one CRF patient was used for one experiment ($n = 6$). Characteristics of the patients (numbers 11–16) are summarized in Table 1. Incubation time was 24 h. Hepatocytes were then harvested as described above, and stored at −80°C till analysis.

Fractionation of CRF serum proteins

In order to characterize the serum proteins responsible for the downregulation of P450, six CRF patients (1, 2, 4, 7, 9 and 10; Table 1) and six control sera were fractionated according to the M_r of the proteins. In a first step, serum proteins were separated by ultrafiltration using a centrifugal concentrator Apollo 30K (Continental Lab Products, San Diego, CA, U.S.A.) to obtain a protein fraction < 30 kDa. This fraction was further resolved by size exclusion high-performance liquid chromatography (HPLC) on a Superose 12 HR 10/300 GL

column (Amersham Biosciences, Baie d'Urfé, Québec, Canada) as previously reported (Bleau *et al*., 2000). Column pressure was maintained below 2.0 MPa, flow rate was set at 0.4 ml min^{-1} and absorbance was measured at 280 nm with a System Gold (Beckman Coulter, Missauga, Ontario, Canada). The mobile phase contained 115 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1 mM HEPES, 25 mM EGTA and 5.5 mM glucose adjusted to pH 7.4 and filtered on a 0.22 μm membrane. HPLC was connected to a fraction collector and 0.5 ml fractions were collected. Fractions of 250 μl of the 30K ultrafiltrate were injected.

A calibration curve, using six standard proteins, was established to calculate the M_r of the proteins contained in each HPLC fraction. Fractions were concentrated on Centricon YM-3 (Millipore, Bedford, MA, U.S.A.), which retains proteins of more than 3 kDa to reach the final volume of 250 μl.

After preincubation, normal rat hepatocytes were incubated for 24 h in William E medium containing 10% of each fraction or whole serum obtained from patients with CRF (at the start of dialysis) or from control volunteers.

Effect of chronic hemodialysis on the inhibitory factors

This experiment was designed to evaluate whether starting chronic hemodialysis and therefore removing potential uremic factors could reverse the decrease in P450 mediated by the serum of patient in predialysis state. The serum of eight patients was thus obtained at the start of chronic dialysis and after 1 and 6 months of chronic hemodialysis. In both cases, serum was obtained prior to a regular dialysis session. The same criteria of inclusion and exclusion, as described above, were applied to these patients. Characteristics of the patients are summarized in Table 2 (patients 17–24). The dialysis parameters were the following: high-flux and polysulfone membrane, 4 h session, three times per week and access blood flow between 350 and 450 ml min^{-1} (depending on the access type).

After preincubation, normal rat hepatocytes were incubated for 24 h in William E medium containing 10% of serum from patients with CRF (at the start of dialysis as well as 1 and 6 months later) or from control volunteers (as described above).

Table 2 Characteristics of the CRF patients with chronic hemodialysis

	Etiology of CRF	*Age*	*Gender*	*GFR*
Patient 17	Diabetes	49	M	6.1
Patient 18	Diabetes	70	M	6.6
Patient 19	Diabetes	63	M	9.1
Patient 20	Diabetes	69	M	10.9
Patient 21	Alport	27	M	5.7
Patient 22	Diabetes	66	F	7.3
Patient 23	Pyelonephritis	52	F	6.6
Patient 24	Diabetes	58	F	10.7

GFR: residual glomerular filtration rate (ml min^{-1} 1.73 m^{-2}) at the time of initiation of chronic hemodialysis. In these patients, measurements were made before the beginning of chronic hemodialysis and after 1 and 6 months on chronic dialysis.

Effect of kidney transplantation on the inhibitory factors

To evaluate whether correction of renal function by transplantation was associated with the loss of the inhibitory effect of CRF serum, the serum of eight patients was obtained prior to the start of chronic hemodialysis and during the second month after successful kidney transplantation. The same criteria of inclusion and exclusion, as described above, were applied to these patients, except that kidney transplant recipients were all receiving low-dose prednisone (i.e. 0.22 ± 0.05 mg kg^{-1} daily) and mofetil mycophenolate. Five patients were on tacrolimus and three on cyclosporine A. Three patients have had an acute rejection episode in the first 3 weeks following transplantation, which was treated with methylprednisolone. Characteristics of the patients (numbers 21, 23 and 25–30) are summarized in Table 3.

After preincubation, normal rat hepatocytes were incubated for 24 h with William E medium containing 10% serum from patients with CRF (at the start of dialysis and after kidney transplantation) or from control volunteers.

Microsome preparation from rat hepatocytes

Microsomes were isolated from freshly harvested rat hepatocytes by differential centrifugation (Tindberg *et al*., 1996). The pellet containing the microsomes was resuspended in a solution of 0.9% NaCl and 0.1 mM PMSF and stored at -80°C till analysis.

Determination of total P450 activity

Protein content was determined by using the Micro BCA Protein Assay (Pierce, Rockford, IL, U.S.A.) using bovine serum albumin as standard protein. Total P450 activity was measured from the difference spectrum of the reduced protein according to a previously published method (Omura & Sato, 1964).

Western blot analysis

Some of the major rat P450 isoforms (CYP1A2, 2C6, 2C11 2D1/2D2, 2E1, 3A2 and 4A1/4A3) were assessed by Western blot analysis as described elsewhere (Leblond *et al*., 2000; 2001). We chose these isoforms for the following reasons: (a) they are implicated in the metabolism of drugs, (b) they are the most abundant isoforms in the rat liver, (c) they could be specifically quantified using available antibodies, and (d) calibration curves as well as specificity test have been performed in our laboratory. Every blot was repeated three times and results are pooled to obtain the final reported values.

Isoforms 1A2, 2C6, 2C11, 2E1, 3A2 and 4A1/4A3 were detected using polyclonal goat anti-rat 1A1/1A2, 2C6, 2C11, 2E1, 3A2 and 4A1, respectively. CYP2D1/2D2 was detected using a rabbit anti-human 2D6. The specificity of each of these antibodies was previously tested against commercial preparation of baculovirus-insect cell-expressed rat CYP450 isoform (Supersomes). Each of these preparations expresses only one isoform of P450 and was used to establish that antibodies efficiently recognized their antigen, has less than 5% cross-reactivity for other isoforms and is used at the optimal dilution (results not shown).

Table 3 Characteristics of the CRF patients with kidney transplantation

	Etiology of CRF	*GFR before dialysis* ($ml\,min^{-1}\,1.73\,m^{-2}$)	*Time on dialysis* (months)	*Time after transplantation* (days)	*GFR after transplantation* ($ml\,min^{-1}\,1.73\,m^{-2}$)
Patient 21	Alport	5.7	10	32	37.5
Patient 23	Pyelonephritis	6.6	12	63	33.1
Patient 25	Glomerulonephritis	10.2	26	59	109.6
Patient 26	Polycystic	5.5	10	34	53.9
Patient 27	Diabetes	8.4	5	62	69.4
Patient 28[a]	FSGS	5.6	27	31	59.9
Patient 29	IgA	6.2	18	47	28.6
Patient 30	Diabetes	7.9	15	38	71.3

GFR: glomerular filtration rate ($ml\,min^{-1}\,1.73\,m^{-2}$). Measurements were made before the first dialysis session or after transplantation.
[a]Peritoneal dialysis.

Immune complexes were revealed by secondary antibody (swine anti-goat IgG and goat anti-rabbit IgG) coupled to peroxidase and the Luminol derivative of Lumi-Light Western blotting substrate. Immune reaction intensity was determined by computer-assisted densitometry on exposed Biomax MR film.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and aspartate amino *S*-transferase (AST), two constitutively expressed proteins, were also measured by Western blot using polyclonal rabbit anti-human GAPDH and sheep anti-pig AST. AST was used as non-P450 proteins, while GAPDH was used as loading control for P450 Western analysis.

For each protein detected, difference between control and CRF sample was confirmed by varying the amount of material loaded on gel (5, 10 and 25 μg) as well as exposure time of chemiluminescence with Biomax film to avoid any risk of signal saturation on film (results not shown).

RNA isolation and quantitative RT–PCR analysis

mRNAs encoding rat P450 isoforms and GAPDH were measured by reverse transcriptase–polymerase chain reaction (RT–PCR) analysis as previously described (Guevin *et al*., 2002). Briefly, total RNA was extracted from frozen samples with RNeasy mini-kit (Qiagen, Mississauga, Ontario, Canada). A 1 μg portion of total RNA was used to prepare cDNA by reverse transcription using the Omniscript RT kit (Qiagen) and random primers from Invitrogen (Burlington, Ontario, Canada). Quantitative PCR was performed using *Taq* DNA polymerase (Qiagen) and the QuantumRNA Classic 18S kit from Ambion (Austin, TX, U.S.A.). This enables the comparison of control and CRF samples by normalization with the 18S ribosomal RNA content. The expression of GAPDH mRNA was measured by using previously published primers and PCR conditions (Robitaille *et al*., 1999).

The primers used for P450 isoforms RT–PCR quantification were designed according to respective Genebank sequence. Specificity of each primer set was tested by sequencing of the resulting PCR products on an ABI Prism 3100 analyser (Applied Biosystems, Foster City, CA, U.S.A.). The sequences of the primers used for PCR are presented in Table 4. Each PCR experiment was repeated three times and results are pooled to obtain the final reported values.

Evaluation of in vitro *metabolic activity in rat hepatocytes*

Metabolic activity of P450 isoforms 1A1/1A2 and 3A1/3A2 was determined on microsomes prepared from rat hepatocytes incubated with serum of five patients (numbers 3 and 5–8; Table 1) and five control sera. To evaluate the metabolic activity of CYP3A, erythromycin N-demethylation was determined as described previously (Guevin *et al*., 2002). Briefly, erythromycin (500 μM) was incubated with 1.5 mg of rat hepatocyte microsomal proteins at 37°C for 30 min, in the presence of an NADPH-generating system consisting of 1 mM NADP, 10 mM glucose 6-phosphate and 0.7 U ml^{-1} glucose 6-phosphate dehydrogenase.

The activity of CYP1A was measured by determination of the 7-ethoxyresorufin-*O*-deethylase (EROD) activity in microsomes as described by Paine *et al*. (1999). Briefly, 7-ethoxyresorufin (25 μM) was incubated with 0.5 mg of rat hepatocyte microsomal proteins in the presence of an NADPH-generating system consisting of 25 mM NADP, 0.25 M glucose 6-phosphate and 25 U ml^{-1} glucose 6-phosphate dehydrogenase (Leblond *et al*., 2002). The fluorescence of resorufin was measured by spectrofluorometry (Ex: 530 nm, Em: 585 nm).

Blood and urine chemistries

Blood (urea, creatinine) and urine (creatinine) chemistries were determined with an Advia 1650 autoanalyser (Bayer Diagnostic, Toronto, Ontario, Canada). Parathyroid hormone (PTH) levels were measured by immunochemiluminescence on an Advia Centaur immunoanalyser (Bayer) using an intact PTH (1–84) specific assay. Creatinine clearances were calculated using the Levey formula (Levey *et al*., 1999).

Statistical analysis

The results are expressed as mean ± standard error over the mean (s.e.m.). Differences between groups were assessed by using either paired or unpaired Student's *t*-test (for difference between two groups) or an ANOVA test when appropriate. Significant ANOVA was followed by Fisher LSD multiple comparisons procedure. The threshold of significance was $P<0.05$.

Drug and chemicals

Heparin, glucose, EGTA, trypsin inhibitor, William E medium, insulin, erythromycin and 7-ethoxyresorufin were

Table 4 Nucleotide sequences of PCR primers

P450 isoforms	*Primer sequence (5′–3′)*	*Predicted product size* (bp)	*Accession number*
1A2	CATCTTTGGAGCTGGATTTG CCATTCAGTGAGGTGTCC	255	K03241
2C6	AGAACATGAAGATTTTGAGCAG CAAATCAGTCACAGTGATTGAT	275	M18336
2C11	TCATTCCCAAGGGTACCAATG GGAACAGATGACTCTGAATTCT	664	J02657
2D1[a]	ACCAATGCTGTCATCCATGAGG CATGAAGGCCTCATGCTTCAC	237	J02867
2E1	AGCACAACTCTGAGATATGG ATAGTCACTGTACTTGAACT	366	AF061442
3A2	ATCCAGAAAGGTTTAGCAAGG GGACGAGGACATGGTTACTATC	589	M13646
4A1[b]	AAAGGCCAATGGCGTCTACAGATTG ATTCAAGTTCCCAATGGCCTGGAT	334	M57718
GAPDH	GTGATGGGTGTGAACCACGAG CTGCTTCACCACCTTCTTGAT	399	NM_017008

Primers for CYP2C11 and 3A2 were synthesized according to Leblond *et al*. (2001). Primers for GAPDH were synthesized according to Robitaille *et al*. (1999). Primers for CYP1A2, 2C6, 2D1, 2E1 and 4A1 were designed based on published cDNA sequences with the aid of the Jellyfish computer program. The resulting PCR product was sequenced on an ABI Prism 3100 analyser (Applied Biosystems, Foster City, CA, U.S.A.) to confirm the specificity of the primers.
[a]Primer for CYP2D1 also recognizes CYP2D2, 2D3, 2D4, 2D5 and 2D18.
[b]Primer for CYP4A1 also recognizes CYP4A10 and 4A22.

purchased from Sigma Chemicals (St Louis, MO, U.S.A.). Percoll was obtained from Amersham Biosciences. Collagenase D was provided by Roche Diagnostics (Laval, Québec, Canada). Fetal calf serum was acquired from Biosources International (Camarillo, CA, U.S.A.). Culture plates coated with type I rat tail collagen were purchased from BD Biosciences Discovery Labware (Oakville, Ontario, Canada). RLT buffer was provided by Qiagen. Primers used for PCR quantification were obtained from Sigma.

Polyclonal goat anti-rat 1A1/1A2, 2C6, 2C11, 2E1, 3A2 and 4A1, as well as rabbit anti-human 2D6, were acquired from Gentest Corporation (Woburn, MA, U.S.A.). Polyclonal rabbit anti-human GAPDH was obtained from Abcam Ltd (Cambridge, U.K.). Polyclonal sheep anti-pig AST was obtained from Rockland (Gilbertsville, PA, U.S.A.). Secondary antibodies (swine anti-goat IgG and goat anti-rabbit IgG) were provided by Biosources International (Camarillo, CA, U.S.A.) while donkey anti-sheep IgG was obtained from Sigma. Lumi-Light Western blotting substrate was purchased from Roche Diagnostics. CYP450 Supersomes for rat isoforms 1A1, 1A2, 2B1, 2C6, 2C11, 2C13, 2D1, 2E1, 3A1 and 3A2 were purchased from Gentest Corporation. For isoform 4A1, we used clofibrate-treated rat liver microsomes as positive control (Gentest Corporation).

Results

Total P450 level in rat hepatocytes incubated with serum from CRF or control subjects

Total P450 level in rat hepatocytes incubated for 24 h with 10% of control human serum was 0.20 ± 0.11 nmol mg^{-1} of proteins. Following 24 h of incubation of normal rat hepatocytes with 10% serum from patients with CRF, total P450 level decreased by 33% ($P<0.01$).

Protein expression of liver P450 isoforms in rat hepatocytes incubated with serum from CRF or control human subjects

Following 24 h of incubation of normal rat hepatocytes with serum (10%) from patients with CRF, several P450 isoforms decreased compared to hepatocytes incubated with serum from control (Figure 1). The levels of CYP1A2, 2C6, 2C11, 2D1/2D2, 3A2 and 4A1/4A3 were reduced by 50% ($P<0.001$), 35% ($P<0.01$), 39% ($P<0.01$), 29% ($P<0.01$), 63% ($P<0.001$) and 42% ($P<0.01$), respectively, while the protein expression of CYP2E1 was not modified by uremic sera. Furthermore, the levels of the control proteins GAPDH and AST were similar in the two groups of rat hepatocytes.

Correlation of liver P450 isoforms and biochemical parameters

All the CRF serum tested produced a significant decrease in the expression of P450 isoforms. Of course, there was a difference in the magnitude of the decrease. This was not due to the etiology of CRF (data not shown). However, we found significant correlations between the P450 protein expression and predialysis glomerular filtration rate (GFR, which is the most accurate marker of uremia; Levey *et al*., 1999), but also serum creatinine and PTH. No correlation was found with serum urea. Figure 2 shows correlations found with CYP3A2 expression. For all parameters except for urea, we found $P<0.001$.

mRNA encoding liver P450 isoforms in rat hepatocytes incubated with serum from CRF or control human subjects

To determine whether the reduction in the protein expression of hepatic P450 isoforms was secondary to a decrease in their synthesis, mRNAs encoding these isoforms (CYP1A2, 2C6,

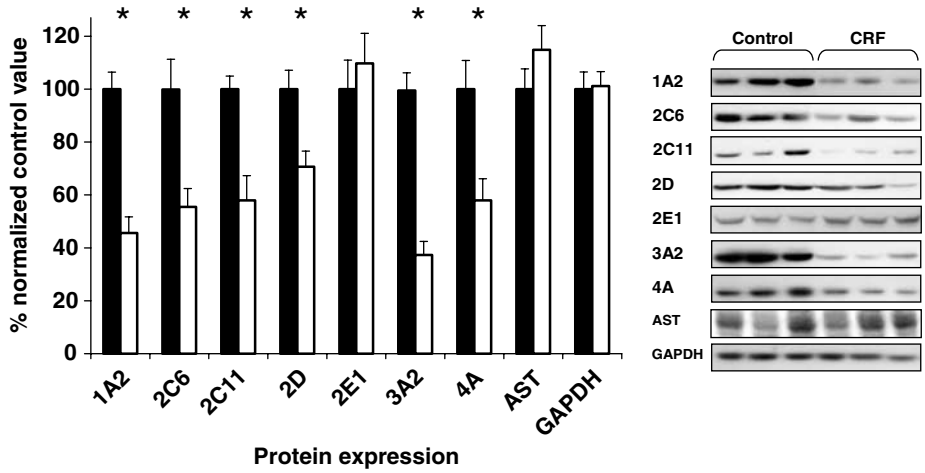

Figure 1 Protein expression of hepatic cytochrome P450 isoforms, AST and GAPDH in rat hepatocytes incubated with serum from control (black bars) and CRF patients (white bars). Protein bands are expressed in densitometry units (%). The densitometry units of control samples were arbitrarily defined as 100%. Data are the mean ± s.e.m. of 10 experiments in each group. $^*P<0.01$ as compared to serum from control. Representative blots are also shown.

2C11, 2D1, 2E1, 3A2 and 4A1) were measured by quantitative RT–PCR analysis. Following 24 h of incubation of normal rat hepatocytes with serum (10%) from patients with CRF, a significant decrease in mRNAs encoding CYP1A2 (31%, $P<0.001$), 2C6 (31%, $P<0.001$), 2C11 (31%, $P<0.001$), 2D1 (23%, $P<0.001$), CYP3A2 (39%, $P<0.001$) and 4A1 (33%, $P<0.001$) isoforms was observed, compared to serum from control subjects, while the mRNA levels of CYP2E1 was similar (Figure 3). Levels of mRNA encoding GAPDH were similar in both groups (Figure 3). Thus, the decrease in protein expression of P450 isoforms mediated by serum from patients with CRF is at least in part secondary to reduced gene expression.

In vitro *metabolism of erythromycin and EROD activity in rat hepatocytes incubated with serum from CRF or control subjects*

To determine the repercussion of P450 reduction in CRF on the metabolism of drugs, the *in vitro* N-demethylation of erythromycin and EROD activity have been assessed in liver microsomes prepared from rat hepatocytes previously incubated for 24 h with serum (10%) from CRF or control human subjects. These enzymatic reactions are mediated primarily by the CYP3A and CYP1A families, respectively. The N-demethylation of erythromycin was decreased by 51% in rat hepatocytes incubated with serum from patients with CRF, compared to serum from control patients ($P<0.001$) (Figure 4a). Similarly, EROD activity was decreased by 59% in rat hepatocytes incubated with serum from patients with CRF ($P<0.001$) (Figure 4b).

Dose–response curves

The reduction in P450 induced by CRF serum was closely related to the concentration of serum used (Figure 5). There was a progressive reduction of CYP3A2 expression when rat hepatocytes were incubated with increasing concentrations of serum from CRF patients (from 1.0 to 20%), compared to serum from control, which produces no modulation at any dose. There was no change in GAPDH expression between both groups (data not shown).

Fractionation of uremic serum

Sera obtained from CRF patients were firstly fractionated by ultrafiltration on a 30 kDa cutoff device. The fractionation by ultrafiltration concentrated the inhibitory activity of the serum in the 0–30 kDa fraction (Figure 6, inset). This fraction was further fractionated by HPLC in seven different fractions according to the size of the constituents. Among them, the ability to decrease CYP3A2 protein expression (Figure 6), as well as mRNA levels (data not shown), was present in the fraction containing proteins of molecular weight between 10 and 15 kDa. The other fractions have no effect on CYP3A2 protein expression.

Effect of chronic hemodialysis on the inhibitory effects of uremic serum

This experiment was designed to evaluate whether starting chronic hemodialysis and therefore removing potential uremic factors could reverse the decrease in P450 mediated by the serum of patient in predialysis state. Compared to controls, serum from CRF patients not on dialysis was associated with a decrease in the protein expression of CYP3A2 (Figure 7). This inhibitory effect on CYP3A2 was similar after a month on chronic hemodialysis. There was no change in GAPDH expression between these groups (data not shown). Similar results were obtained after 6 months on hemodialysis (data not shown).

Effect of kidney transplantation on the inhibitory effects of uremic serum

In this experiment, we evaluated the effect of serum from patients with CRF at the start of their chronic hemodialysis and after successful kidney transplantation (serum was obtained during the second month after transplantation).

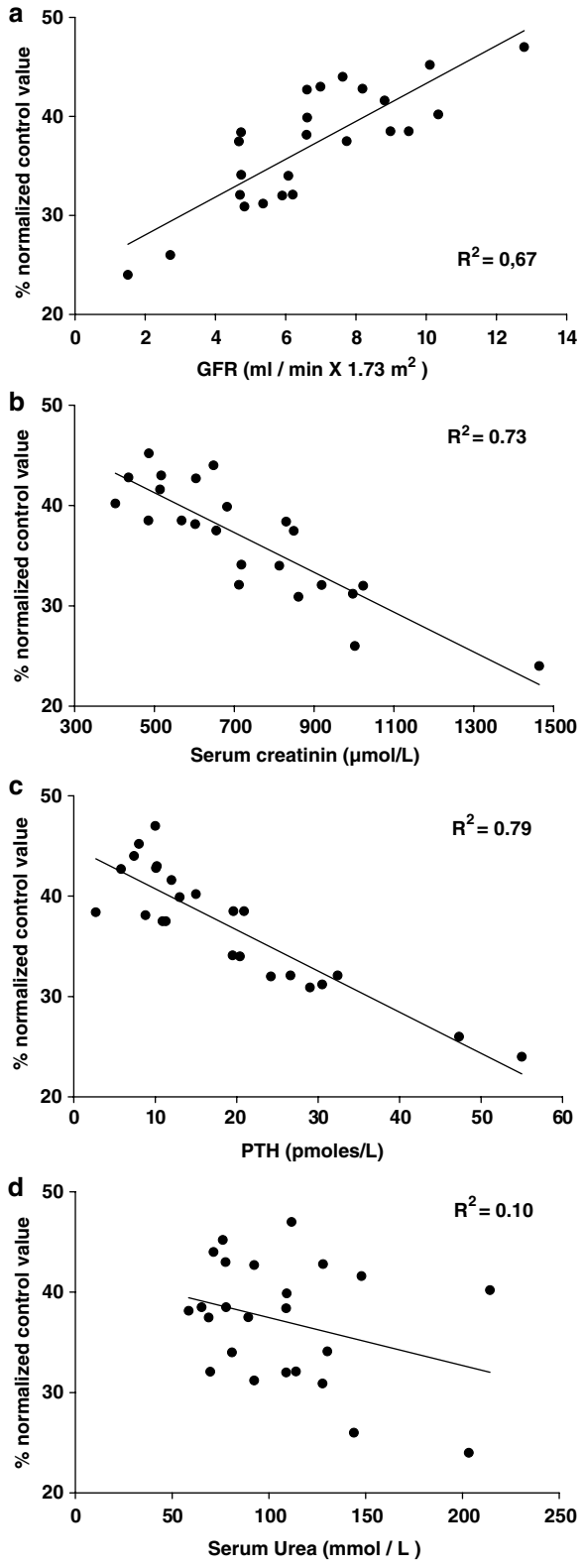

Figure 2 Protein expression of CYP3A2 in rat hepatocytes incubated with serum from CRF patients was plotted against patient GFR, serum creatinine, PTH and serum urea concentration obtained at the time of the first dialysis. Protein bands are expressed in densitometry units (%). The densitometry units of control samples were arbitrarily defined as 100%. Correlation coefficients are 0.67, 0.73, 0.79 and 0.10. $P<0.001$ as compared to serum from control for GFR, creatinine and PTH.

Compared to control, serum from CRF patients not on dialysis was associated with a decrease in the protein expression of CYP2D1/2D2 and 3A2 as shown in Figure 8. CYP2D was tested because this isoform is not induced by corticosteroids (Edwards *et al*., 2003). The sera of the same patients were then tested after the kidney transplantation and P450 expression was restored to control levels (Figure 8). As shown in Table 3, GFRs were nearly normal in these patients. There was no change in GAPDH expression before and after transplantation (data not shown).

Discussion

The present study shows that in humans, the serum of patients with severe CRF contains mediator(s) able to reduce hepatic P450 content and activity, secondary to a reduction in protein expression of several P450 isoforms. Furthermore, once on chronic hemodialysis, the inhibitory effect of uremic serum on hepatic P450 persists, while it is reversed following a successful renal transplantation. The mechanism underlying this decrease is a reduction in the concentration of specific mRNA encoding these proteins indicating that pretranslational mechanisms were largely operative for the decreased P450 expression.

Several studies have demonstrated that patients with CRF present decreased hepatic drug metabolism (Gibson, 1986; Matzke & Frye, 1997). The results of these studies show that in CRF patients there is a 19–80% decrease in the metabolic clearance of several xenobiotics (Matzke & Frye, 1997; Pichette & Leblond, 2002; Dreisbach & Lertora, 2003). The vast majority of these drugs are metabolized by the liver P450, suggesting a decrease in liver total P450. Indeed some authors have shown a decrease in the metabolic clearance of sparteine in CRF, reflecting a diminution in CYP2D6 activity (Kevorkian *et al*., 1996; Rostami-Hodjegan *et al*., 1999). Moreover, a decrease in CYP2C9 and 3A activity has been described in patients with renal failure (Dowling *et al*., 2003; Dreisbach *et al*., 2003). Animal studies have also clearly shown a decrease in P450 activity and expression in CRF rats (Leber & Schutterle, 1972; Van Peer & Belpaire, 1977; Leber *et al*., 1978; Patterson & Cohn, 1984; Uchida *et al*., 1995; Leblond *et al*., 2001). We have demonstrated that in rats with CRF, liver mRNAs of P450 were significantly decreased, suggesting that there is reduced gene expression (Leblond *et al*., 2001).

In the rat, the decrease of liver P450 appears to be caused by circulating factors present in uremic serum. Terao & Shen (1985) reported that when livers from healthy rats where perfused with plasma of uremic rats, the extraction of l-propranolol was reduced, suggesting the presence of an inhibitory factor in the uremic blood that could modify the biotransformation of drugs. We recently demonstrated that the serum of rat with CRF contains mediator(s) able to decrease rat hepatic P450 (Guevin *et al*., 2002).

In humans, on the other hand, mechanisms underlying the decrease in drug metabolism, as well as P450 activity, remain poorly understood. There is some preliminary data (published as an abstract) supporting the presence of hepatic P450 inhibitors in CRF serum. It has been shown that serum of CRF patients could decrease the *in vitro* hepatic metabolism of midazolam and tolbutamide, which are biotransformed by P450 (mainly CYP3A4 and 2C9) (Taburet *et al*., 1996). In the present study, we clearly demonstrate that uremic serum from

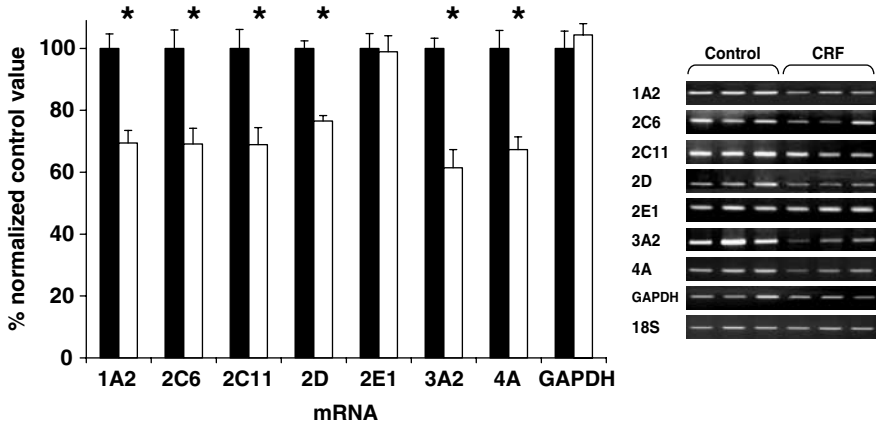

Figure 3 mRNA encoding hepatic cytochrome P450 isoforms and GAPDH in rat hepatocytes incubated with serum from control (black bars) and CRF patients (white bars). mRNA bands are expressed in standardized densitometry units (%). The densitometry values for cytochrome P450 isoforms were standardized by dividing these values by the values for 18S ribosomal RNA. The standardized densitometry units of control samples were arbitrarily defined as 100%. Data are the mean ± s.e.m. of 10 experiments in each group. $*P<0.001$ as compared to serum from control subjects. Representative blots are also shown.

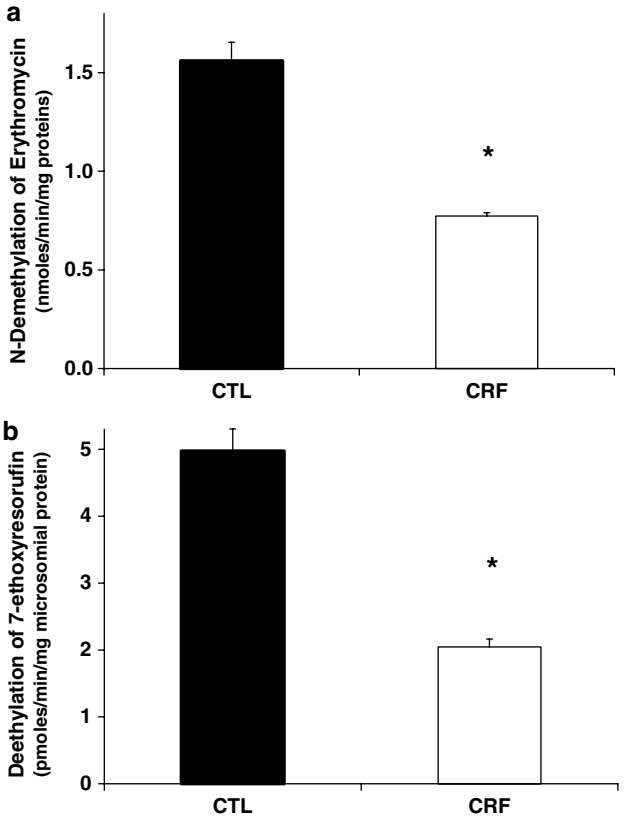

Figure 4 *In vitro* metabolism of erythromycin (a) and EROD activity (b) in microsomes prepared from rat hepatocytes incubated with serum from control (black bar) and CRF patients (white bar). Data are the mean ± s.e.m. of five experiments in each group. $*P<0.001$ as compared to serum from control.

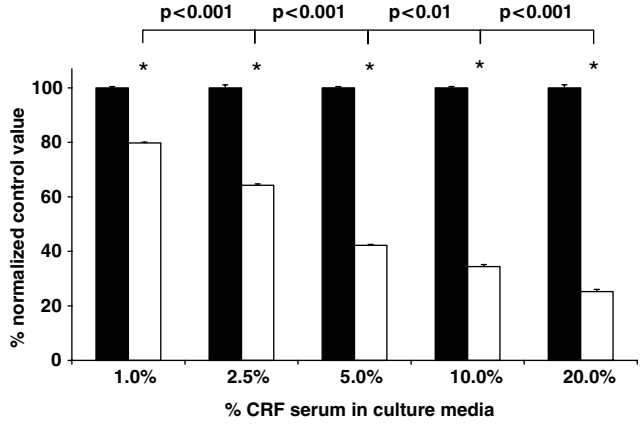

Figure 5 Level of CYP3A2 expression as a function of the concentration of serum used for the incubation of rat hepatocytes. Hepatocytes were incubated for 24 h with progressive concentration of serum from control (black bar) and CRF patients (white bar). Protein bands are expressed in densitometry units (%). The densitometry units of control samples were arbitrarily defined as 100%. Data are the mean ± s.e.m. of six experiments. $*P<0.001$ as compared to control. *P*-value for the difference between each dose is reported directly in the figure.

CRF patients contains mediators able to decrease liver P450. Indeed, we found a decrease of 33% in total rat hepatic P450 and a marked decrease in the protein expression of several P450 isoforms: CYP1A2, 2C6, 2C11, 2D1/2D2, 3A2 and 4A1/4A3. The repercussions on the hepatic metabolism of drugs are important, since we observed a reduction in erythromycin biotransformation and in EROD activity, mediated by CYP3A and CYP1A, respectively. These results closely mimic what has already been published *in vivo* in rats and patients with CRF (Leblond *et al*., 2000; 2001; Dowling *et al*., 2003; Dreisbach *et al*., 2003).

The decrease in P450 activity and expression mediated by uremic sera appears to be rather specific to P450 isoforms since we did not observe any modification in the expression of GAPDH and AST, two non-P450 liver proteins. Although all sera tested produced a reduction in P450, the magnitude of the decrease greatly differs between sera and this seems to be related to the severity of CRF. Indeed, we found a strong correlation between the severity of CRF (evaluated by the levels of creatinine and the GFR) and the decrease in liver P450 (Figure 2). Furthermore, we found a correlation between

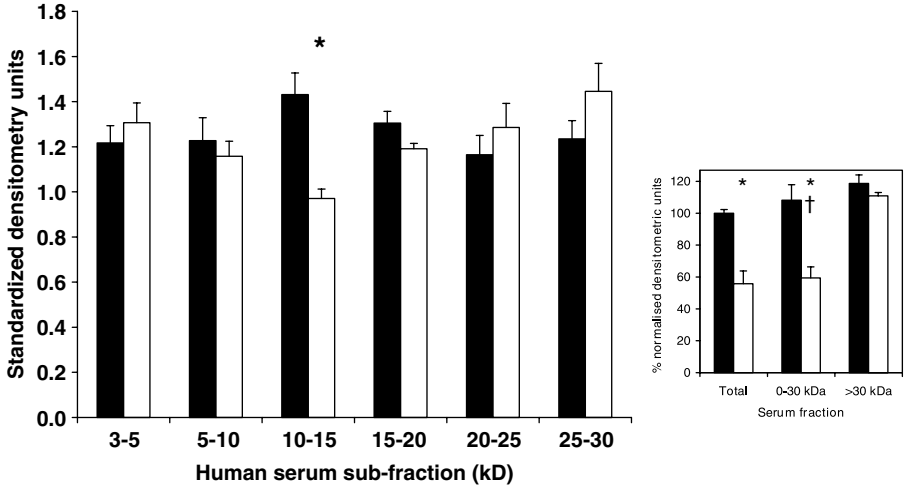

Figure 6 Level of CYP3A2 expression in rat hepatocytes incubated with subfractions of serum from control (black bars) and CRF patients before the first dialysis treatment (white bars). Protein bands are expressed in densitometry units. The inset shows the inhibitory activity of fractions obtained by ultrafiltration. The densitometry units of control sera were arbitrarily defined as 100%. Data are the mean ± s.e.m. of six experiments. $^{*}P<0.05$ as compared to serum from control. $^{\dagger}P<0.01$ as compared to the same fraction in control.

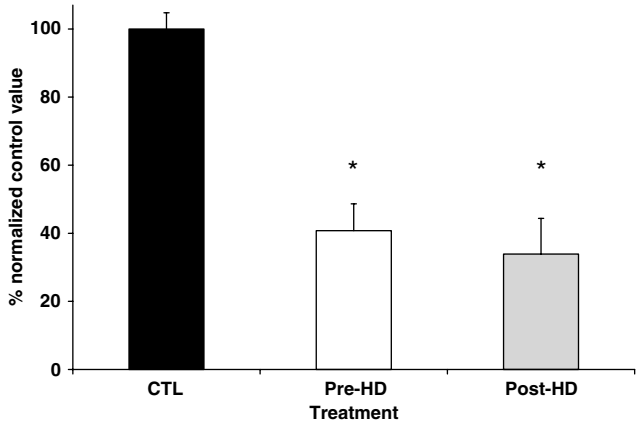

Figure 7 Level of CYP3A2 expression in rat hepatocytes incubated with serum from control (black bars) and CRF patients before the first dialysis treatment (white bars) and a month later (gray bars). Protein bands are expressed in densitometry units (%). The densitometry units of control samples were arbitrarily defined as 100%. Data are the mean ± s.e.m. of eight experiments. $^{*}P<0.001$ as compared to serum from control subjects.

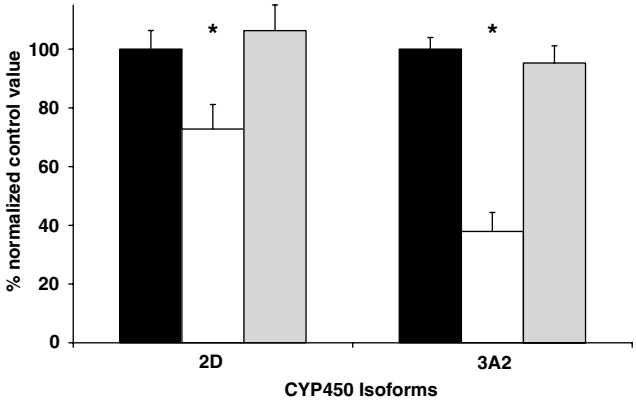

Figure 8 Level of CYP2D and 3A2 expression in rat hepatocytes incubated with serum from control (black bars) and CRF patients before the first dialysis treatment (white bars) and during the second month after successful kidney transplantation (gray bars). Protein bands are expressed in densitometry units (%). The densitometry units of control samples were arbitrarily defined as 100%. Data are the mean ± s.e.m. of eight experiments. $^{*}P<0.01$ as compared to serum from control subjects.

the dose of mediator(s) (serum concentration) and the decrease in P450 expression (Figure 5). Similar correlation between the decrease in the metabolism of drugs and the levels of renal failure has been described in humans (Konishi, 1986). These results suggest that as CRF worsens, patients are at risk of drug accumulation and toxicity secondary to reduction in their metabolism. Our study did not show that a particular renal disease (e.g. diabetes) was a risk factor for P450 decrease.

In an attempt to identify the mediators in uremic sera, responsible for the decrease in P450 activity and expression, we fractionated the serum. Our results show that the inhibitory effect of uremic serum is confined to proteins of M_r between 10 and 15 kDa. Although several uremic toxins have been described, we hypothesized, based on M_r, that PTH or cytokines could be putative molecules implicated in the decrease of liver P450. First, in CRF, PTH has been shown to decrease protein synthesis (in the liver, heart and skeletal muscle) secondary to reduced gene expression (Ding *et al*., 1996; Klin *et al*., 1996; Qing *et al*., 1999). Interestingly, the present study shows that there is an association between lower levels of mRNAs and protein expression (Figure 3) in rat hepatocytes incubated with serum from patients with CRF. This suggests that gene expression is reduced by mediator(s) present in uremic serum. Whether PTH is implicated in this phenomenon remains to be defined. However, the M_r of PTH is between 10 and 15 kDa and we found a significant correlation between the decrease in P450 expression and the levels of PTH (Figure 2). On the other hand, cytokines could also be implicated in the reduction of P450. Several cytokines having an M_r around 15 kDa are potent inhibitors of P450 (Morgan, 1997; Renton, 2004) and are increased in CRF (Raj *et al*., 2003).

Our results demonstrate that chronic dialysis, even after 6 months, does not affect the inhibitory effect of uremic serum (as it corrects many metabolic disturbances induced by uremia), suggesting that uremic mediators are still present even after the initiation of dialysis. Pharmacokinetics studies have shown a decrease in the metabolic clearance of several drugs in patients on hemodialysis (Pichette & Leblond, 2002; Dreisbach & Lertora, 2003). However, the repercussions of dialysis on P450 have been poorly studied. There are animal data suggesting that plasticizer exposure in hemodialysis (leached from dialysis tubings) could be associated with P450 induction. Indeed, di-(2-ethylhexyl)-phthalate (DEPH), a plasticizer, has been shown to induce total P450 in the liver as well as increase antipyrine clearance (Pollack & Shen, 1984). This effect has not been observed with the serum of our patients. On the other hand, recent data obtained in hemodialysis patients demonstrated that CYP2C9 and 3A activity is significantly reduced (Dowling *et al*., 2003; Dreisbach *et al*., 2003). Our data suggest that this could be secondary to circulating factors. An interesting question is whether a dialysis session normalized the inhibitory effect of serum obtained before the start of dialysis, as in the current study.

Normalizing renal function with a kidney transplant completely reversed the inhibitory effects of serum on liver P450 compared to predialysis serum (Figure 6). Although no studies have been performed on liver P450 and renal transplantation, some authors have reported an increase in the metabolic clearance of drugs after successful kidney transplantation compared to dialysis (Wright *et al*., 1988; Kim *et al*., 1993). For instance, the nonrenal clearance of isoniazid and metoclopramide markedly increase following kidney transplantation compared to the clearance observed in hemodialysis (Wright *et al*., 1988; Kim *et al*., 1993).

In conclusion, the serum of patients with CRF contains mediator(s) that decrease the P450 of normal rat hepatocytes. These mediator(s) decrease protein expression of several P450 isoforms (mainly, CYP1A2, 2C6, 2C11, 2D1/2D2, 3A2 and 4A1/4A3), secondary to reduced mRNA levels. Drug metabolism, assessed by the biotransformation of erythromycin and EROD, was greatly reduced by serum mediator(s). These results suggest that uremic mediator(s) could be responsible for the reduction in *in vivo* drug metabolism observed in CRF patients. Normalizing renal function by kidney transplantation reverses this inhibitory effect.

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