Expression of multidrug resistance-associated protein 2 in small intestine from pregnant and postpartum rats

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Although the primary function of the small intestine is to absorb food and water, it also serves as a major portal of entry for many chemicals including drugs and toxic compounds in the environment. It therefore has one of the greatest exposures to xenobiotics in the body. It has been shown that the gastrointestinal absorption of amphipathic neutral or cationic compounds is decreased by the presence of the multidrug resistance 1 P-glycoprotein, a member of the ATP-binding cassette (ABC) family of transporters (19, 47), the first ATP-dependent transporter identified in the intestine (16). Clearly, conjugating enzymes and mrp2 may act coordinately to metabolize and secrete xenobiotics into the intestinal lumen. Biliary excretion of several mrp2 substrates is decreased in the latter stages of pregnancy (45). For example, the hepatic transport maximum for bilirubin glucuronides is decreased in pregnant rats (35). Bilirubin glucuronides are mrp2 substrates (21), and transport across the canalicular membrane of the hepatocyte rather than metabolism is likely the rate-limiting step in the overall hepatic elimination of bilirubin, suggesting that mrp2 expression is downregulated during pregnancy. It has been observed recently that mrp2 expression in plasma membrane from maternal liver is decreased in the latter stage of pregnancy (5). Several studies have also shown a decrease in liver UDP-glucuronosyltransferase (UGT)-specific activity in pregnant rats compared with that in nonpregnant control rats, affecting substrates such as bilirubin, phenol derivatives, estradiol, and estrone (3, 35, 46). The decreased capacity to metabolize and secrete glucuronide derivatives into bile in these animals could...


increase the toxicity of several endogenous and exogenous compounds to the maternal liver and, in addition, increase the risk of exposure to the fetuses. Conjugating enzymes, together with secretion of the corresponding derivatives across the apical membrane of the proximal intestinal cells, may represent an alternative pathway for preventing toxicity of these compounds, particularly those entering the body via the intestinal tract. Whether intestinal mrp2 expression is preserved in pregnant rats despite its downregulation in liver membranes is not known.

Food intake is slightly increased in pregnant rats (~50%) but is greatly increased in postpartum rats (2- to 4-fold), particularly at the latter stage of lactation (14–21 days after delivery) (12, 14, 15). This implies an adaptation of the intestinal tract to satisfy the increased need for absorption of nutrients. During lactation, the maternal small intestine increases in length, weight, and villus development (12, 15) so that the mucosal surface of the intestine is expected to increase significantly. The absorption and interaction of potentially toxic compounds with intestinal cells, particularly dietary xenobiotics, is also expected to increase. Because the intestine has a large capacity for metabolizing xenobiotics, intestinal metabolism serves as a presystemic strategy for coping with the excessive xenobiotics present in the diet. This point is illustrated by the presence of intestinal UGT activity, which can detoxify the widely occurring phenolic constituents of plant material in experimental animals (1, 24) and in humans (7, 37). Similarly, glutathione-S-transferase (GST) activity is involved in the metabolism of a large variety of food contaminants (38), particularly the inactivation of carcinogens (11). The activity of the conjugating enzymes UGT and GST is significantly increased in the small intestine of postpartum female rats (31). Fourteen and twenty-one days after delivery, lactating rats exhibit an increase in the activity of UGT toward p-nitrophenol and in GST activity toward 1-chloro-2,4-dinitrobenzene (CDNB), primarily because of an increase in expression of specific isozymes. A parallel increase in the activity of the transport proteins involved in the secretion of conjugated derivatives into the intestinal lumen would seem to be essential to prevent absorption of dietary xenobiotics in postpartum animals. The expression and activity of members of the ABC family of membrane transporters, particularly those involving transport of conjugated metabolites at the apical level of the intestine, have not been determined in postpartum rats.

In the present study, we analyzed the expression of mrp2 in the small intestine of female rats in late pregnancy and during lactation. Transport activity of a conjugated xenobiotic in the everted intestinal sac model was also analyzed. The data indicate that the expression of the transport protein was preserved in pregnant rats and increased in postpartum rats with respect to control animals. Mrp2 mRNA levels correlated well with protein levels. The data on transport activity also agreed with the data on expression of mrp2. Additional studies conducted to determine whether prolactin is involved in mrp2 protein upregulation in lactating rats revealed no effect of this hormone on intestinal expression of mrp2.

**MATERIALS AND METHODS**

**Chemicals**

Leupeptin, phenylmethylsulfonyl fluoride, pepstatin A, CDNB, 1-fluoro-2,4-dinitrobenzene, glutathione, and acivicin were obtained from Sigma (St. Louis, MO). Bromocriptine (2-Br-α-ergocriptine methane sulfate) was a gift from Sandoz Research Institute (East Hanover, NJ). Ovine prolactin (oPRL) (NIDDK-oPRL-19 and AFP-9221A) was kindly provided by the National Institute of Diabetes and Digestive and Kidney Diseases, the National Hormone and Pituitary Program, and Dr. A. F. Parlow. The specific antibodies against the COOH-terminus of rat mrp2 and mrp2 cDNA were generous gifts from Dr. Peter Meier (University Hospital, Zurich, Switzerland). The horseradish peroxidase-linked secondary antibody used in the Western blot studies was from Amersham Pharmacia Biotech (Piscataway, NJ), and a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) was used for the immunohistochemical studies. All other chemicals were of analytical grade purity and were used as supplied.

CDNB was recrystallized from ethanol and water (3:2 vol/vol) before use (17). The glutathione-conjugated derivative of CDNB, S-glutathione-dinitrophenol (DNP-SG), was synthesized with the use of 1-fluoro-2,4-dinitrobenzene and glutathione as described by Sokolovsky et al. (42).

**Animals**

Female Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) were used throughout the experiments. The pregnant and postpartum rats were timed according to the first day that sperm were detected (day 0). The rats had free access to food and water and were maintained on a 12:12-h automatically timed light-dark cycle. All procedures involving animals were conducted in accordance with National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

The following groups were used for Western blot analysis of mrp2: nonpregnant rats (180–210 g), which served as controls; rats at 19–20 days of pregnancy (late pregnant; 360–400 g); and rats at 2–4 (early lactation; 240–270 g), 10–14 (midlactation; 250–280 g), and 21 (late lactation; 260–290 g) days postpartum. Litter sizes ranged from 8 to 10 pups. Two groups of ovariectomized (OVX) rats weighing 190–230 g were implanted with osmotic minipumps (ALZET 2001, Alza, Palo Alto, CA) attached to an intravenous catheter as described by Liu et al. (28). The minipumps were filled with solvent (0.4 M NaHCO₃, 1.6% glycerol, and 0.02% sodium azide; OVX group) or with solvent plus oPRL to yield an infusion rate of 300 or 600 μg of oPRL/day for 7 days (PRL300 and PRL600 groups). The minipumps were immersed in saline at 37°C to ensure that flow had started. To suppress endogenous prolactin secretion, all the rats were implanted with bromocriptine pellets (7.5 mg, 10-day release, Innovative Research of America, Toledo, OH) subcutaneously at the time of implantation of the minipumps. Adult female mutant (TR⁻) Wistar rats bred in our animal facility, hereditarily deficient in mrp2 and weighing 180–230 g, were also used for...
Western blot studies of mrp2. It has been demonstrated that these animals do not express mrp2 in plasma membrane from the liver (36) or in apical membrane from the small intestine (33).

A group of control and pregnant rats and a group of lactating rats (14–21 days postpartum [pp (14–21 days)]; 250–290 g) were used for immunohistochemistry, Northern blot analysis, and transport activity determinations. Litter size in the latter group ranged from 8 to 10 pups. TR− rats were also used in the immunohistochemistry analysis.

**Specimen Collection**

The whole small intestine was divided into nine equal segments, carefully rinsed with ice-cold saline, and weighed. The most proximal segment, starting from the pylorus, was given the number 1, whereas the most distal segment, close to the ileocecal valve, was given the number 9. The segments were placed in saline at 4°C until preparation of mucosal tissue or isolation of the intestinal sacs. For Western blot analysis, the intestinal segments were opened lengthwise, the mucus layer was carefully removed, and the mucosa was obtained by scraping (6) and weighed. The tissue thus obtained was used for total homogenate or brush-border membrane (BBM) preparation.

For the transport studies, 3-cm segments of intestine were isolated from two different regions of the small intestine corresponding to jejunum (segments 3 and 4) and distal ileum (segments 8 and 9) and from the whole colon. The 3.5-cm segments were carefully rinsed with ice-cold saline and placed in saline at 4°C until use in the everted sac preparations.

For intestinal total RNA extraction, the segment corresponding to the proximal jejunum (segment 2) was rinsed with ice-cold saline, and the whole tissue was immediately placed in liquid nitrogen. A liver sample from the normal rats was also collected, and the whole plasma membrane from the hepatocytes (mixed membranes) was prepared as described (32).

**Preparation of BBM**

Total homogenates were prepared from mucosa samples as previously described (33). BBMs were prepared from total homogenates with the use of a divalent cation precipitation method (23) with some modifications (33). Aliquots of the homogenates and BBM preparations were placed in liquid nitrogen and used within 2 days for Western blot analysis and alkaline phosphatase activity determination. Protein concentration in homogenate and BBM preparations was measured (29) with bovine serum albumin used as the standard. Alkaline phosphatase activity was determined with p-nitrophenylphosphate as substrate (kit G01245-K; Sigma). The apical membrane enrichment was estimated by calculating the ratio of the alkaline phosphatase activity in the BBM to that in the homogenate.

**Western Blot Studies**

Western blotting for mrp2 was performed with BBM with an amount of protein (15 μg) in the gels that was found to give a densitometric signal in the linear range of the response curve for the anti-mrp2 antibody (data not shown). All procedures were performed as previously reported (33). Immunoreactive bands were quantified by densitometry (Shimadzu CS-9000, Shimadzu, Japan) and expressed in arbitrary units.

**Northern Blot Studies**

Total RNA was isolated from mucosal samples frozen in liquid nitrogen by a guanidinium-thiocyanate lysis procedure (8). Total RNA (15 μg) was denatured, electrophoresed through a 1.2% agarose-formaldehyde gel, transferred to a nylon membrane (Duralon-UV, Stratagene, La Jolla, CA) overnight by capillary blotting, and cross-linked by ultraviolet light (FB-UVXL-1000, Fisher Scientific, Westbury, NY). Hybridization with the mrp2 cDNA probe and the 28S rRNA oligoprobe and quantitation of the Northern blots were performed as previously described (33). The relative optical densities of mrp2 mRNA were adjusted by those of the 28S rRNA.

**Immunohistochemistry**

For in situ immunodetection of mrp2, rats were anesthetized with diethyl ether and perfused via transcardiac puncture with 0.1 M Dulbecco’s phosphate-buffered saline (pH 7.4; Gibco BRL, Grand Island, NY) followed by 4% paraformaldehyde in the same buffer. Two different segments corresponding to duodenum (segment 1) and proximal jejunum (segment 2) were used for the immunohistochemistry study. The segments were removed and kept overnight at 4°C in the above fixative, which contained 30% sucrose. Forty-micrometer-thick freezing microtome sections were washed for 30 min in Tris-HCl buffer (0.05 M; pH 7.6) containing 10% normal horse serum, 0.1% sodium azide, and 0.2% Triton X-100 for 1 h and then incubated overnight at room temperature with the primary antibody (1:100). After being rinsed with Tris-HCl buffer, the sections were incubated for 1 h with the secondary biotinylated antibody (1:400), washed in Tris-HCl buffer, and exposed for 1 h to the avidin-biotin-peroxidase complex (Elite, Vector Laboratories). Sections were stained with a solution containing 50 mg of 3,3′-diaminobenzidine tetrahydrochloride (Aldrich, Milwaukee, WI) and 5 μl of H2O2 in 100 ml of Tris-HCl buffer. To distinguish between specific and nonspecific staining, immunohistochemistry was also performed in the same rats but incubation with the primary antibody was omitted. We also performed the immunohistochemistry analysis in TR− rats, which are genetically deficient in mrp2 (36) so only nonspecific staining is expected. To evaluate possible differences in jejunal villus length between control and postpartum rats, a computer morphometrical analysis of immunohistochemistry preparations was performed with an NIH image analyzer program.

**Transport Activity in Intestinal Sacs**

The model of everted intestinal sacs was used throughout (39, 48). Two different strategies were used to characterize DNP-SG transport either as efflux from the intracellular compartment to the lumen or as uptake from the lumen into the cellular and serosal compartments.

**Efflux Studies.** Three-centimeter segments from jejunum were everted and filled with Krebs-Henseliti buffer (40 mM glucose, pH 7.4) that had been previously gassed with 95% O2-5% CO2, and incubated for 0, 10, 20, 30, and 60 min. The mucosal compartment was continuously gassed with 95% O2-5% CO2. After incubation, the exterior of the intestinal sacs was carefully washed with drug-free buffer, and the entire serosal solution was collected. Intestinal tissue was homogenized in 5 ml of the same buffer. Aliquots of mucosal, serosal, and homogenate samples were collected. Intestinal tissue was homogenized in 5 ml of the same buffer. Aliquots of mucosal, serosal, and homogenate samples were collected.
were treated with 70% (wt/wt) HClO₄ (50 μl/ml sample) and centrifuged at 3,500 g for 5 min. DNP-SG content was determined in the supernatants by HPLC.

Uptake studies. Three-centimeter segments from the jejunum, distal ileum, and colon were everted and filled with Krebs-Henseleit buffer as described in Efflux studies. DNP-SG (100 μM) was added to the mucosal compartment, and the DNP-SG content was determined in tissue and in the serosal compartment after 0, 10, 20, 30, and 60 min of incubation.

Because of gastric secretions, the pH in the intestinal lumen, particularly at the proximal level, is lower than intracellular pH (40). In some experiments, to mimic the in vivo situation, the pH of the mucosal side was decreased from 7.4 to 6.0. CDNB or, alternatively, DNP-SG was incorporated to 6.0. CDNB or, alternatively, DNP-SG was incorporated into the mucosal compartment, and the transport activity and tissue accumulation of DNP-SG were measured at 0, 10, 20, 30, and 60 min.

HPLC Analysis

The HPLC system consisted of a Waters model M-6000 (Waters, Milford, MA). Isocratic elution was performed with a C18 column (μBondapak, Waters) with a mobile phase of acetonitrile:0.01% H₃PO₄ (1:3, vol/vol) at a flow rate of 1.0 ml/min (18). DNP-SG was detected at 365 nm and was quantified by the external standard method by the height of the peak.

Statistical Analysis

Data are presented as means ± SD. Comparison among groups was performed with one-way ANOVA followed by the Newman-Keuls multiple-range test if ANOVA reached statistical significance (43). Values of P < 0.05 were considered statistically significant.

RESULTS

Intestine Mass, BBM Protein Recovery, and Alkaline Phosphatase Activity in Pregnant, Lactating, and oPRL-Treated Rats

Parameters describing the hypertrophy of the intestine at the macroscopic level (intestine and mucosa mass) as well as the quality of the BBM preparations (protein recovery and alkaline phosphatase activity and enrichment) were measured in all segments from the different groups. Because mrp2 is maximally expressed in proximal jejunum (33), we present the data corresponding to all of these parameters for segment 2 (Table 1). Segments corresponding to proximal jejunum from pp (10–14 days) and pp (21 days) rats were longer than those from the other groups. Because all segments within each rat were the same length, it is clear that the whole intestine from pp (10–14 days) and pp (21 days) groups was ~30% (on average) longer than that from control, pregnant, or pp (2–4 days) groups. The intestine and mucosa mass corrected per unit of length also increased (~80% on average) in pp (10–14 days) and pp (21 days) groups, indicating hypertrophy in these animals. Intestine and mucosa mass slightly decreased from the most proximal to the most distal segment (~20% decrease between segments 1 and 9), presumably because of a similar decrease in the intestinal wall thickness (12, 15). All of the other segments studied (segments 1 and 3–9) presented a pattern of increase in mass in pp (10–14 days) and pp (21 days) rats similar to that reported for segment 2 (data not shown). oPRL administration, at the dose of either 300 or 600 μg/day, did not affect intestine size

Table 1. Tissue mass, mucosa mass, BBM protein recovery, and BBM alkaline phosphatase activity and enrichment in proximal jejunum

<table>
<thead>
<tr>
<th>Segment length, cm</th>
<th>Control</th>
<th>Pregnant</th>
<th>pp (2–4 days)</th>
<th>pp (10–14 days)</th>
<th>pp (21 days)</th>
<th>OVX</th>
<th>PRL300</th>
<th>PRL600</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestine mass, g/cm</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>14</td>
<td>15</td>
<td>11</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Mucosa mass, g/cm</td>
<td>0.071 ± 0.015</td>
<td>0.077 ± 0.012</td>
<td>0.082 ± 0.010</td>
<td>0.123 ± 0.012*</td>
<td>0.130 ± 0.020*</td>
<td>0.070 ± 0.017</td>
<td>0.073 ± 0.009</td>
<td>0.080 ± 0.015</td>
</tr>
<tr>
<td>BBM protein recovery, mg/g</td>
<td>0.046 ± 0.012</td>
<td>0.051 ± 0.008</td>
<td>0.045 ± 0.015</td>
<td>0.081 ± 0.016†</td>
<td>0.090 ± 0.022†</td>
<td>0.047 ± 0.012</td>
<td>0.055 ± 0.012</td>
<td>0.052 ± 0.025</td>
</tr>
<tr>
<td>BBM AP activity, μmol·min⁻¹·mg protein⁻¹</td>
<td>2.3 ± 0.5</td>
<td>3.0 ± 1.0</td>
<td>2.1 ± 0.5</td>
<td>1.9 ± 1.1</td>
<td>1.8 ± 0.6</td>
<td>3.5 ± 1.6</td>
<td>2.5 ± 0.5</td>
<td>1.8 ± 0.7</td>
</tr>
<tr>
<td>AP enrichment</td>
<td>6.2 ± 1.8</td>
<td>4.7 ± 3.0</td>
<td>5.2 ± 2.2</td>
<td>10.3 ± 2.1</td>
<td>14.5 ± 3.4†</td>
<td>5.1 ± 0.9</td>
<td>7.0 ± 3.2</td>
<td>4.9 ± 2.5</td>
</tr>
</tbody>
</table>

Values (except for segment length) are means ± SD for n = 3 experiments/groups; segment lengths are approximate values. Statistical analysis was performed separately in the perinatal and in the OVX groups. BBM, brush-border membrane; AP, alkaline phosphatase; pp (days), days postpartum/lactation; OVX, ovariectomized; PRL300, group given 300 μg ovine prolactin/day for 7 days; PRL600, group given 600 μg ovine prolactin/day for 7 days. The whole small intestine was divided into 9 equal segments. Data in the table are from segment 2, corresponding approximately to the proximal jejunum. Each segment was rinsed and weighed, and the mucosa was immediately removed by scraping and weighed. The corresponding weights were corrected per unit of length. Although the mass of the whole intestine was not determined directly, by taking into account the increase in size (30%) and weight (80%) of segments in pp (2–4 days) and pp (10–14 days) groups, it was possible to estimate the increase in mass of the small intestine to be ~130%. BBM protein mass from each segment was corrected per mucosal tissue mass. AP activity in BBM preparations was assayed with p-nitrophenylphosphate as substrate. Apical membrane enrichment was estimated by calculation of the ratio of AP activity in BBM to the AP activity in homogenate. Prolactin treatment did not affect any of the parameters analyzed. *Significantly different from control, pregnant, and pp (2–4 days) animals (P < 0.01). †Significantly different from control, pregnant, and pp (2–4 days) animals (P < 0.05).
and weight (Table 1), in agreement with a previous study reporting no trophic effect of hyperprolactinemia on mucosal structure (34).

BBM protein recovery for segment 2 was the same in all the groups studied (Table 1). This parameter also exhibited a gradient between the most proximal and the most distal segments (~20% decrease) and did not differ among groups for any of the regions of the intestine analyzed (data not shown). Alkaline phosphatase activity was measured in all BBM and homogenate samples, and the corresponding ratio was calculated to ensure comparable apical membrane enrichment in the different segments. Although the absolute enzyme activity values decreased from the most proximal to the most distal of the nine segments analyzed (~80% decrease; data not shown), the ratio was similar for all samples in normal and OVX rats and was not affected by pregnancy, lactation, or oPRL administration (data not shown). Representative values of alkaline phosphatase enrichment are shown in Table 1 and correspond to segment 2. Interestingly, alkaline phosphatase activity in BBM from proximal jejunum tended to increase in pp (10–14 days) and significantly increased in pp (21 days) rats (Table 1). A similar increase was observed in BBM from segments 1 and 3–5 at mid- and late lactation (data not shown), the average increase being ~120% among the five most proximal segments.

**Expression of mrp2 Protein in Pregnant and Lactating Rats**

Because mrp2 expression varies along the small intestine (33), we analyzed mrp2 protein expression along the entire length of the small intestine. Figure 1A shows expression of mrp2 in mixed plasma membrane from liver and in BBM from small intestine (9 equal segments) in the female control rats and from duodenum and proximal jejunum (segments 1 and 2, respectively) in the TR- rats. As we previously reported (33), mrp2 expression was significantly lower in the intestine than in the liver, and a gradient between the proximal and distal regions of the intestine was observed, with maximal expression in segments 1–5. As expected, mrp2 was not detected in the TR- rats. Figure 1B shows the level of mrp2 in the five most proximal segments from control and pregnant rats and from postpartum rats at three different stages of lactation. Densitometric analysis of the immunoreactive bands revealed that expression of mrp2 was preserved in pregnant rats and increased in postpartum rats with

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**Fig. 1. Western blot analysis of multidrug resistance-associated protein (mrp)2. A:** Typical immunoblot performed with mixed membranes from liver and with brush-border membrane prepared from 9 segments of the small intestine in a control rat and from the 2 most proximal segments in a female mutant (TR-) Wistar rat, hereditarily deficient in mrp2. The most proximal segment (segment 1) started from the pylorus, whereas the most distal (segment 9) was close to the ileocecal valve. B: Mrp2 levels in the 5 most proximal segments from the different groups. Densitometry was performed in 3 animals/group and expressed in arbitrary units. Data are means ± SD. aSignificantly different from pregnant rats; bsignificantly different from control rats; csignificantly different from rats 2–4 days postpartum (pp (2–4 days)) and pp (10–14 days) rats.
respect to control animals, the increase being maximal at late lactation (~100% increase among the 5 segments). The pattern of change in mrp2 level in response to lactation was similar for the five segments.

**mrp2 mRNA Levels**

The mrp2 mRNA level was measured in control and pregnant rats and in postpartum rats at late lactation (pp (14 to 21 days)) to determine the molecular basis of the postpartum increase in mrp2 protein expression. mrp2 mRNA levels were determined in the proximal jejunum (segment 2), where it is maximally expressed, and are shown in Fig. 2. Although the level of mrp2 mRNA was not affected by pregnancy, it increased in postpartum animals (~100% over controls), correlating well with the increase in expression of mrp2 protein.

**In Situ Localization of mrp2**

mrp2 protein is preferentially expressed in the apical membrane of the enterocyte. Expression is maximal in the upper region of the villi from the duodenum and proximal jejunum (33). We analyzed mrp2 localization in intestinal specimens from control, pregnant, and pp (14 to 21 days) rats. Figure 3 shows immunoreactivity on the surface of the villi in slices prepared from segments 1 and 2, corresponding approximately to duodenum and proximal jejunum, respectively. Figure 3, D and E, representing low-magnification views, shows clearly that intestinal structures, and particularly the villi in the proximal jejunum, were hypertrophic in postpartum rats compared with control rats (Fig. 3, A–C). By image analysis of the jejunum performed in six preparations per group, the villus in lactating rats was ~100% longer than that in control animals (690 ± 150 vs. 340 ± 90 µm; means ± SD; P < 0.05). Thus hypertrophy of the villus agrees well with the literature in which increases between 50 and 100% were observed (12, 15, 34) and may explain, at least in part, the increases in intestine and mucosa mass reported in Table 1. Although no quantitative analysis of mrp2 expression was performed with this methodology, Fig. 3, D and E, shows clearly that localization of mrp2 staining in the upper versus lower villus regions in pp (14–21 days) animals did not differ from that in control rats (Fig. 3, A–C) and was maximal at the villus tip level. Pregnant rats did not differ from control rats either in the size of villi or in the distribution of mrp2 along the surface of the villus (images not shown). As a negative control of the immunostaining procedure, we analyzed expression of mrp2 in preparations from normal rats, in which incubation with the primary antibody was omitted, and in TR rats in which mrp2 expression is expected to be absent (33, 36). Figure 3F (no primary antibody) shows a very weak staining of the villus structure and a more intense staining of lymphocytes. When the villus from a TR rat was incubated with the primary and secondary antibodies, the internal structure of the villus presented a staining similar to that in normal rats (Fig. 3G); however, no staining was present on its external surface.

**Transport Activity in Evvert Intestinal Sacs**

To establish a correlation between mrp2 expression and its transport activity, we evaluated secretion of DNP-SG, a typical substrate for mrp2, to the mucosal compartment in evverted intestinal sacs. Three-centimeter-long intestinal sacs were prepared from jejunum (segments 3 and 4) and incubated with CDNB or DNP-SG. HPLC analysis of CDNB derivatives in the mucosal and serosal compartments and in tissue homogenates showed two major peaks identified as DNP-SG (~11-min retention time) and as an unidentified peak (7 min-retention time) that likely represents cysteinyl DNP (17, 18). Quantitative analysis of the peaks revealed that DNP-SG always represented >85% of the total compounds absorbing at 365 nm, indicating that conversion of the glutathione derivative to alternative metabolites (e.g., those mediated by γ-glutamyl transferase) was minimal under the given experimental conditions.

Figure 4 shows the time course of DNP-SG accumulation in the mucosal side of the intestinal sacs when CDNB was used as substrate. A linear increase in the
conjugated derivative secretion was observed for all experiments. When CDNB concentration was increased from 50 to 100 μM, the content of DNP-SG in the mucosal compartment also increased for all three experimental groups; it was significantly different only for postpartum rats (Fig. 4A). A difference in the DNP-SG accumulation for the two different CDNB concentrations among the three groups was only observed at the highest dose of CDNB at 30 and 60 min of incubation (Fig. 4B). Postpartum animals exhibited a 30% higher capacity to secrete DNP-SG to the mucosal side than control and pregnant rats. In contrast to the time course of mucosal secretion, cumulative tissue uptake exhibited a steady state after 30 min of incubation (Fig. 5). When the dose of CDNB increased from 50 to 100 μM, the DNP-SG concentration increased similarly for the three experimental groups (Fig. 5A). No differences were observed among the three groups at either of the concentrations studied (Fig. 5B). DNP-SG was also found in the serosal compartment of the intestinal sacs. Transport into this compartment was lower in magnitude than mucosal secretion and did not differ among groups (data not shown). Together, these data indicate that CDNB is efficiently

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**Fig. 3. Immunohistochemical analysis of mrp2 expression.** Duodenum (A and B) and jejunum (C) show brown staining that represents mrp2 on the surface of the villus in a control rat. Duodenum (D) and jejunum (E) show similar intensity of immunostaining on the surface of the villi in a postpartum rat. Staining is more evident at the tip of the villus (arrowheads). A villus preparation from jejunum of a normal rat in which incubation with the primary antibody was omitted (F), as well as a villus preparation from jejunum of a TR2 rat (G), show no staining (arrowheads). Bar, 50 μm; original magnification, ×100.
taken up by the intestinal cells, converted into its glutathione derivative, and secreted to the mucosal and serosal compartments. Secretion to the mucosal compartment was significantly different only in lactating rats.

The efficiency of the secretory process may be neutralized by hydrolysis of the conjugated derivative back to the parent compound followed by absorption or, alternatively, by the absorption of the conjugate itself. Absorption of the conjugated compound may occur at the proximal or distal level of the small intestine or in the colon. In a second set of experiments, we evaluated DNP-SG absorption from the mucosal to the serosal compartment using everted sacs prepared from jejunum (segments 3 and 4), from distal ileum (segments 8 and 9), and from the whole colon. Cumulative secretion of DNP-SG to the serosal side of the everted sacs and tissue uptake are shown in Fig. 6. Transport of DNP-SG to the serosal compartment was similar in magnitude for jejunum and colon and slightly higher for the distal ileum. The transport process did not differ in control, pregnant, and postpartum rats except at the proximal level, where postpartum rats showed a decreased capacity for DNP-SG absorption (Fig. 6, top). Tissue uptake was slightly higher for ileum than for jejunum and colon and exhibited a tendency to decrease in postpartum rats, which was statistically significant only for the jejunal sacs (Fig. 6, bottom). Thus variations in serosal secretion of DNP-SG among tissues and groups were consistent with variations in tissue uptake, suggesting that transport across the apical membrane is the rate-limiting step in the overall absorption of the conjugated compound.

Additional experiments were performed to determine whether a change in the pH of the mucosal compartment from 7.4 to 6.0 affected transport activity in these same experimental models. No substantial differences were observed in the transport of DNP-SG or in its tissue accumulation in either of the protocols or experimental groups analyzed (data not shown).

Expression of mrp2 Protein in oPRL-Treated Rats

oPRL was administered in a range of doses that was shown to increase the expression of Na\(^+\)-taurocholate cotransport polypeptide (27) and 4-nitrophenol UGT (30) in the rat liver and to increase the P1 subunit of GST in the rat intestine (31), thus accounting for the increases observed in postpartum rats. After Western blot analysis of the five most proximal segments of OVX (n = 4), PRL300 (n = 4), and PRL600 (n = 5) rats,
Fig. 5. Cumulative uptake of DNP-SG by intestinal tissue. Tissue accumulation of DNP-SG was determined in intestinal samples corresponding to the experiments described in Fig. 4. The time course of accumulation of DNP-SG is shown for each dose (A) and as a comparison among groups (B). Data are means ± SD; n = 3 rats/group. No difference was detected among groups. *Significantly different from 50 μM CDNB.

Fig. 6. Absorption of DNP-SG in intestinal sacs. DNP-SG (100 μM) was added to the mucosal side. Top: transport of the conjugated compound into the serosal compartment. Bottom: uptake of DNP-SG in the intestinal tissue. Data are means ± SD; n = 3 rats/group. *Significantly different from control and pregnant animals.
no significant changes were observed in mrp2 expression in response to oPRL treatment at any of the doses administered (data not shown).

**DISCUSSION**

Transport systems for conjugated derivatives at the apical level of the enterocyte play a crucial role in the elimination of potentially toxic compounds including drugs, toxins, carcinogens, and other xenobiotics that are orally introduced into the intestine. mrp2, an important member of the ABC family of transporters (20), is a candidate for pumping conjugated compounds out of the intestinal cells. mrp2 is principally expressed in the proximal small intestine and follows a pattern of distribution similar to that of conjugating enzymes (10, 25, 33, 38, 41). Regulation of mrp2 expression during perinatal events may determine the fate and, consequently, the toxicity of xenobiotics introduced into the maternal intestine. We report here that mrp2 expression in small intestine was preserved in pregnant rats and increased in postpartum rats by late lactation. Because the expression of mrp2 is decreased in liver membranes from pregnant rats and unchanged in lactating animals (5), we postulate that intestinal mrp2 plays a role, compensating for the liver in elimination of orally ingested xenobiotics perinatally.

Biliary excretion of mrp2 substrates is decreased during pregnancy (45), consistent with decreased expression of mrp2 protein in liver in pregnant rats (5). However, mrp2 mRNA expression was preserved in liver in pregnancy, suggesting that posttranscriptional factors are involved in the downregulation of protein expression. Treatment of female rats with ethynylestradiol also decreased the mrp2 protein level in liver and was able to mimic dissociation between protein and mRNA (44). These data suggest that hormonal factors (e.g., sex steroids) affect mrp2 expression posttranscriptionally, thus leading, for example, to an impairment of protein synthesis, a redistribution of protein molecules to a compartment distinct from the canalicular membrane, or an increase in protein degradation. In the present study, we demonstrated that expression of mrp2 protein as well as mRNA is preserved during pregnancy. The data clearly indicate a differential regulation of mrp2 expression between liver and intestine.

The current data also indicate a significant increase in expression of mrp2 in intestine from postpartum rats, which was maximal during the last week of lactation. Significantly increased food intake and hypertrophy of the intestine have also been noted throughout lactation, reaching maximal values in late lactation (12, 14, 15). We report here increases in weight of the whole small intestine of ~130% (Table 1), which were much greater than the increases observed in body weight (~40%, see Animals), indicating a disproportionate development of the intestinal tract at the latter stages of lactation. The intestinal development agreed most closely in magnitude with the increases (100–300%) in food intake (12, 14, 15). It is reasonable to speculate that the increase in mrp2 expression is an adaptive mechanism to prevent absorption of dietary xenobiotics.

Immunohistochemistry studies indicated that mrp2 protein was preferentially expressed at the tip of the villus in control rats as previously described (33) and in postpartum rats and that villi from lactating animals exhibited a marked hypertrophy. We did not analyze quantitatively the in situ expression of mrp2; however, no substantial differences were observed in the intensity of the immunostaining between groups. Because the size of the villus and thereby the villus surface changed substantially between control and postpartum rats, it is possible that the increased expression of mrp2 protein observed postpartum in Western blot studies resulted from preferential development of the regions where mrp2 is better expressed, i.e., the upper villus. The parallel increase in mRNA level in postpartum animals indicated possible increased gene transcription. Interestingly, alkaline phosphatase activity in BBM was also increased in pp (14–21 days) rats compared with control, pregnant, or pp (2–4 days) rats (Table 1).

Immunohistochemistry analysis of alkaline phosphatase in jejunum from control and lactating rats demonstrated similar intensity of staining on the surface of the villi in both groups (4). Thus mrp2 and alkaline phosphatase, which follow a similar gradient along the intestine and along the villus-crypt axis (25, 33), exhibited a similar relative enrichment in their expression and/or activity with respect to BBM total protein in proximal segments from lactating rats. Together, these data suggest that the increase in mrp2 protein and mRNA levels results from preferential hypertrophy of the villus tip rather than from a specific induction of the transport protein. The level of mRNA encoding mrp2 is higher in the upper villus than in the lower villus or in the crypt (33), so the increased level of mrp2 mRNA observed in lactating rats could result from an enrichment of total RNA preparation in enterocytes belonging to the upper villus relative to the entire enterocyte population.

Because prolactin is a major hormone secreted postpartum, we investigated its role in mrp2 upregulation. Western blot studies failed to demonstrate any effect on mrp2 expression in BBM. Similarly, oPRL treatment had no readily detectable effect on intestinal development at any of the doses administered (Table 1), in agreement with a previous report (34). Other factors such as xenobiotics present in the food or the large increase in food intake that is partially responsible for the intestinal hypertrophy (14) may be involved. Treatment of Caco-2 cells with the polyphenolic antioxidants quercitin and t-butylhydroquinone was recently shown to lead to increased expression of mrp2 (2). The isoform of UGT-conjugating phenolic compounds (i.e., UGT1A6) was also induced in these cells, leading the authors to suggest that coordinate induction of UGT and mrp2 may facilitate chemoprotection against phenolic toxins and excretion of conjugates into the gut.
the intestinal lumen. We (31) recently reported that certain specific UGT and GST activities are increased in jejunum from postpartum rats. Because food intake is substantially increased in these animals, increased expression of phase II enzymes and mrp2 may act coordinately to prevent the absorption of dietary xenobiotics. Interestingly, the factors involved in UGT, GST, and mrp2 regulation during lactation are likely to differ to some extent because phase II enzymes, but not mrp2, were induced by prolactin administration (31). Additional studies are necessary to clarify the mechanism of increased mrp2 expression during lactation.

Because it is not possible to obtain inside-out vesicles from the apical membrane of the enterocyte (13), different strategies, such as the use of Caco-2 cells, isolated intestinal sacs, Ussing chambers, and preloading of BBM vesicles, have been used to study intestinal secretion that is mediated by ABC transporters (16, 17). To determine whether there was a correlation between expression of mrp2 and transport activity of conjugated compounds in our experimental groups, we studied secretion of DNP-SG to the mucosal compartment in the isolated and everted intestinal sac model. The parent compound of DNP-SG, CDNB, was added to the mucosal compartment of the intestinal sac, and the conjugated derivative generated endogenously was detected in the same compartment. This procedure allowed us to obtain intracellular concentrations of DNP-SG approximately five times greater than those obtained by adding the same concentration of DNP-SG (i.e., 100 μM) to the mucosal compartment (Figs. 5 and 6). In addition, the coordinate action of GST and mrp2 also closely mimics the in vivo situation because it is most likely that dietary xenobiotics follow a similar pathway. Under these experimental conditions, cumulative uptake of DNP-SG did not differ among the groups or doses. Thus differences in the content of DNP-SG in the mucosal compartment are most likely a result of the activity of the secretory process across the apical membrane of the enterocyte. We found that postpartum rats exhibited an increased secretion of DNP-SG with respect to control and pregnant rats, in agreement with the increase in mrp2 expression. It is noteworthy that the difference in secretory activity was observed only for the highest concentration of CDNB (Fig. 4B). These data suggest that the maximal rate for transport was significantly increased in postpartum animals, consistent with increased expression of mrp2. Together, the evidence indicates that mrp2-mediated transport of DNP-SG from the cell to the intestinal lumen, rather than uptake and metabolism of the parent compound, is the rate-limiting step of the secretory process in this model. The increased secretion of DNP-SG observed in lactating rats may be relevant in vivo, particularly when xenobiotics are introduced into the intestinal lumen under saturating conditions. Secretory activity in pregnant rats was unchanged, consistent with preserved expression of mrp2, reinforcing the postulate that mrp2 is responsible for DNP-SG transport.

The presence of conjugated xenobiotics in the intestinal lumen may result from their biliary secretion or from transport across the apical membrane of the enterocyte. Bacterial metabolism (e.g., hydrolysis mediated by β-glucuronidase) may reverse conjugation reactions and allow reabsorption of the parent compounds, thus leading to futile cycles. It is also possible that the conjugated xenobiotic could be absorbed intact from the intestine. Our results indicate that expression of mrp2 is maximal at the proximal level of the small intestine. The proximal small intestine also has the greatest exposure to xenobiotics that enter the intestine after oral ingestion. Because phase II enzymes are also maximally expressed in the proximal intestine, it is likely that xenobiotics preferentially undergo absorption, conjugation, and secretion to the lumen at the proximal level. Conjugated xenobiotics thus secreted into the lumen could subsequently be reabsorbed at distal sites. The glucuronide derivative of 1-naphthol was recently shown to be substantially absorbed in the rat intestine, particularly in the colon, when added to the mucosal side of perfused everted sacs (20). Absorption of 1-naphthol glucuronide was about eight times higher for the colon than for the small intestine. We analyzed the transport of DNP-SG from the mucosal to the serosal compartment at three different levels of the intestine, the jejunum, ileum, and colon, and found that DNP-SG was absorbed to a similar extent by all three segments in contrast to 1-naphthol glucuronide. The data from Figs. 5 and 6 also indicate that cellular uptake was substantially lower for DNP-SG than for the parent compound, suggesting a low probability of the absorptive process for DNP-SG. In these studies, postpartum rats exhibited a reduced accumulation of DNP-SG in jejunum and transport to the serosal side, consistent with the increased expression of mrp2 in this region.

In conclusion, these studies show that the intestinal expression of mrp2 protein and mRNA was the same in control and pregnant rats but was significantly increased in postpartum rats. Quantitation of intestinal transport in everted sacs confirmed studies on mrp2 expression. Additional studies conducted to determine whether prolactin mediated increased mrp2 expression in lactating rats failed to demonstrate any effect. The increased postpartum intestinal expression of mrp2 may represent an adaptive mechanism that minimizes absorption of dietary toxins and thus protects this tissue, and possibly the organism, against chemical injury.

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