Probenecid Protects against In Vivo Acetaminophen-Induced Nephrotoxicity in Male Wistar Rats

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ABSTRACT

Renal effects of acetaminophen (APAP) were studied in rats pretreated with probenecid to analyze whether acute APAP-induced nephrotoxicity could be related to a probenecid-sensitive transport system for APAP or its S-derived conjugates. The administration of probenecid (200 mg/kg b.wt. i.p.) 30 min before APAP administration (1000 mg/kg b.wt. i.p.) improved urine flow rate and protected against the alterations in glomerular filtration rate and urea and creatinine plasma levels induced by APAP. Fewer epithelial cells and granular casts and a decrease in the urinary excretion of protein and glucose were observed in rats pretreated with probenecid. Probenecid pretreatment promoted an elevation in the urinary 16-hr excretion of APAP and a diminution in the plasma levels attained by APAP. These results suggest that protection afforded by probenecid in vivo could be a consequence of the inhibition of APAP S-conjugate renal uptake and/or an increase in APAP renal clearance. The effects of APAP in presence of probenecid were studied with the isolated perfused kidney model. Perfusion with probenecid (0.1 mM) before APAP (10 mM) did not change APAP direct renal effects, APAP urinary excretion, or APAP renal clearance relative to glomerular filtration rate. Our results suggest that protection afforded by probenecid in vivo could be the result of the inhibition of the uptake of nephrotoxic APAP metabolites and/or a diuresis-induced enhanced APAP renal excretion.

APAP is a widely used analgesic/antipyretic drug. Even though APAP is considered a safe drug, in overdose situations it produced hepatic necrosis and renal failure in both humans (Boyer and Rouff, 1971; Cobden et al., 1982; Prescott et al., 1971) and experimental animals (McMurtry et al., 1978; Mitchell et al., 1973).

In previous work, we reported the development of APAP-induced acute nephrotoxicity in male Wistar rats. The nephrotoxic dose used produced a diminution in hepatic GSH levels (Trumper et al., 1992), so important formation of GSH conjugates could be assumed. The contribution of the GSH-derived APAP metabolites formed in the liver and the involvement of the renal γGT-dependent transport of these conjugates also were reported. However, despite a significant inhibition of renal γGT activity by acivicin pretreatment, only a partial protection of APAP renal effects was observed (Trumper et al., 1996). This could be the result of other mechanisms participating in the renal incorporation of the GSH conjugates. In this regard, Lash and Jones (1984) characterized a PROB-sensitive transport system for GSH and GSH conjugates in renal basolateral vesicles. Proximal tubular toxicity of hexachloro-1,3-butadiene to the rat kidney was related to a PROB-sensitive transport process (Lock and Ishmael, 1985). PROB also protected against acute toxicity of hexachloro-1,3-butadiene and methyl mercury to the mouse kidney (Ban and Décaux, 1988).

The aim of the present study was to examine whether acute APAP nephrotoxicity could be related to a PROB-sensitive transport system. The renal effects of APAP in rats pretreated with PROB were studied in in vivo experiments. To assess whether PROB protects against direct renal effects of APAP or modifies its renal clearance, the effects of APAP in presence of PROB were studied with the IPK model.

Methods

Animals

Male Wistar rats (3 months; 250–350 g b.wt.) were used. They were housed in rooms with controlled temperature (21–23°C), humidity and regular light cycles (12 hr) and maintained on a standard diet and water ad libitum.

ABBREVIATIONS: APAP, acetaminophen; IPK, isolated perfused kidney; GSH, glutathione; GFR, glomerular filtration rate; γGT, γ-glutamyl transpeptidase; PAH, p-aminophenolic acid; CrPAH, clearance of p-aminophenolic acid; CaAPAP, clearance of acetaminophen; FENO, fractional excretion of sodium; FEH2O, fractional excretion of water; FEGLU, fractional excretion of glucose; UFR, urine flow rate; PROB, probenecid; PF, perfusion flow; PP, perfusion pressure.
Effects of PROB on APAP-Induced Nephrotoxicity In Vivo

Rats were fasted for 17 hr (5:00 p.m. to 10:00 a.m.) before the experiments. They were always allowed free access to water. They were kept singly in stainless steel metabolic cages (La Técnica CI, Buenos Aires, Argentina) during a 16-hr (6:00 p.m. to 10:00 a.m.) urinary collection period. A 2-day acclimation period to this regimen was allowed before initiation of the experiment. On the third day, several experimental groups were studied: (1) animals that received a single dose of APAP (Sigma Chemical, St. Louis, MO), 1000 mg/kg b.wt. i.p. at 5 ml/kg in propylene glycol (APAP, n = 4), before the collection period; this dose of APAP was described previously as nephrotoxic in male Wistar rats (Trumper et al., 1992); (2) animals injected with PROB (Sigma Chemical), 200 mg/kg b.wt. i.p. at 10 ml/kg in isotonic saline (the solution was made alkaline and then adjusted to pH 7.4), 30 min before the administration of APAP (PROB-APAP, n = 4); (3) animals injected with PROB, 200 mg/kg b.wt. i.p., 30 min before the collection period (PROB, n = 4); and (4) animals that received the corresponding volume of APAP vehicle (control, n = 5). The dose of PROB used is similar to the used by others (Ban and de Cáceres, 1988; Fowler et al., 1993).

At the end of the 16 hr-collection period, animals were anesthetized with sodium thiopental (70 mg/kg b.wt. i.p.), and blood was collected from inferior vena cava into a syringe containing heparin. Livers were promptly removed to study the GSH content on tissue homogenates as an index of S-conjugate formation. Plasma was separated immediately through centrifugation for the determination of creatinine, urea, glucose and APAP concentrations. Urine volume was recorded before urine centrifugation. Urine sediment was examined by light microscopy. The activity of γGT was determined on fresh urine samples. Urinary creatinine, protein, glucose and APAP also were determined. Hepatic GSH content was measured on tissue homogenates.

Based on previous experiments, we found that plasma creatinine levels (control = 3.5 ± 0.2 mg/ml) were elevated after 1 hr of APAP treatment (10.4 ± 0.3 mg/ml) and these levels remained elevated for the following 16 hr (10.6 ± 1.4 mg/ml), GFR was estimated as the creatinine clearance.

Effects of APAP in the Presence of PROB in the IPK

Perfusion procedure and apparatus. Rats were anesthetized with sodium thiopental (70 mg/kg b.wt. i.p.). The right kidney was prepared and perfused as described previously (Elías et al., 1981; Trumpet et al., 1995). The perfusion medium (pH 7.4) consisted of Ringer-Krebs solution containing dextran 2% (Sigma Chemical; average molecular weight, 82,200) as a colloid osmotic agent, glucose (10 mM), sodium pyruvate (5 mM), sodium lactate (5 mM), creatinine (100 mg/liter) and PAH (6 mg/liter). The medium also contained 0.5 mM cysteine, 0.5 mM glutamic acid and 2.3 mM glycine to prevent the loss of GSH from specific regions of the kidney (Breis et al., 1983; Epstein et al., 1982; Torres et al., 1986). The medium was constantly bubbled with 95% O2/5% CO2. The whole system operated under thermostatic control at 37°C. The total volume of perfusate used in the recirculating system of each experiment was 100 ml. PF through the isolated kidney in situ was performed with a peristaltic pump (Masterflex; Cole-Parmer Instrument) at a constant rate measured with a flowmeter (Gilmont Instruments, Barrington, IL) inserted in the arterial line. PF was continuously measured at the tip of the arterial cannula by a pressure transducer (P231D; Gould, Glen Burnie, MD) and recorded with a multifen recorder (Rikadenki; Kogyo Co., Tokyo, Japan).

Experimental design. The isolated kidney was perfused for ~30 min (equilibration time) until PP and PF remained constant. The subsequent experimental time was 40 min, during which urine was collected over 5-min periods. Perfusate was sampled at the midpoint of each 5-min urine collection interval. At the end of the experiment, the kidney was removed, gently blotted on filter paper and weighed.

Four experimental groups were studied. (1) Kidneys were perfused with Ringer-Krebs solution to assess the viability of the preparations during the experimental time (control, n = 7). (2) Kidneys were perfused with APAP (perfusate concentration = 10 mM) (APAP, n = 7). APAP was dissolved in a small volume of perfusate and added to the recirculating medium (total volume, 100 ml) at the end of the second period after the equilibration time. The concentration of APAP examined is in the range of the concentration obtained in vivo 1 hr after the administration of APAP 1000 mg/kg (Trumper et al., 1995). (3) Kidneys were perfused with 0.1 mM PROB from the moment of isolation (PROB, n = 4). The concentration of PROB in the perfusate was used by others (Newton et al., 1986) to inhibit the secretion of APAP mercapturate in the IPK. (4) Kidneys were perfused with APAP (10 mM) added as stated in (2) to the perfusion medium containing 0.1 mM PROB (PROB-APAP, n = 4).

Functional data from the two first experimental periods after the equilibration time were averaged and considered to be control values for each preparation. All the subsequent results were expressed as percentage change relative to control values.

The following parameters were evaluated as a criteria of overall kidney function during the course of the perfusion: PF, PP, UFR, GFR (estimated on the basis of the clearance of creatinine), C_pail, FEGlu, FE_Na, and FE_H2O.

Analytical Methods

The volume of urine was estimated gravimetrically. Creatininemia was determined by the Jaffé reaction with a commercial kit (Henry et al., 1980a; Wiener Laboratories, Rosario, Argentina), glucose by the glucose oxidase method (Henry et al., 1980b; Wiener Laboratories), and PAH by Brun’s method as modified by Waugh and Beall (1974). Urea was measured with a commercial kit (Henry et al., 1980c; Wiener Laboratories). Sodium was measured by flame photometry. Determination of liver nonprotein sulfhydryls (roughly representing GSH) was carried out in whole tissue homogenates prepared in cold 5% trichloroacetic acid in 0.01 M HCl and measured as described by Ellman (1959). Total urinary proteins were measured with the EDTA/Cu reagent (Biuret’s method, Henry et al., 1980d, Wiener Laboratories). APAP was determined by a spectrophotometric method (Glynn and Kendal, 1975). Urinary γGT activity was determined by use of γ-glutamyl-p-nitroanilide as substrate with a commercial kit according to the manufacturer’s directions (Wiener Laboratories).

Fractional excretion of water (urine volume/min/GFR), sodium (clearance of sodium/GFR) and glucose (clearance of glucose/GFR) were calculated with the use of conventional formulae. APAP clearance relative to GFR also was calculated.

Statistical Analysis

Results are expressed as mean ± S.E.M. In vivo data were analyzed using the one-way analysis of variance followed by Newman-Keuls comparisons. IPK data were analyzed by two-way analysis of variance and Bonferroni’s comparisons. The .05 level of probability was used as the criterion of significance in all cases.

Results

Effects of PROB on APAP-Induced Nephrotoxicity In Vivo

Tissue measurements. APAP administration induced a significant diminution in hepatic GSH content, whereas dual treatment did not produce any change in the APAP-induced diminution of hepatic GSH levels (control = 3.83 ± 0.1, PROB = 3.48 ± 0.2, APAP= 2.51 ± 0.3, * PROB-APAP = 2.63 ± 0.5 μmol/g wet tissue; *P < .05 compared with control).

Renal function. PROB treatment promoted an increase in UFR. UFR showed a trend to decrease in APAP-treated rats. In rats that received the dual treatment PROB-APAP,
UFR was improved. PROB did not alter GFR, plasma creatinine or urea levels compared with control values. APAP treatment significantly decreased GFR, associated with an increase in creatinine and urea plasma levels. Prior treatment with PROB followed by APAP protected against APAP-induced diminution of GFR. Plasma creatinine levels of rats that received the dual treatment PROB-APAP were significantly lower than those of animals treated with APAP alone and were not different from control values. Urea plasma levels, although different from control values, were significantly lower than those observed in APAP-treated rats. All data are shown in figure 1.

APAP promoted a marked proteinuria and glucosuria and a significant increase in the urinary excretion of γGT (fig. 2). PROB treatment had no effect on protein and glucose excretion and urinary γGT activity. Total urinary protein, glucose and γGT excretion of rats that received the dual treatment of PROB and APAP were significantly decreased compared with animals treated with APAP alone and did not differ from control levels.

Urinary sediments of APAP-treated animals contained many granular casts and epithelial cells. In the urinary sediment of PROB-pretreated rats, very few casts or cells were observed. These data combined could provide evidence that PROB protects against APAP-induced nephrotoxicity.

Plasma APAP levels 16 hr after APAP administration were significantly higher in rats treated with APAP alone than in rats pretreated with PROB. APAP urinary excretion during the 16-hr urinary collection period was higher in rats pretreated with PROB (fig. 3). These results suggest that PROB increases APAP renal clearance, and this could explain its protective effects.

Effects of APAP in the Presence of PROB in the IPK

Perfusion with PROB decreased the C_{PAH} in the IPK (table 1), showing an important level of inhibition of the organic anion transport system. Other functional parameters evalu-
TABLE 1

Functional parameters of control periods of IPK

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 7)</th>
<th>PROB (n = 4)</th>
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<tbody>
<tr>
<td>$C_\text{APAP}$ (ml/min/g)</td>
<td>2.5 ± 0.3</td>
<td>0.9 ± 0.4**</td>
</tr>
<tr>
<td>$UFR$ (ml/min/g)</td>
<td>0.11 ± 0.02</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>$GFR$ (ml/min/g)</td>
<td>0.30 ± 0.03</td>
<td>0.23 ± 0.06</td>
</tr>
<tr>
<td>$F\text{E}_{\text{H}_2O}$ (%)</td>
<td>22.0 ± 5</td>
<td>22.3 ± 5</td>
</tr>
<tr>
<td>$F\text{E}_{\text{Na}}$ (%)</td>
<td>18.0 ± 4.6</td>
<td>22.2 ± 5.0</td>
</tr>
<tr>
<td>$F\text{E}_{\text{glu}}$ (%)</td>
<td>11.3 ± 2.6</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>$\text{PP}$ (mm Hg)</td>
<td>124 ± 3.4</td>
<td>129 ± 4.8</td>
</tr>
<tr>
<td>$\text{PF}$ (ml/min)</td>
<td>22 ± 0.3</td>
<td>21 ± 0.37</td>
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Kidneys were perfused with Ringer-Krebs alone (Control) or with the addition of probenecid 0.1 mM from the moment of isolation (PROB). Values are expressed as mean ± S.E.M. of control periods.

**P < .05 compared with control preparations.

Effects of APAP (10 mM) alone or in presence of PROB were studied with the IPK. The time course of the fractional excretion of water (A), sodium (B) and glucose (C) and GFR (D) are shown. At time = 0 min, APAP was added as a single dose to the perfusate. In PROB-APAP, kidneys were perfused with PROB from the moment of isolation. Values are expressed as percentage change relative to control period. Each point represents mean ± S.E.M. *P < .05 compared with previous time. †P < .05 compared with time = 5 min.

**Fig. 4.** Effects of APAP (10 mM) alone or in presence of PROB (0.1 mM) in the IPK. The time course of the fractional excretion of water (A), sodium (B) and glucose (C) and GFR (D) are shown. At time = 0 min, APAP was added as a single dose to the perfusate. In PROB-APAP, kidneys were perfused with PROB from the moment of isolation. Values are expressed as percentage change relative to control period. Each point represents mean ± S.E.M. *P < .05 compared with previous time. †P < .05 compared with time = 5 min.

Discussion

Based in our previous work, we can postulate that APAP-induced nephrotoxicity may involve the contribution of at least two components: direct effects of APAP, which were confirmed with the IPK model (Trumper et al., 1995), and the effects of GSH-derived APAP metabolites (Trumper et al., 1996). In this regard, S-conjugates in systemic circulation may be accumulated in the kidney via several carrier-mediated mechanisms, including the organic anion transporter and degradation by membrane bound γGT (Commandeur et al., 1996; Koob and Dekant, 1991). In our previous work, we showed that an almost complete inhibition of renal γGT activity only partially protected against APAP renal effects.

In the present work, PROB was used as another possible tool to modify active tubular transport of APAP-S-conjugates. PROB has shown to decrease the renal uptake of S-(1,2-dichlorovinyl) GSH (Lash and Jones, 1985) and block the nephrotoxicity of S-2-chloroethyl) GSH (Kramer et al., 1987).

This study presents evidence that PROB pretreatment affords protection of APAP renal effects in vivo. PROB treatment before APAP intoxication protected against APAP-induced decrease of GFR and elevations in creatinine and urea plasma levels. PROB pretreatment also protected against the elevations of the urinary excretion of protein, glucose and γGT induced by APAP. Fewer epithelial cells and granular casts were observed in rats that received PROB pretreatment compared with the abundant cells and casts observed in rats treated with APAP alone. It is noticeable that PROB alone promotes an enhanced diuresis. The trend to decrease UFE by APAP treatment was blunted when rats were pretreated with PROB. Moreover, APAP plasma levels 16 hr after APAP administration were significantly higher in rats treated with APAP alone compared with the level attained in rats pretreated with PROB. APAP urinary excretion during a 16 hr urinary collection period was higher in rats pretreated with PROB. It is noteworthy that the colorimetric method for APAP determination is specific for unchanged drug (Glynn and Kendal, 1975).

APAP administration induced a significant diminution in hepatic GSH content, while dual treatment PROB-APAP did not produce any change in the APAP-induced diminution of hepatic GSH levels, so we could assume the same level of hepatic GSH derived conjugate formation in both experimental conditions.

Taken together, these results may suggest that the protection afforded by PROB may be a consequence of an inhibition of the uptake of APAP-S-conjugates and/or an increase of APAP renal clearance.

The effects of APAP in presence of PROB were studied with the IPK model to assess whether PROB protects against direct renal effects of APAP or modifies its renal clearance. The IPK provides a suitable experimental model in which no hepatically derived metabolites are present. Our clearance studies in the IPK suggest that APAP is reabsorbed from the tubular lumen. In this regard, it is known that APAP excretion involves filtration and reabsorption by passive diffusion...
of the nonionic form (Duggin, 1980; Duggin and Mudge, 1978).

Perfusion with PROB decreased the clearance of PAH, showing an important level of inhibition of the organic anion transport system, as PROB and PAH share a common mechanism in isolated kidney cells (Lash and Anders, 1989).

Perfusion with PROB before APAP did not change the increases in FEH₂O, FENa, and FEglu, or the decrease in GFR induced by APAP. These results show that in this model, PROB does not modify APAP renal effects, so it could be suggested that the delivery of APAP to the renal cells is not modified. This suggestion is reinforced by the fact that APAP urinary excretion and APAP renal clearance relative to GFR were not modified by PROB. This suggests that a PROB-sensitive system does not participate in the accumulation of APAP in the renal cell. However, UFRs attained in the IPK model may be too high to test the hypothesis of a PROB-sensitive reabsorption system.

In summary, protection afforded by PROB in vivo could be the result of the inhibition of a PROB-sensitive transport system for GSH-derived APAP conjugates. PROB did not modify APAP renal uptake as shown in the IPK experiments. PROB in vivo promoted an enhancement of APAP urinary excretion. Walker and Duggin (1988) stated that diuresis results in an increased clearance of APAP. In our study, the improvement in urine flow occurred together with an enhancement in APAP urinary excretion. The enhanced APAP renal excretion also could account for the protection observed. The participation of a PROB-inhibitable reabsorption system should not be disregarded.

Acknowledgments
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References


