Role of Parathyroid Hormone in the Downregulation of Liver Cytochrome P450 in Chronic Renal Failure

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Chronic renal failure (CRF) is associated with a decrease in drug metabolism secondary to a decrease in liver cytochrome P450 (P450). The predominant theory to explain this decrease is the presence of factors in the blood of uremic patients. This study tested the hypothesis that parathyroid hormone (PTH) could be this factor. The objectives of this study were to determine (1) the role of PTH in the downregulation of hepatocyte P450 induced by rat uremic serum, (2) the role of PTH in the downregulation of liver P450 in rats with CRF, and (3) the effects of PTH on P450 in hepatocytes. For this purpose, (1) hepatocytes were incubated with serum from rat with CRF that was depleted with anti-PTH antibodies or with serum from parathyroidectomized (CRF-PTX) rat with CRF, (2) the effect of PTX on liver P450 was evaluated in rats with CRF, and (3) the effects of PTH on P450 in hepatocytes were determined. The depletion of PTH from CRF serum completely reversed the downregulating effect of CRF serum on P450 in hepatocytes. Addition of PTH (10^{-9} M) to depleted CRF serum induced a decrease in P450 similar to nondepleted CRF serum. The serum of CRF-PTX rats had no effect on P450 in hepatocytes compared with CRF serum. Adding PTH to CRF-PTX serum induced a similar decrease in P450 as obtained with CRF serum. Finally, PTX prevented the decrease of liver P450 in rats with CRF. In summary, PTH is the major mediator implicated in the downregulation of liver P450 in rats with CRF.


C hronic renal failure (CRF) interferes with the elimination of many drugs because of the reduction in GFR and tubular secretion (1). However, renal failure also diminishes the metabolic clearance of selected drugs secondary to decrease of hepatic and intestinal metabolism of these drugs (2–6). The major determinant for these metabolic changes is a reduction in enzymatic activity.

Cytochrome P450 (P450) is the major catalyst of drug biotransformation. Several animal studies have shown that liver and intestinal P450 are reduced in CRF (7–10). These studies demonstrated that CRF is associated with a decrease in the activity as well as in the expression of liver and intestinal P450 isoforms secondary to reduced mRNA levels (9,10). The main hypothesis to explain P450 activity and expression downregulation is the presence in the blood of uremic animals of endogenous inhibitors that modulate the P450. Indeed, we have shown that in normal hepatocytes that were incubated for 24 h with serum from rats with CRF, total P450 level and protein expression of several P450 isoforms decreased by 45% compared with serum from control animals (11). This decrease in protein expression of P450 isoforms was secondary to reduced gene expression (11). Similar results have been shown with serum of patients with severe CRF (12). The next step was to find which factor in the uremic blood downregulates P450 in CRF.

CRF is associated with multiple metabolic disturbances. As a consequence, numerous molecules are increased in CRF. However, taking into account the changes that are induced by CRF (metabolic, hormonal, and retention of toxins) and the factors that are known to affect the P450, two main mediators are most likely to be associated with downregulation of P450 in CRF: parathyroid hormone (PTH) and proinflammatory cytokines (6). Although the potency of cytokines to downregulate P450 have been established in inflammatory disease (13), we hypothesized that PTH could be implicated in the downregulation of P450 in CRF for the following reasons: (1) secondary hyperparathyroidism is frequent in CRF (14); (2) PTH is known to downregulate the mRNA of many proteins, particularly in the liver but also in other tissue such as the heart (15–17); and (3) we have found a strong correlation between the levels of PTH and P450 reduction induced by serum of patients (12).

The objectives of this study were to determine (1) the role of PTH in the in vitro downregulation of liver P450 that is induced by rat uremic serum, (2) the role of PTH in the in vivo downregulation of liver P450 in CRF rats, and (3) the in vitro effects of PTH on P450 in cultured hepatocytes. For this purpose, (1) we incubated normal rat hepatocytes with CRF rat serum that was preadsorbed on immobilized anti-PTH antibodies or with serum from CRF parathyroidectomized (CRF-PTX) rat, (2) we
evaluated the effect of PTX on liver P450 activity and expression in rats with CRF, and (3) we determined the effects of PTH on P450 in cultured hepatocytes.

Materials and Methods

Experimental Model
Male Sprague-Dawley rats (Charles River, Saint-Charles, QC, Canada) that weighed 200 to 300 g were housed in the Research Centre animal care facility and maintained on Purina rat pellets (Ralston-Purina, St. Louis, MO) and water ad libitum. An acclimatization period of 3 d was allowed before any experimental work was undertaken. All of the experiments were conducted according to the Canadian Council on Animal Care guidelines for care and use of laboratory animals.

As shown in Table 1, rats were divided in four groups. Hepatocytes were isolated from normal rats (n = 35), and the sera that were used for incubation experiments were obtained from CRF, CRF-PTX, control, or control-PTX rats at the time of sacrifice.

CRF was induced by two-stage five-sixths nephrectomy as previously published (9,18). After surgery, rats with CRF were fed Purina rat chow and water ad libitum. Rats from the control group also underwent two sham laparatomies (days 1 and 8). Control rats were pair-fed the same amount of rat chow that was ingested by the rats with CRF on the previous day. At day 41 after the nephrectomy, the rats were housed in metabolic cages and urine was collected for 24 h to determine the clearance of creatinine. Rats were killed by decapitation at 42 d. Blood was collected and stored rapidly on ice. After coagulation, serum was recovered by centrifugation (600 × g for 10 min at 4°C), and samples were kept for the measurement of serum creatinine and urea. The remaining sera were stored at −80°C.

Total parathyroidectomy (PTX) was performed as described previously (16). Briefly, surgical PTX was carried out under a surgical microscope, without removal of the thyroid tissue. The success of the PTX was ascertained by a significant decrease of calcium after PTX. To avoid hypocalcemia, PTX rats then were supplemented in calcium by adding calcium gluconate to drinking water (control 5%; CRF-PTX, or control rats). The serum calcium was measured before and after the addition of calcium gluconate to drinking water (control 5%; CRF-PTX, or control rats). The serum calcium was measured before and after the addition of calcium gluconate to drinking water (control 5%; CRF-PTX, or control rats).

Immunoadsorption of PTH in Uremic Serum

To deplete PTH in the sera of rats with CRF, we used polystyrene beads (8.4 mm) coated with goat antibodies that were specific to the N-terminal (1-34) region of rat PTH (Alpco Diagnostics, Windham, NH). Each of these beads can bind at least 400 pg of PTH. Rat sera were incubated at 4°C for 16 h with beads (7 beads/ml serum). After depletion, treated sera were added to culture medium (10%) and filtered on 0.22-μm filter. Samples of depleted sera were reserved for quantification of PTH after depletion. Nondepleted control and CRF sera were incubated concomitantly at 4°C and treated in the same way.

Hepatocyte Isolation and Culture
Hepatocytes were isolated from normal rats according to the two-step liver perfusion method of Seglen as previously published (11). Collagenase type 4 (Worthington, Lakewood, NJ) was used.

After preincubation, the medium was changed for 2 ml of William E medium that contained 10% of serum from rats with CRF, CRF-PTX, or control rats. The serum of one rat was used for one experiment. Thereafter, the hepatocytes were incubated for another 24 h. Hepatocytes then were harvested by scraping in PBS. For mRNA analysis, cells were harvested in RLT buffer (Qiagen, Mississauga, Ontario, Canada). Samples were stored at −80°C until analysis.

For assessment of whether liver P450 could be downregulated by PTH, a dosage-response curve was obtained by measurement of the ability of a wide-range PTH (rat synthetic 1-34 PTH; Sigma, St. Louis, MO) concentrations (10⁻¹² to 10⁻³ M) to depress the P450 of normal hepatocytes. Incubation time was 24 h. PTH was dissolved in 0.15 N acetic acid. Hepatocytes then were harvested as described in the previous paragraph and stored at −80°C until analysis.

Effect of Inhibition of the NF-κB Pathway on PTH-Induced Downregulation of Liver CYP3A2
For assessment of whether the blockade of the NF-κB pathway could prevent the effect of PTH on liver P450, two inhibitors of NF-κB were used: pyrrolidine dithiocarbamate (PDTC) (19) and Andrographolide (20) (Calbiochem, San Diego, CA). Normal hepatocytes were incubated for 24 h in the presence of either PDTC (50 μM) or Andrographolide (30 μM) or in absence of these inhibitors with and without PTH (10⁻³ M).

Microsome Preparation from Hepatocytes and Liver
Hepatocyte microsomes were isolated by differential centrifugation (21). The pellet that contained the microsomes was resuspended in 0.9% NaCl and stored at −80°C until analysis. Liver microsomes were isolated by differential centrifugation according to Cinti et al. (22). The pellet that contained the microsomes was stored at −80°C in 0.1 M Tris (pH 7.4), 20% glycerol, and 10 mM EDTA until analysis.

Western Blot Analysis
Although several cytochrome P450 isoforms are implicated in the metabolism of drugs, we assessed only CYP1A1, CYP2C11, CYP2E1, and CYP3A2, which are the more abundant isoforms in the rat liver and are most affected by CRF as previously reported (9). These isoforms were assessed by Western blotting as described elsewhere (9). P450 were detected using polyclonal goat anti-rat 1A1, 2C11, 2E1, and 3A2

Table 1. Physiologic characteristics of rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CRF</th>
<th>Control PTX</th>
<th>CRF-PTX</th>
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<tr>
<td>Rats (N)</td>
<td>22</td>
<td>20</td>
<td>11</td>
<td>12</td>
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<tr>
<td>Body weight (g)</td>
<td>376 ± 15</td>
<td>318 ± 12</td>
<td>377 ± 17</td>
<td>324 ± 15</td>
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<td>Serum creatinine (μmol/L)</td>
<td>56.6 ± 1.3</td>
<td>216 ± 13b</td>
<td>53.4 ± 0.8</td>
<td>211 ± 29b</td>
</tr>
<tr>
<td>Creatinine clearance (μl/100 g per min)</td>
<td>390 ± 16</td>
<td>78.2 ± 9.9b</td>
<td>371 ± 24</td>
<td>79.1 ± 12.4b</td>
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<tr>
<td>PTH (pg/ml)</td>
<td>50.4 ± 7.0</td>
<td>1730 ± 560b</td>
<td>19.3 ± 2.5b</td>
<td>18.2 ± 3.8b</td>
</tr>
<tr>
<td>Plasma calcium (mmol/L)</td>
<td>1.26 ± 0.03</td>
<td>1.20 ± 0.07</td>
<td>0.99 ± 0.05</td>
<td>1.17 ± 0.06</td>
</tr>
<tr>
<td>Phosphate (mmol/L)</td>
<td>3.14 ± 0.08</td>
<td>3.91 ± 0.41</td>
<td>3.70 ± 0.35</td>
<td>2.88 ± 0.25</td>
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aCRF, chronic renal failure; PTH, parathyroid hormone; PTX, parathyroidectomy.
bp < 0.05 versus control group.
RNA Isolation and Real-Time Quantitative PCR Analysis
RNA extractions were done on either the liver or the hepatocytes with the RNeasy Midi and Mini Kit (Qiagen), respectively. One microgram of total RNA was used to prepare cDNA by reverse transcription using Omniscript RT kit (Qiagen) and random primer (Invitrogen, Burlington, Ontario, Canada). Quantitative PCR analysis was performed using Platinum SYBR green qPCR (Invitrogen) on the iCycler real-time detection system (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Specific primer sets were designed for each of the two tested mRNA (3A2 and glyceraldehyde-3-phosphate dehydrogenase) on the basis of published cDNA sequences with the aid of the Jellyfish computer program (LabVelocity Inc., Los Angeles, CA) and are reported in Table 2. All primers were obtained from Sigma, and their specificity was confirmed by sequencing the resulting PCR product on an ABI Prism 3100 analyzer (Applied Biosystems, Foster City, CA). Used PCR conditions were optimized to 95°C for 15 s, 59°C for 30 s, and 72°C for 60 s. The respective PCR products were cloned in the pCR 2.1 vector using TA cloning Kit (Invitrogen). The resulting plasmids were purified with Hispeed Plasmid Midi Kit (Qiagen), quantified at 260 nm and diluted to make a standard curve.

Evaluation of CYP3A Activity
For evaluation of the metabolic activity of CYP3A in microsome of either treated hepatocytes or the livers microsomes of the different groups of rats, a selective fluorescence probe, 3-[(3,4-difluorobenzyl)oxy]-5,5-dimethyl-4-[4-methylsulfonyl]phenoxyfuran-2(5H)-one (DFB), which is specifically metabolized by rat CYP3A2, was used as previously reported (23). In the case of microsomes, 25 μg of protein was used. The fluorescence of the metabolite 3-hydroxy-5,5-dimethyl-4-[4-(methylsulfonyl)phenyl]-furan-2(5H)-one (DFH) is read on the cytometer (Cytoflor 4000/TR, Perspective Biosystems, Framingham, MA) using appropriate wavelength (excitation filter 360/40 nm; emission filter 460/40 nm). Standard curve was prepared with known dilution of DFH. For hepatocytes, substrate was added directly to the culture after removal of the culture medium and replacement by Krebs buffer that contained 12.5 mM HEPES.

Blood and Urine Chemistries
Blood (urea, creatinine, calcium, and phosphate) and urine (creatinine) chemistries were determined with a Hitachi 717 autoanalyzer (Roche). PTH was measured by using the Rat intact PTH ELISA Kit (Alpco Diagnostics), which measure the intact 1-84 PTH. The lowest detectable level is 15 pg/ml.

Table 2. Nucleotide sequences of PCR primers

<table>
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<th>mRNA</th>
<th>Primer sequence (5’-3’)</th>
<th>Predicted Product Size</th>
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<tr>
<td>3A2</td>
<td>GATTCTAAGCATAAGGCACCGAGT</td>
<td>ACAGGGCTTTATGAGACACTTCTCTT</td>
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<tr>
<td>GAPDH</td>
<td>TAAAGGGCATCCTGGGCTACACT</td>
<td>CTTACTCCTTGAGGGCCATGAG</td>
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</table>

*Primers for 3A2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed on the basis of published cDNA sequences with the aid of the Jellyfish computer program. The resulting PCR product was sequenced on an ABI Prism 3100 analyzer (Applied Biosystems, Foster City, CA) to confirm the specificity of the primers.
in vitro liver P450, we incubated during 24 h normal hepatocytes with synthetic 1-34 rat PTH.

**Dosage-Response Curve of PTH on Liver CYP3A in Cultured Hepatocytes.** Figure 4 depicts the effects of various concentrations of PTH (10\(^{-12}\) to 10\(^{-7}\) M) on the protein expression as well as on the mRNA levels of CYP3A2 of hepatocytes. As the concentration of PTH increased, there was a dosage-dependent decrease in CYP3A2 levels at both the protein and the mRNA level that tended to plateau at 10\(^{-8}\) M. It is interesting that the \textit{in vivo} concentrations that were obtained in our rats with CRF were between 10\(^{-10}\) and 10\(^{-9}\) M (see Table 1). There was no effect of PTH on other control hepatocyte proteins (glyceraldehyde-3-phosphate dehydrogenase, aspartate aminotransferase, or \(\beta\)-actin; data not shown).

**Effects of PTH on Other P450 Isoforms.** Because CRF as well as uremic serum was associated with a decrease in several P450 isoforms, we were interested to determine whether PTH could modulate other P450 isoforms in cultured hepatocytes. Figure 6 shows that besides CYP3A, PTH (10\(^{-9}\) M) could decrease the protein expression of CYP1A1 and CYP2C11 and had no effect on CYP2E1.

**Role of PTH in the \textit{In Vivo} Downregulation of P450 Induced by CRF**

These experiments were to determine whether PTH is implicated in the \textit{in vivo} decrease of P450 that is found consistently in CRF. As shown in Figure 3, we confirmed that in CRF, there was a 72% decrease in liver CYP3A protein expression. However, preventing the development of secondary hyperparathyroidism by PTX partly reverses the negative effect of CRF on CYP3A. The mRNA coding for CYP3A2 was significantly reduced in rats with CRF. We also observed a significant increase in mRNA levels in CRF-PTX rats. The magnitude of this increase was similar (approximately 300%) for both protein expression and mRNA. Similar results were obtained when we evaluated the metabolic activity of CYP3A by the DFB assay.

**In Vitro Effect of PTH on P450 in Cultured Hepatocytes**

To confirm that PTH not only was implicated in the downregulation of liver P450 in uremia but also could regulate in...
Effects of NF-κB Inhibitors on PTH-Induced Decrease of P450. PDTC and Andrographolide are known to be specific inhibitors of the NF-κB pathway (19,20). Figure 7 shows that the decrease in CYP3A protein expression, mRNA, and activity by PTH (10^{-9} M) was prevented by the addition of PDTC and Andrographolide to hepatocytes’ culture media.

**Discussion**

This study demonstrates that PTH is the major mediator implicated in the downregulation of liver P450 in rats with CRF. Indeed, we showed that the depletion of CRF serum with
CRF is associated with an increase in PTH (secondary or tertiary hyperparathyroidism), which causes several uremic complications (25–29). More specific, PTH downregulates the mRNA of many proteins, such as hepatic lipase, as well as the receptors for vasopressin, angiotensin II in hepatocytes, and IGF-1 in cardiomyocytes (15–17). Recently, CRF-induced resistance to IGF-1 was attenuated by PTX, suggesting a role of PTH in this resistance (17). The mechanisms underlying these effects of PTH seem to be related to an increase of cAMP and/or an increase in intracellular calcium (26).

The effects of PTH on the isoforms of P450 that are implicated in the metabolism of drugs, as found in this study, also could be secondary to activation of cAMP and increase in [Ca²⁺]. PTH signaling pathway includes the generation of cAMP with activation of protein kinase A and subsequent phosphorylation of proteins (30). In hepatocytes, PTH increases cAMP production (31), which could cause a phosphorylation of P450 by a cAMP-dependent protein kinase, leading to a decrease in the activity of selected isoenzymes of the P450 (32,33) or a downregulation of the expression of genes (CYP1A1, 2B1, 2B6, and 3A1) (34,35). However, some data also suggest that PTH may activate protein kinase C (30), and that also could lead to P450 inhibition (36). However, the role of [Ca²⁺], in the regulation of P450 remains poorly defined (35,37,38).

Aside from these well-characterized PTH-signaling pathways, recent data suggest a third hypothesis through which PTH could inhibit P450. This is via activation of the NF-κB. NF-κB is a pleiotropic transcription factor that plays an important role in the regulation of physiologic processes, including immune responses, inflammatory reactions, cell proliferation, apoptosis, and developmental processes (39). Several studies have shown that NF-κB plays an important role in mediating the suppression of P450 expression by inflammatory agents, such as inflammatory cytokines and LPS (40,41). Moreover, it has been reported that PTH and PTH-rP cause dosage- and time-related increases in NF-κB in human and rat osteoblastic cells (42,43). Our results support the hypothesis that PTH may act via NF-κB to downregulate P450. Indeed, we have shown that the inhibition of the NF-κB pathway prevents the decrease in CYP3A2 expression and activity (Figure 7). Further studies are ongoing to identify the precise mechanism of PTH-induced P450 downregulation.

In this study, we also evaluated whether PTX could prevent the downregulation of liver P450 that is induced in vivo by CRF. As shown in Figure 3, P450 decreased by 72% in liver of rats with CRF but only by 28% in liver of CRF-PTX rats. This reduction, although less important, still was significant compared with control rats. These results also suggest that PTH is a major factor not only in vitro but also in vivo but that in vivo, other factors could be implicated in the downregulation of liver P450. One of these factors could be cytokines. Indeed, several studies have demonstrated that CRF is associated with a chronic activation of inflammatory response (44,45). Patients with CRF show an increase in plasma levels of many cytokines, such as IL-1, monocyte chemotactic and activating factor, IL-6, granulocyte inhibitory protein, and TGF (46–50). However, cytokines are able to downregulate hepatic P450 in vitro and in vivo.
vivo (13). These observations support the hypothesis that, besides PTH, cytokines could downregulate the liver P450 in rats with CRF.

Conclusion
CRF is associated with a decrease in liver P450 secondary to reduced mRNA levels. The main reason for the decrease in P450 is the presence of uremic factors that accumulate in CRF. In this study, we demonstrated that PTH is one of these factors. Preventing secondary hyperparathyroidism by PTX precludes the downregulation of liver P450 in rats with CRF. Finally, we have identified a new hormone (PTH) that modulates the major drug-metabolizing system, the P450.

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References
26. Massry SG, Smogorzewski M: Parathyroid hormone,


