Riboflavin transport by isolated perfused rabbit renal proximal tubules

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Yanagawa, Norimoto, Remi N. G. Shih, Oak D. Jo, and Hamid M. Said. Riboflavin transport by isolated perfused rabbit renal proximal tubules. Am J Physiol Cell Physiol 279: C1782–C1786, 2000.—Rabbit renal proximal tubular transport of riboflavin (RF) was examined by using the in vitro isolated tubule perfusion technique. We found that proximal tubules actively reabsorbed \( J_{\text{in}} \) and secreted \( J_{\text{out}} \) RF. At 0.1 \( \mu \)M RF concentration, \( J_{\text{in}} \) was significantly higher than \( J_{\text{out}} \), resulting in a net secretion. This net secretion of RF was decreased at 0.01 \( \mu \)M RF concentration and increased at 1 \( \mu \)M RF concentration. Both \( J_{\text{in}} \) and \( J_{\text{out}} \) were inhibited by lowering temperature or by adding iodoacetate, a metabolic inhibitor, and lumichrome, an RF analog, suggesting the involvement of carrier-mediated transport mechanisms. \( J_{\text{in}} \) was inhibited by probenecid, an anion transport inhibitor, and by para-aminobiphrpic acid, an anorganic anion, suggesting the relevance of RF secretion to renal organic anion transport. \( J_{\text{out}} \) was also inhibited by alkaline pH (8.0) and by the calmodulin inhibitor trifluoperazine, indicating the influence of pH and \( Ca^{2+} \)/calmodulin-dependent pathway on RF secretion. Finally, we found that addition of chlor promazine, a phenothiazine derivative, inhibited both \( J_{\text{in}} \) and \( J_{\text{out}} \), raising the concern about the nutritional status in patients receiving such a type of medication.

Riboflavin (RF) is an essential vitamin required for normal cellular functions. It serves in key metabolic reactions in the body, which include carbohydrate, amino acid, and lipid metabolism, and the conversion of folic acid and pyridoxine into their coenzyme forms (4, 14, 15). RF deficiency can thus cause a series of clinical abnormalities, including growth retardation, degenerative changes in the nervous system, anemia, skin lesions, and an increase in the susceptibility to carcinogens (4, 8, 16). Mammals cannot synthesize RF but obtain the vitamin from the diet through intestinal absorption. Elimination of RF from the body takes place mainly through the kidneys, so that kidneys play a key role in the regulation of normal body RF homeostasis. In attempts to elucidate the mechanisms whereby kidneys excrete RF, clearance studies have been performed in chickens (20), rats (3), dogs (10), and humans (9), which showed that RF is filtered through glomeruli and followed by tubular reabsorption. On the basis of the finding that RF excretion rate exceeded glomerular filtration rate, a component of tubular secretion was also implicated (3, 9, 10). Furthermore, stop-flow studies suggested proximal tubules to be the site where RF reabsorption and secretion take place (10). However, the limitations in these classical clearance studies made it difficult to disclose the individual transport process from this complex bidirectional RF transport. The aim of our present study is to examine proximal tubule RF transport by using the in vitro isolated tubule perfusion technique, an experimental system that allows unidirectional transport processes to be examined in a defined tubular segment. We found that both RF reabsorption and secretion occurred in rabbit proximal tubules with a net secretion of RF. These transport processes are likely to occur through carrier-mediated transport mechanisms, under the regulation of RF availability, the prevailing pH, and the intracellular \( Ca^{2+} \)/calmodulin-mediated pathway. Our results also show that chlor promazine, a widely used psychotropic agent, interfered with these RF transport processes.

METHODOLOGY

Animals. New Zealand White male rabbits, weighing 1.5–2.0 kg, were used in these studies. The animals were maintained on an ad libitum diet of standard rabbit chow with free access to water. This study protocol was approved by the local animal care committee.

In vitro isolated tubule perfusion. Superficial proximal straight and convoluted tubules were dissected from rabbit renal cortex free-hand under the dissecting microscope and perfused in vitro using the conventional method originally described by Burg et al. (2). The luminal perfusate contained (in mM) 125 NaCl, 25 NaHCO\(_3\), 5 KCl, 1.2 MgSO\(_4\), 2 NaH\(_2\)PO\(_4\), and 1.5 CaCl\(_2\), pH 7.4. The peritubular bath was maintained at 37°C, pH 7.4, and the perfusate flow rate was 1.0 ml/min.

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solution was the identical solution plus 6% BSA. The bath pH was adjusted after being pre-equilibrated with 95% O₂-5% CO₂ at 37°C. The perfusate and bath were made isomolar with NaCl. Twenty- to thirty-minute equilibration periods were allowed before the first sample collection and between each collection period. To minimize the potential photodecomposition of RF, the lights of the room and the microscope were dimmed, and the duration of these studies was limited to <2 h.

Measurements of RF fluxes. Extensively dialyzed [³H]riboflavin (15 μCi/ml) was added to the perfusate as the impermeant volume marker for the measurements of perfusion rate (Vᵢ) and collection rate (Vₒ). The lumen-to-bath RF flux rate (J₀b) was measured by adding [³H]RF (15 μCi/ml) to the perfusate, whereas the bath-to-lumen RF flux rate (Jₐb) was measured by adding [³H]RF (15 μCi/ml) to the bath solution. J₀b and Jₐb were calculated as J₀b = [(VₒCₒ - VᵢCᵢ)/Vₒ]RF/Cᵢ and Jₐb = VᵢCᵢ/XblL, where Vᵢ is the perfusion rate (nl/min), Vₒ is the collection rate (nl/min), L the tubule length (mm), Cᵢ is the [³H]RF concentration in the perfusate (cpm/ml), Cₒ is the [³H]RF concentration in the collected samples (cpm/ml), RF is the RF concentration (fmol/ml), and Xb is the specific activity of [³H]RF in the bath (cpm/fmol/ml). It is not known to what extent RF is metabolized by the proximal tubules in these studies. Our computation of Jₐb based on the rate of RF appearance from the peritubular bath into the luminal perfusate thus does not include the amount of RF metabolized by the proximal tubules.

Data analysis. Data presented are means ± SE of separate determinations on separate tubules. Statistic P values were analyzed by using Student’s t-test for paired or unpaired data as appropriate.

RESULTS

J₀b and Jₐb across the isolated perfused proximal tubules. We first measured J₀b and Jₐb across the isolated perfused proximal straight tubules (PST; S2-S3 segments) with both perfusate and bath solution containing 0.1 μM RF. As shown in Fig. 1, we found that Jₐb was significantly higher than J₀b (0.58 ± 0.06 vs. 0.22 ± 0.02 fmol·mm⁻¹·min⁻¹, n = 8, P < 0.01). The net RF transport across PST thus occurred in the direction of RF secretion from bath to lumen at a rate of ~0.36 fmol·mm⁻¹·min⁻¹. Similar results were also obtained with superficial proximal convoluted tubules (PCT; S1-S2 segments), where Jₐb was also significantly higher than J₀b (0.90 ± 0.15 vs. 0.45 ± 0.1 fmol·mm⁻¹·min⁻¹, n = 9, P < 0.03), with minimal net RF secretion, whereas raising RF concentration from 0.1 to 1 μM significantly increased both J₀b and Jₐb with increased net RF secretion (n = 6, *P < 0.01 vs. J₀b).

Effects of RF concentrations. We next examined the effect of varying RF concentrations on J₀b and Jₐb across PST. As shown in Fig. 2, lowering the perfusate and bath RF concentration from 0.1 to 0.01 μM significantly reduced both J₀b and Jₐb with minimal net RF secretion. In contrast, raising the perfusate and bath RF concentration from 0.1 to 1 μM significantly increased both J₀b and Jₐb. The net transport of RF across PST at this high RF concentration occurred in the direction of RF secretion at a rate (~0.45 fmol·mm⁻¹·min⁻¹) not significantly different from that of PST.

Effects of temperature and lumichrome. We found that both J₀b and Jₐb across PST were temperature sensitive. Thus lowering bath temperature from 38 to 18°C significantly lowered J₀b (from 0.14 ± 0.02 to 0.04 ± 0.01 fmol·mm⁻¹·min⁻¹, n = 4, P < 0.01) and Jₐb (from 0.32 ± 0.08 to 0.09 ± 0.05 fmol·mm⁻¹·min⁻¹, n = 4, P < 0.05). In separate studies, we also found that addition of the metabolic inhibitor iodoacetate (10 mM) to the bath medium completely abolished RF fluxes. In addition, we found that adding lumichrome (50 μM), an RF analog, to either the perfusate or the bath solution significantly

Fig. 1. Riboflavin (RF) reabsorption from the lumen to bath (J₀b) and secretion from the bath to lumen (Jₐb) across proximal straight tubules (PST) and proximal convoluted tubules (PCT). J₀b and Jₐb across PST and PCT were measured with both perfusate and bath solution containing 0.1 μM RF. In these studies, J₀b and Jₐb were measured in sequence with the same tubule. Jₐb was significantly higher than J₀b in both PST (n = 8, *P < 0.01) and PCT (n = 9, *P < 0.03), with a net Jₐb at comparable rates between PST and PCT.

Fig. 2. Effects of RF concentrations. The influence of RF levels on J₀b and Jₐb was examined by changing RF concentrations in the perfusate and bath. Lowering RF concentration from 0.1 to 0.01 μM significantly reduced both J₀b and Jₐb with minimal net RF secretion, whereas raising RF concentration from 0.1 to 1 μM significantly increased both J₀b and Jₐb with increased net RF secretion (n = 6, *P < 0.01 vs. J₀b).
lowered $J_{lb}$ (from $0.16 \pm 0.03$ to $0.10 \pm 0.01$ fmol·mm$^{-1}·min^{-1}$, $n = 4$, $P < 0.05$) or $J_{bl}$ (from $0.44 \pm 0.03$ to $0.21 \pm 0.06$ fmol·mm$^{-1}·min^{-1}$, $n = 4$, $P < 0.05$), respectively. Taken together, these results indicate the involvement of carrier-mediated active transport mechanisms in both $J_{lb}$ and $J_{bl}$ across PST.

**Effects of probenecid and PAH.** Because organic anion transport mechanism plays an important role in the secretion of organic metabolites by proximal tubules, we examined the involvement of this transport system in RF transport by testing the effects of probenecid, an anion transport inhibitor, and PAH, an organic anion. As shown in Fig. 3, we found that $J_{lb}$ was significantly inhibited when 1 mM of probenecid (from $0.51 \pm 0.12$ to $0.31 \pm 0.06$ fmol·mm$^{-1}·min^{-1}$, $n = 5$, $P < 0.05$) or PAH (from $0.52 \pm 0.12$ to $0.42 \pm 0.12$ fmol·mm$^{-1}·min^{-1}$, $n = 5$, $P < 0.05$) was added to the bath solution. In contrast, no effect on $J_{bl}$ was found with the addition of 1 mM of probenecid (from $0.19 \pm 0.03$ to $0.24 \pm 0.04$ fmol·mm$^{-1}·min^{-1}$, $n = 4$) or PAH (from $0.19 \pm 0.04$ to $0.17 \pm 0.03$ fmol·mm$^{-1}·min^{-1}$, $n = 4$) to the perfusate. These results thus indicate that the organic anion transport system is involved in RF secretion by PST.

**Effect of pH.** In another set of studies, we examined the effects of altering luminal and peritubular pH on RF transport across PST. Changes in perfusate pH from 7.4 to 7.0 or 8.0 did not affect $J_{lb}$ significantly (from $0.16 \pm 0.02$ to $0.13 \pm 0.02$ or $0.14 \pm 0.01$ fmol·mm$^{-1}·min^{-1}$, $n = 6$, respectively). In contrast, whereas lowering bath medium pH from 7.4 to 7.0 did not affect $J_{bl}$ (from $0.39 \pm 0.12$ to $0.35 \pm 0.07$ fmol·mm$^{-1}·min^{-1}$, $n = 6$), raising bath medium pH from 7.4 to 8.0 significantly lowered $J_{bl}$ (from $0.39 \pm 0.12$ to $0.19 \pm 0.03$ fmol·mm$^{-1}·min^{-1}$, $n = 6$, $P < 0.05$).

**Effects of cAMP, phorbol ester, and trifluoperazine.** Because protein kinase systems are known to regulate solute transport processes in proximal tubules, we examined the possible role played by these pathways in regulating the RF transport across PST. For PKA- or PKC-mediated pathways, we tested the effect of adding 8-bromo-cAMP (0.1 mM) or phorbol 12-myristate 13-acetate (PMA) (50 μM), respectively, to the bath solution. For the involvement of Ca$^{2+}$/calmodulin-mediated pathways, we tested the effect of the calmodulin inhibitor trifluoperazine (TFP). We found that neither 8-bromo-cAMP nor PMA significantly affected $J_{lb}$ (from $0.16 \pm 0.03$ to $0.17 \pm 0.04$ fmol·mm$^{-1}·min^{-1}$, $n = 6$) or $J_{bl}$ (from $0.32 \pm 0.08$ to $0.33 \pm 0.05$ fmol·mm$^{-1}·min^{-1}$ and from $0.75 \pm 0.10$ to $0.57 \pm 0.11$ fmol·mm$^{-1}·min^{-1}$, respectively, $n = 6$). However, addition of TFP (250 μM) to the bath solution significantly lowered $J_{bl}$ (from $0.45 \pm 0.04$ to $0.19 \pm 0.05$ fmol·mm$^{-1}·min^{-1}$, $n = 6$, $P < 0.05$) but not $J_{lb}$ (from $0.12 \pm 0.02$ to $0.13 \pm 0.03$ fmol·mm$^{-1}·min^{-1}$, $n = 6$). These results therefore indicate that RF secretion, but not reabsorption, by PST is under the influence of Ca$^{2+}$/calmodulin-mediated pathway.

**Effect of chlorpromazine.** Because a variety of psychotropic agents have been found to share structural similarity with RF and may thus affect RF transport/metabolism, we also tested the effect of chlorpromazine, a phenothiazine derivative, on RF transport across PST. As shown in Fig. 4, addition of chlorpromazine (1 mM) to the perfusate inhibited $J_{lb}$ significantly (from $0.38 \pm 0.03$ to $0.17 \pm 0.07$ fmol·mm$^{-1}·min^{-1}$, $n = 5$, $P < 0.02$). Similarly, addition of chlorpromazine (1 mM) to the bath solution also caused a significant inhibition of $J_{lb}$ (from $0.65 \pm 0.06$ to $0.22 \pm 0.05$ fmol·mm$^{-1}·min^{-1}$, $n = 5$, $P < 0.01$).

DISCUSSION

The aim of our present study is to examine RF transport in rabbit renal proximal tubules by using the in vitro isolated tubule perfusion technique. This experimental system is ideal for studying bidirectional renal tubular transport processes because it allows unidirectional fluxes to be measured separately in a
defined tubule segment. We found that rabbit renal proximal tubules reabsorbed and secreted RF. In PST, the capacity of $J_{lb}$ was significantly greater than that of $J_{bl}$, so that the net RF transport across PST occurred in the direction of RF secretion. Compared with PST, PCT exhibited higher $J_{lb}$ and $J_{bl}$, probably due in part to their larger luminal and peritubular surface area per tubular length. However, the net RF transport across PCT also occurred in the direction of RF secretion at a rate not significantly different from that of PST. It thus appears that RF secretion occurs throughout different segments of proximal tubules at a comparable rate. These results are in agreement with in vivo clearance studies, where RF infusion induced a component of tubular secretion as implicated by the greater RF excretion rate than the glomerular filtration rate (3, 9, 10, 23).

In our present study, we found that both $J_{lb}$ and $J_{bl}$ were influenced by RF concentration. Thus, compared with the corresponding transport rates at 0.1 μM RF concentration, both $J_{lb}$ and $J_{bl}$ were significantly reduced when RF concentrations in the perfusate and bath solution were lowered to 0.01 μM. At this lower RF concentration, $J_{lb}$ was not significantly different from $J_{bl}$, so that there was no net RF transport across PST. In contrast, both $J_{lb}$ and $J_{bl}$ were significantly increased when RF concentrations in the perfusate and bath solution were raised to 1 μM, leading to a significantly greater net RF secretion. Because the physiological plasma RF concentration has been found to be 0.01–0.03 μM in humans (23) and in rats (3), the secretion of RF by proximal tubules at higher RF concentrations may thus constitute an adaptive mechanism to maximize urinary RF excretion during RF-sufficient states. A similar adaptive change in urinary RF excretion according to blood RF concentration was also found in vivo clearance studies in human (9) and rats (3). Our studies therefore lend further support to these previous observations and clarify the mechanism underlying this phenomenon.

To delineate the nature of RF transport mechanisms, we found that both $J_{lb}$ and $J_{bl}$ were energy dependent as indicated by the abolishment of these transport activities when energy supply was blocked by low temperature or iodoacetate. In separate studies, we found that changes in peritubular bath osmolality did not affect $J_{lb}$ or $J_{bl}$. It is therefore likely that the RF transport across the proximal tubule occurs through transcellular, rather than paracellular, pathways. We also found that both $J_{lb}$ and $J_{bl}$ were inhibited by lumichrome (50 μM), an RF structural analog, suggesting the involvement of carrier-mediated transport mechanisms. In further support of this notion, we found that $J_{bl}$ was inhibited by probenecid and PAH, indicating the relevance of RF secretion to renal organic anion transport. RF has been reported to behave as an anion when transported across biological membranes (9, 13), and probenecid was found to inhibit renal excretion of RF in rats (3), dogs (10), and humans (9). However, in vitro studies using kidney slices or isolated kidney cells have produced conflicting results with regard to the involvement of organic anion transport system. Thus, by using rabbit kidney slices, Spector (22) found that RF transport was inhibited by organic anions such as PAH and penicillin G. In contrast, by using isolated kidney cells in suspension, Bowers-Komro and McCormick (1) found no inhibition of RF uptake by these organic anions. Because we also found that both probenecid and PAH had no effect on $J_{lb}$, it is conceivable that the contradictory reports from these previous in vitro studies could have resulted from their inability to separate RF reabsorption from secretion. Nevertheless, the finding that organic anions may interfere with RF secretion in renal proximal tubules could be of clinical importance, because many commonly used drugs are organic anions and may thus have the potential of interacting with renal RF excretion.

In our further studies, we examined the regulatory mechanisms of RF transport in renal proximal tubules. We found that changes in luminal pH over the physiological range (7.0–8.0) did not affect $J_{lb}$ whereas changes in bath pH caused an inhibition of $J_{lb}$ when it was increased to 8.0. It thus appears that changes in systemic acid-base balance may affect urinary excretion of RF by inhibiting proximal tubule RF secretion during systemic alkalemia. As for the intracellular regulatory pathways, we focused on the potential roles of protein kinase systems known to affect solute transports in renal proximal tubules (12). We found that activation of PKA by cAMP, or PKC by PMA, did not affect $J_{lb}$ or $J_{bl}$, whereas activation of Ca$^{2+}$-calmodulin-dependent pathway by TFP inhibited $J_{bl}$ but not $J_{lb}$. These results therefore indicate that the intracellular Ca$^{2+}$-calmodulin-dependent pathway may play an important role in mediating the regulation of proximal tubule RF transport through its effect on RF secretion. A similar result was also found in our recent studies using human proximal tubular cells (11) and colonic cells (21).

In a series of studies, Gabay and Harris (5–7) called attention to the structural similarities between RF and phenothiazine derivatives and showed the interference of these drugs with RF metabolism. Subsequent studies by Pinto and associates (17–19) also showed that treatment of rats with these drugs caused an increase in RF losses in the urine and accelerated the development of RF deficiency in animals fed RF-deficient diet. In support of these notions, we found in our present study that chlorpromazine, a phenothiazine derivative, caused a significant inhibition of both $J_{lb}$ and $J_{bl}$. However, results of our study would indicate that the net effect of chlorpromazine was to reduce RF secretion by proximal tubules. This may appear to contradict the riboflavinuric effect of these drugs found in vivo animal studies (17–19). Although the reason for this discrepancy is not immediately clear, it is conceivable that the inhibitory effect of these drugs on systemic RF metabolism may lead to increased levels of RF in the blood to be filtered through glomeruli. With the concomitant inhibition of proximal tubule RF reabsorption by these drugs, the increase in RF filtered may exceed...
the decrease in RF secretion and result in riboflavinuria. Regardless of the underlying mechanism, our studies did confirm the interference of proximal tubule RF transport by chlorpromazine and reiterate the concern about the nutritional status of psychiatric patients receiving these medications.

In summary, our current studies confirm the bidirectional RF transport activity in isolated perfused rabbit proximal tubules. Our results suggest the involvement of carrier-mediated transport systems in RF transport, which are under the influence of RF availability, pH, and intracellular Ca\(^{2+}\)/calmodulin-dependent pathway. In view of the differing sensitivities between \(J_{\text{sl}}\) and \(J_{\text{lb}}\) to these transport modulators, it is likely that different transport systems are involved in the reabsorption and secretion of RF by renal proximal tubules. Further studies with eventual cloning and isolation of these RF transporters will help to further our understanding on their exact natures and provide new strategies to manage patients with compromised RF homeostasis.

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