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.
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 laparotomies (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. 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.

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**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 a 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 were then 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 10−7 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 (10−9 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>
</tr>
<tr>
<td>Body weight (g)</td>
<td>376 ± 15</td>
<td>318 ± 12</td>
<td>377 ± 17</td>
<td>324 ± 15</td>
</tr>
<tr>
<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>
</tr>
<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 ± 0.8</td>
<td>3.91 ± 0.41</td>
<td>3.70 ± 0.35</td>
<td>2.88 ± 0.25</td>
</tr>
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</table>

*CRF, chronic renal failure; PTH, parathyroid hormone; PTX, parathyroidectomy.

*b p < 0.05 versus control group.*
(Gentest Corp., Woburn, MA), respectively. β-Actin was detected using a mouse anti-chicken β-actin (Neo-Markers, Fremont, CA). Immune complexes were revealed by secondary antibodies (swine anti-goat IgG coupled to peroxidase [Biosource International, Camarillo, CA] or goat anti-mouse IgG coupled to peroxidase [Sigma]) and the Luminol derivative of Lumi-Light Western blotting substrate (Roche Diagnostics, Laval, Quebec, Canada). Immune reaction intensity was determined by computer-assisted densitometry on Fuji (Stamford, CT) LAS-3000 LCD camera coupled to the analysis program MultiGauge (Fuji).

RNA Isolation and Real-Time Quantitative PCR Analysis
RNA extractions were done on either the liver or the hepatocytes with the RNaseasy 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 of the resulting PCR product on 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-(3,4-difluorobenzyl)oxy]-5,5-dimethyl-4-[4-methylsulfonyl]phenyl]furan-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]furane-2(5H)-one (DFH) was read on the cytofluorometer (Cytofluor 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.

Statistical Analyses
The results are expressed as mean ± SEM. Differences between groups were assessed by using an unpaired t test or an ANOVA test. Significant ANOVA was followed by a post hoc Scheffe analysis (Dunnett for Figure 4). The threshold of significance was P < 0.05.

Results
Biochemical Parameters and Body Weight in Rats
Table 1 presents the biochemical parameters and body weight of the four groups of animals studied. Compared with control rats, rats with CRF had higher levels of plasma creatinine and lower values of creatinine clearance, which was reduced by 80% (P < 0.001). Body weights in control rats and rats with CRF were similar. The serum PTH was increased 10-fold in rats with CRF, reflecting the magnitude of secondary hyperparathyroidism. PTH levels were undetectable in PTX rats. Although, there was a small decrease of plasma calcium in PTX rats, it was not statistically different from that in control rats (Table 1).

Role of PTH in the Downregulation of Liver P450 Induced by Uremic Serum
Two sets of experiments were conducted. We first neutralized PTH in uremic serum. As shown in Figure 1, we confirmed our previous results as CRF serum induced a 34 and 43% decrease in CYP3A protein expression and mRNA levels, respectively. CRF serum that was depleted of PTH lost its inhibitory capacity on CYP3A expression and activity (Figure 1). PTH concentrations in CRF serum were reduced by 85 to 99% by depletion with immobilized anti-PTH. Addition of PTH (at a concentration similar to CRF serum, which is 10−9 M) into depleted CRF serum induced a similar decrease in CYP3A levels as nondepleted CRF serum, confirming that the depletion procedure was specific for PTH (Figure 1). Similar results were obtained for CYP3A activity that was evaluated by the DFB assay. We also evaluated another major isof orm in the rat (CYP2C11), and as shown for CYP3A, the depletion in PTH of CRF serum reverses the downregulation of CYP2C11 that is induced by uremic serum (data not shown).

To confirm further the role of PTH in uremic serum, we evaluated the effect of serum from PTX rats. As shown in Figure 2, the sera of CRF-PTX rats have no downregulating effects on CYP3A protein and mRNA expression. Again, addi-

Table 2. Nucleotide sequences of PCR primers

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primer sequence (5'-3')</th>
<th>Predicted Product Size</th>
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<tbody>
<tr>
<td>3A2</td>
<td>GATTCTAAGCATAAGCCACCGAGT</td>
<td>ACAGGGGCTTTATGAGACACTTCGCTTT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TAAAGGGCCATCCTGGGCTACACT</td>
<td>CTTACTCTTTGGAGGCATGAG</td>
</tr>
</tbody>
</table>

aPrimers 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.
tion of PTH (10^{-9} M) to CRF-PTX sera induced a similar decrease in CYP3A levels as obtained with CRF sera (Figure 2). Similar results were obtained when we evaluated the activity of CYP3A by the DFB assay and also for the expression of CYP2C11 (data not shown).

**Role of PTH in the In Vivo Downregulation of P450 Induced by CRF**

These experiments were to determine whether PTH is implicated in the 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 down-regulation of liver P450 in uremia but also could regulate 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 dosagedependent 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 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 β-actin; data not shown).

**Effects of PTH on CYP3A Activity in Cultured Hepatocytes.** The effects of PTH (at a concentration of 10^{-9} M) on the CYP3A activity are shown on Figure 5. When hepatocytes were incubated with PTH, the activity of CYP3A was decreased by approximately 40%, which is a decrease similar to that obtained with uremic serum or in rats with CRF (Figures 1 and 2).
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 serum. Concentrations that are found in CRF (10^{-9} M) to depleted CRF serum induced a similar decrease in P450 as nondepleted CRF serum. The serum of CRF-PTX rats had no effect on P450 compared with CRF serum. Again, addition of PTH to CRF-PTX serum induced a similar decrease in P450 as that obtained with CRF serum. Finally, PTX partly prevented the decrease of liver P450 in rats with CRF.

Renal failure generally has been thought to decrease only the renal clearance of drugs (24). However, several studies have demonstrated that CRF also decreases hepatic drug metabolism secondary to a decrease in liver P450 (9). The main reason for decreased P450 activity and expression in the liver seems to be the presence of uremic factors that accumulate in CRF (11). Indeed, we have shown that in normal hepatocytes that were incubated serum in the presence or absence of andrographolide or pyrrolidine dithiocarbamate (PDTC). CTL hepatocytes were defined as 100%. Representative blots were shown in insert. *P<0.001 when compared with CTL hepatocytes.

Figure 7. Expression of CYP3A2 protein measured by Western blot (■), and drug metabolizing activity measured by DFB assay (■) in normal rat hepatocytes incubated for 24 h with or without PTH in presence of andrographolide or pyrrolidine dithiocarbamate (PDTC). CTL hepatocytes were defined as 100%. Representative blots were shown in insert. 

 anti-PTH antibodies completely reverses the inhibitory effect on P450 of CRF serum in cultured hepatocytes. Addition of PTH (10^{-9} M) to CRF serum rendered similar decreases in P450 as that obtained with serum from control rats. These results also suggest that PTH is a major factor not only in P450 downregulation but that 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 isozymes 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 [Ca^{2+}]. 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 [Ca^{2+}], 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 doseage- 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 vitro 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 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.

Acknowledgments

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References

26. Massry SG, Smogorzewski M: Parathyroid hormone,


