Effects of Chronic Renal Failure on Brain Drug Transporters in Rats

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ABSTRACT:

Studies demonstrated that chronic renal failure (CRF) affects the expression and activity of intestinal, hepatic, and renal drug transporters. Such drug transporters are expressed in brain cells and at the blood-brain barrier (BBB), where they limit the entry and distribution of drugs in the brain. Perturbations in brain drug transporter equilibrium by CRF could lead to central drug toxicity. This study evaluates how CRF affects BBB drug transporters using a 5/6 nephrectomized rat model. Protein and mRNA levels of organic anion-transporting polypeptide (Oatp), organic anion transporter (Oat) and efflux transporters [P-glycoprotein (P-gp), multidrug resistance-related protein (Mrp), breast cancer resistance protein (Bcrp)] were measured in CRF and control rat brain. Intracerebral accumulation of radiolabeled benzylenicillin, digoxin, doxorubicin, and verapamil was used to evaluate BBB drug permeability. Protein expression of the transporters was evaluated in rat brain endothelial cells (RBECs) and astrocytes incubated with control and CRF rat serum. We demonstrated significant decreases (30–50%) in protein and mRNA levels of Bcrp, Mrp2 to -4, Oat3, Oatp2 and -3, and P-gp in CRF rat brain biopsies, as well as in astrocytes and RBECs incubated with CRF serum. These decreases did not correlate with in vivo changes because BBB permeability of benzylenicillin was decreased by 30% in CRF rats, whereas digoxin, doxorubicin, and verapamil permeabilities were unchanged. It thus seems that even with decreased drug transporters, BBB integrity and function is conserved in CRF.

Introduction

In recent years, many studies have demonstrated that chronic renal failure (CRF) significantly affects the expression and activity of intestinal, hepatic, and renal drug transporters via uremic mediators, thus altering the pharmacokinetics of many drugs (Huang et al., 2000; Laouari et al., 2001; Sun et al., 2004, 2006; Naud et al., 2007, 2008). Such drug transporters are also expressed in brain cells and at the blood-brain barrier (BBB) where they limit the entry and distribution of drugs in the brain (Fig. 1) (Löschler and Potschka, 2005a,b,c; Ohtsuki and Terasaki, 2007). Two major drug transporter families have been identified: ATP-binding cassette (ABC) transporters and solute carriers. The most important ABC transporters at the BBB are multidrug resistance related proteins (Mrp), breast cancer resistance protein (Bcrp), and P-glycoprotein (P-gp). Bcrp, Mrp2 to Mrp5, and P-gp are expressed at the luminal side, and they mediate the efflux of molecules from the brain capillary cell into the blood (Kusuhara and Sugiyama, 2005; Löschler and Potschka, 2005c; Deeken and Löschler, 2007; Oswald et al., 2007; Eyal et al., 2009). Mrp4 was also found on the abluminal side of bovine brain capillaries, where it is thought to mediate the efflux of molecules from the capillary cell into the brain, but this was not reported in rat and human brain (Kusuhara and Sugiyama, 2005; Löschler and Potschka, 2005c; Deeken and Löschler, 2007; Oswald et al., 2007). For solute carriers, the most important at the BBB are the organic anion transporter 3 (Oat3) and the organic anion-transporting proteins 2 and 3 (Oatp2–3). Oat3 is located on both membranes and mediates drug efflux from the brain into the blood (Kikuchi et al., 2003; Mori et al., 2003, 2004; Löschler and Potschka, 2005c; Deeken and Löschler, 2007; Oswald et al., 2007). Furthermore, it was recently hypothesized that Oat3 is also implicated in the influx of drugs from the blood to the brain (Ose et al., 2009). Oatp2 is expressed at the luminal and abluminal membranes, whereas Oat3 was found only at the luminal membrane of brain capillary cells, and they both mediate bidirectional transport of drugs (Löschler and Potschka, 2005c; Deeken and Löschler, 2007; Ohtsuki and Terasaki, 2007; Ose et al., 2010).

Thus, to date, there are no studies reporting the effects of CRF on brain drug transporters. However, indirect evidence suggests that CRF...
Animals were allowed an acclimatization period of at least 7 days before the diet (Harlan Teklad Global Diets, Montreal, QC, Canada) and water ad libitum. Research Center animal care facility and maintained on Harlan Teklad rodent Laboratories, Portage, MI), weighing 176 to 225 g, were housed in the rats; and 3) the in vivo intracerebral accumulation of [14C]benzylpenicillin, [3H]digoxin, [14C]doxorubicin, and [3H]verapamil for substrates of brain drug transporters. Western blot analysis following a previously described protocol (Leblond et al., 2001). Every animal had ad libitum access to water, but to limit the effects of CRF-induced malnutrition, control pair-fed rats were fed the same amount of chow that CRF rats ate on the previous day. We have previously demonstrated that the caloric reduction in control animals induced by pair-feeding did not influence the expression of drug transporters (our unpublished data). At day 41 after nephrectomy, the rats were housed in metabolic cages, and urine was collected for 24 h to determine the clearance of creatinine. Rats were sacrificed by decapitation 42 days after nephrectomy for organ and blood collection or underwent a radiolabeled drug accumulation study. After the rats were sacrificed, the brain was immediately excised, rinsed in ice-cold saline, and flash-frozen in liquid nitrogen. Samples were stored at −80°C until membrane preparation or mRNA extraction. Blood was collected for the measurement of serum creatinine and urea and for bioassays with cultured RBECs and astrocytes. Preparation of Crude Brain Membranes. Frozen rat brain biopsies (500 mg) were homogenized in 3 ml of homogenizing buffer (250 mM sucrose, 10 mM HEPES, and 10 mM Tris-HCl, pH 7.4) containing 0.1 mM PMSF using a Potter-Elvehjem tissue grinder (Wheaton Science Products, Millville, NJ). Differential centrifugation was used to obtain a crude membrane fraction (Chow et al., 2010). In brief, the brain homogenates were centrifuged at 9000g for 10 min at 4°C. The supernatant was then spun at 33,000g for 60 min at 4°C, and the resulting pellet was resuspended in phosphate-buffered saline containing 0.1 mM PMSF. Samples were then sonicated on ice for 10 s to ensure homogeneity. Protein concentration was determined using the method of Lowry et al. (1951), using bovine serum albumin as a reference protein. Aliquots were stored at −80°C up to Western blot analysis.

Western Blot Analysis. Major brain drug transporters were assessed by Western blot analysis following a previously described protocol (Leblond et
al., 2002; Naud et al., 2007, 2008, 2011). Protein expression in control animals was arbitrarily defined as 100%.

P-gp and Mrp2 were detected using C219 and MIII-6 monoclonal antibodies (ID Labs Inc., London, ON, Canada), respectively. Oat3, Oatp2, and Oatp3 were detected using antibodies from Alpha Diagnostic International (San Antonio, TX). Anti-Bcrp was from Abbiotec (San Diego, CA), anti-Mrp4 was from Novus Biologicals, Inc. (Littleton, CO), and anti-Mrp5 was from Abnova (Walnut, CA). Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH), used as a loading control, was detected using rabbit anti-GAPDH (Abcam Inc., Cambridge, MA).

mRNA Analysis. Total RNA was extracted from frozen tissue using TRZol reagent (Invitrogen Canada Inc., Burlington, ON, Canada) according to the manufacturer’s protocol. RNA concentration was determined by measuring absorbance at a wavelength of 260 nm. One microgram of total RNA was used to prepare cDNA by reverse transcription using the SuperScript VILO cDNA Synthesis kit (Invitrogen Canada Inc.). The mRNA encoding for P-gp (mrd1a), Mrp2, and Oatp2 was measured by quantitative real-time PCR as described previously (Naud et al., 2007, 2008, 2011). Other genes were measured using appropriate Taqman gene expression assays from Applied Biosystems (Foster City, CA). Table 1 shows the Taqman gene expression assays used for the quantification of mRNA for each transporter. PCR products were analyzed using the ΔΔCt method (Livak and Schmittgen, 2001) using GAPDH as the standard gene.

In Vivo Accumulation of Radiolabeled Drugs in Brain. The intracerebral accumulation of radiolabeled drugs was used to determine the activity of the blood-brain barrier drug transporters. Four different drugs were tested: [14C]benzylpenicillin, an antibiotic and a substrate for Oat3 and Mrps (Kikuchi et al., 2003; Choi et al., 2009); [3H]digoxin, a cardiac glycoside and a substrate for Oatps and P-gp (Goralski et al., 2003); [3H]doxorubicin, an anthracycline antibiotic used in cancer chemotherapy and a substrate of P-gp (Chan et al., 2004); and [3H]verapamil, a calcium channel blocker and substrate of P-gp (Chan et al., 2004). Digoxin (0.5 mg/kg) was administered via intraperitoneal injection, benzylpenicillin [control (CTL), 5 mg/kg; CRF, 1 mg/kg] was administered via intramuscular injection, and doxorubicin (5 mg/kg) (Hsieh et al., 2008) and verapamil (0.1 mg/kg) (Hendriks et al., 1998) were administered in the tail vein. All were injected with an approximate 1:1500 hot/cold ratio so the milligram amount of radioactive compound would not factor into the final dosage calculation. To evaluate cerebral accumulation, similar plasma concentrations of drugs need to be attained, and therefore CRF rats received a lower dose of benzylpenicillin because of its reduced clearance in CRF. Rats were anesthetized using isoflurane 30 min (doxorubicin), 60 min (verapamil), or 2 h (benzylpenicillin and digoxin) after injection, and a cardiac puncture was performed to recover the maximum volume of blood in heparinized tubes to obtain plasma. Brains were harvested, rinsed in cold phosphate-buffered saline, weighted, minced, and placed in 2 ml of Solvable solution (PerkinElmer Life and Analytical Sciences, Waltham, MA) at 56°C overnight. Dissolved brain volumes were noted, and radioactivity was counted on a Wallac 1409 β-counter (PerkinElmer Life and Analytical Sciences) in 1 ml of dissolved brain tissue and 2 ml of plasma after the addition of 4 ml of ScintiSafe Plus Scintillant (Thermo Fisher Scientific, Waltham, MA). The results were calculated as a ratio between the radioactivity count (in counts per minute) for 1 g of brain tissue and the radioactivity count for 1 ml of plasma. The mean for the CTL groups was arbitrarily defined as 100%.

Effects of Uremic Serum on Brain Drug Transporters. To determine whether uremic serum could affect the expression and function of blood-brain barrier drug transporters, we incubated BBECS and astrocytes with serum from nephrectomized rats and evaluated the drug transporters’ protein expression.
mRNA Expression of Brain Drug Transporters in CRF Rats. Figure 3 presents results from the analysis of mRNA coding for brain drug transporters. There were significant decreases in the expression of Bcrp (35%), Mrp2 (40%), Mrp4 (30%), Oat3 (40%), Oatp2 (35%), Oatp3 (70%), and P-gp (35%) mRNA in CRF rat brain compared with controls. Again, only Mrp5 mRNA remained stable between the groups.

In Vivo Accumulation of Radiolabeled Drugs in the Brain of Rats with Surgically Induced Chronic Renal Failure. Two hours after administration, digoxin plasma radioactivity was similar in control and CRF rats, whereas the plasma radioactivity of benzylpenicillin was slightly elevated by 17% in CRF rats compared with control animals (Fig. 4). There was 33% less [14C]benzylpenicillin in the
brain of CRF rats. On the other hand, no significant differences in 
\[^{3}H\]digoxin, \[^{14}C\]doxorubicin, or \[^{3}H\]verapamil brain accumulation were observed between CTL and CRF rats. These data were obtained via a ratio of radioactivity for 1 g of brain tissue/1 ml plasma.

**Protein Expression of Drug Transporters in RBECs Incubated with Serum from CRF Rats.** Protein expression of drug transporters was measured in rat brain endothelial cells incubated for 48 h with serum from control or CRF animals (Fig. 5). There was a significant decrease in the expression of Bcrp (40%), Mrp2 (25%), Mrp4 (45%), Oat3 (35%), Oatp2 (35%), Oatp3 (25%), and P-gp (30%) protein when incubated with CRF serum compared with controls. On the other hand, the expression of Mrp5 was unaltered. These results were similar to those obtained in vivo in CRF rats.

**Protein Expression of Drug Transporters in Rat Astrocytes Incubated with Serum from CRF Rats.** Protein expression of drug transporters was measured in rat astrocytes incubated for 48 h with serum from control or CRF animals (Fig. 6). Significant decreases in the expression of Bcrp (25%), Mrp2 (25%), Mrp4 (30%), Oat3 (60%), Oatp2 (40%), Oatp3 (50%), and P-gp (30%) protein were observed when incubated with CRF serum compared with controls. The expression of Mrp5 remained unaltered. These results were similar to those obtained in vivo in CRF rats.

**Discussion**

This study demonstrated a significant (20–50%) decrease in protein and mRNA expression of Bcrp, Mrp2 and 4, Oat3, Oatp2 and 3, and P-gp in crude rat brain membranes from CRF rats compared with CTL rats, whereas Mrp5 was unchanged. Similar reductions were observed in RBECs and astrocytes incubated with serum from nephrectomized rat serum, compared with control. Influx and efflux drug transporter...
expression at the BBB and in the brain thus seem equally affected by CRF. For BBB permeability, we observed a 30% decrease in the BBB permeability to benzylpenicillin in nephrectomized rats and no change in the BBB permeability to digoxin, doxorubicin, and verapamil as measured by their brain/plasma radioactivity ratio.

We found significant correlations between the clearance of creatinine and the expression of all down-regulated transporters at the protein level, indicating that the changes in transporters are related to the severity of the renal failure. In addition, we have demonstrated that the modifications in drug transporter expression occurred in response to one or many factors present in the sera from uremic rats in at least two brain cell types, as demonstrated by the changes in protein expression of transporters in astrocytes and BBECs, which are similar to those observed in brain biopsies of CRF rats.

To our knowledge, no group has studied the impact of renal failure on the expression of brain drug transporters. However, many studies have reported a change in drug transporters in various organs in CRF (Huang et al., 2000; Laouari et al., 2001; Sun et al., 2004, 2006; Naud et al., 2007, 2008, 2011). In some of these studies, uremic circulating factors seemed involved in the expression and activity changes. Although no specific factor has been identified, many potential factors include uremic toxins such as CMPF, IS, or PAH and proinflammatory cytokines that are known to be elevated in CRF (Oettinger et al., 1994; Sunder-Plassmann et al., 1994; Ziesche et al., 1994; Akahoshi et al., 1995; Higuchi et al., 1997). Indeed, uremic toxins have been shown to inhibit drug uptake by Oat1, Oat3, and Oatp2 in the rat liver, kidney, and brain (Deguchi et al., 2004, 2006). In addition, proinflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor α can modulate drug transporter expression and activity in various organs including at the BBB (Petrovic et al., 2007; Morgan et al., 2008). For example, in vitro studies have demonstrated that IL-1β and IL-6 can decrease the expression of P-gp and Bcrp in brain capillary endothelial cells and that IL-6 can decrease P-gp expression in cultured rat astrocytes (Ronaldson and Bendayan, 2006; von Wedel-Parlow et al., 2009; Poller et al., 2010). Proinflammatory cytokines could thus play a role in the BBB drug transporter expression down-regulation observed in CRF.

Despite significant decreases of brain transporter expression in CRF rats, we did not observe in vivo brain accumulation of three different substrates, namely digoxin, doxorubicin, and verapamil. Such a discrepancy has also been observed in inflammation studies. Indeed, previously published data demonstrated that systemic inflammation, as seen in CRF, moderately down-regulated (~24%) the protein expression of P-gp at the BBB 6 h after the induction of inflammation by endotoxin (Zhao et al., 2002). In that study, P-gp expression returned to normal 24 h after inflammation (Zhao et al., 2002). They observed increased plasma and brain concentrations of doxorubicin in mice 6 h after the induction of inflammation compared with control. However, endotoxin-induced systemic inflammation did not significantly change the brain/plasma concentration ratio of doxorubicin (Zhao et al., 2002). The authors thus conclude that endotoxin-induced systemic inflammation has no effect on BBB integrity and doxorubicin transport across the blood-brain barrier in mice (Zhao et al., 2002). In addition, another study demonstrated that central nervous system inflammation produces a 50% decrease in P-gp mRNA expression and an almost 100% decrease in Oatp2 mRNA in the rat brain 6 h after induction (Goralski et al., 2003). This group used [3H]digoxin as a probe for P-gp function in vivo and observed, 6 and 24 h after inflammation induction, an increase in brain and plasma [3H]-radioactivity (Goralski et al., 2003). However, the brain/plasma radioactivity ratio was unchanged (Goralski et al., 2003). Based on these results, and because CRF is a state of chronic inflammation, it could explain why, like Zhao et al. (2002) and Goralski et al. (2003), we observe decreased protein and mRNA expression of P-gp and Oatp2 in the brain of nephrectomized rats but no intracerebral accumulation of digoxin, doxorubicin, or verapamil.

We also observed a 30% decrease in the BBB permeability to benzylpenicillin in nephrectomized rats compared with controls as measured by the total brain/plasma radioactivity ratio. In vitro, studies showed that benzylpenicillin is a substrate of Mrp2 and Oat3, but no blood-to-brain transporter has been identified (Kikuchi et al., 2003; Choi et al., 2009). As mentioned previously, Oat3 is involved in the transport of uremic toxins from the brain (Ohtsuki et al., 2002; Kikuchi et al., 2003; Tahara et al., 2005; Deguchi et al., 2006). In fact, this transporter is typically thought to play a role in the efflux of drugs.

**Fig. 6.** Protein expression of various drug transporters in astrocytes incubated with the serum of CRF rats. Protein bands are expressed in densitometry units. The densitometry units measured for transporters in CTL (open bars) and CRF (filled bars) rats were standardized by dividing them by the value obtained for GAPDH. The standardized densitometry units of control rats were arbitrarily defined as 100%. Shown is the mean expression in rats expressed as percentage of controls ± S.D. of 10 different rat sera in each group, *, p < 0.05, **, p < 0.01, and ***, p < 0.001 compared with control rats.
from the brain. However, a recent study hypothesized that it is also involved in the uptake of \(3R,4R,5S-4\)-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate phospho (\(\text{Ro 64-0802}\)), a pharmaco-logically active form of oseltamivir, from the blood to the brain (Ose et al., 2009). Thus, it could be responsible for the blood-to-brain uptake of substrates, such as benzylpenicillin, or uremic toxins and for their brain-to-blood transport. This hypothesis is sup-ported by a study performed in the 1960s that demonstrated that probenecid, a well known Oat3 inhibitor, limits the entry of ben-zylpencillin into rat brain as shown by a decrease in brain/plasma concentration ratio of benzylpenicillin when coadministered with probenecid (Fishman, 1966). The 30% decrease in the brain/blood radio-activity ratio of benzylpenicillin observed in the brain of nephrecto-mized rats indicates a more efficient efflux of the drug compared with its uptake. However, Mrp2 (efflux) and Oat3 (influx and efflux) were equally reduced by 40% in the brain of nephrectomized rats compared with controls. If the hypothesis of Oat3 involvement in the blood-to-brain influx of benzylpenicillin and uremic toxins is correct, the greater functional reduction in benzylpenicillin brain uptake could be attributed to competition with accumulating uremic toxins such as CMPF, IS, and PAH for blood-to-brain transport by Oat3, preventing benzylpenicillin entry into the brain. However, it is also possible that Mrp2 plays a more important functional role in the BBB permeability of benzylpenicillin, causing a greater efflux of the drug compared with its influx. In conclusion, although we reported significant reductions in the expression of influx and efflux brain drug transporters, it seems that the BBB integrity and permeability to drugs is preserved in renal failure as shown by the unchanged intracerebral accumulation of digoxin, doxorubicin, and verapamil and by the reduced accumulation of benzylpenicillin.

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**References**


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**Authorship Contributions**

**Participated in research design:** Naud, Laurin, and Pichette.

**Conducted experiments:** Naud, Laurin, Michaud, Beauchemin, and Leblond.

**Performed data analysis:** Naud, Leblond, and Pichette.

**Wrote or contributed to the writing of the manuscript:** Naud, Leblond, and Pichette.

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