Peritubular interaction of unconjugated bilirubin in isolated perfused rat kidney

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Summary

1. The interaction of unconjugated bilirubin with peritubular cell membranes of the rat kidney was studied by means of an isolated rat-kidney preparation applying the multiple-indicator-dilution technique.

2. Inulin was used as an extracellular marker and p-aminohippuric acid as a model of organic anion that interacts with the peritubular membrane.

3. A single renal artery injection of a mixture containing inulin and unconjugated bilirubin was followed by the appearance of the two compounds in the venous effluent. The unconjugated bilirubin curve was always under the curve of inulin and its mean transit time was always less than that for inulin.

4. The cumulative venous recovery of inulin was higher than that of unconjugated bilirubin.

5. When unconjugated bilirubin uptake was plotted against the injected dose of pigment the relationship suggested a saturation phenomenon.

6. The recovery of p-aminohippuric acid was significantly increased when unconjugated bilirubin was added.

7. The results provide evidence for the interaction of unconjugated bilirubin with the peritubular cell membranes of rat kidney.

Key words: bilirubin, isolated perfused rat kidney, organic anion transport.

Introduction

Jaundiced patients with conjugated bilirubin in their plasma excrete bilirubin in their urine, whereas patients whose plasma bilirubin is mainly unconjugated usually have no bilirubinuria [1]. Post-mortem examinations of icteric infants and homozygous Gunn rats have shown yellow staining in the inner medulla of the kidney, particularly in the renal papilla [2]. It has been reported that bile pigments accumulate in the renal tubular cells of dogs with experimental obstructive jaundice [3]. Additional information obtained in jaundiced infants and rats demonstrated cell necrosis and bilirubin staining of the tubular epithelium [4, 5]. Furthermore, bilirubin, in high concentrations, may interfere with such important renal functions as sodium reabsorption [2] and urinary concentrating ability [6, 7]. In addition, the kidney may also be able to conjugate bilirubin [8–10] and glucuronyltransferase of Wistar rat kidney seems involved in the phenomenon [11]. Therefore it is likely that bilirubin enters the renal tubular cells where it may also be metabolized.

Bilirubin handling by the kidney has not been completely clarified. It has been demonstrated that the small fraction of plasma conjugated bilirubin, which is not protein bound, may be filtered by the glomeruli [12, 13] and then largely reabsorbed in the tubules [14]. Conversely, secretion of conjugated bilirubin by the renal tubules was considered unlikely in this study [14, 15]. The mechanism of urinary excretion of bilirubin in the rat, involving glomerular filtration of unconjugated pigment and tubular reabsorption, was also described for the unconjugated pigment.
The reabsorption mechanism of unconjugated bilirubin seemed to be located at the proximal tubular level [18] and was not completely explained by diffusion [17]. Since unconjugated bilirubin may exert a toxic effect on the cells [19] it is important to investigate other possible routes of access of the pigment to the renal tubular cells. For example, unconjugated bilirubin that was not filtered by the glomeruli and circulates within the peritubular capillaries may interact with pericapillary membranes. This interaction should depend on the equilibrium concentration of non-protein-bound bilirubin. This concentration is of the order of 1 nmol/l in normal human blood plasma [20]. However, in cases of pronounced unconjugated hyperbilirubinaemia, or if the binding site on albumin is partly occupied by other substances, concentration of unbound bilirubin may be elevated to the order of 1–10 pmol/l [20]. In the present investigation we have studied the interaction of unconjugated bilirubin with peritubular membranes in an isolated rat-kidney preparation where the pigment was incorporated in the perfusion medium mainly as a diffusible or unbound form.

Methods

Chemicals

All chemicals were of the highest grade commercially available. Unconjugated bilirubin, creatinine, inulin, p-aminohippuric acid and bovine albumin were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.)

Perfusion procedure and apparatus

Male Wistar rats weighing 250–350 g were used as kidney donors. The animals were anaesthetized intraperitoneally with pentobarbital sodium (40 mg/kg body weight). The right kidney was prepared by techniques as described previously [17, 21, 22], modified for catheterization of the mesenteric artery. Urine was collected from an ureteral catheter. Venous effluent drained into a graduated reservoir and was not recirculated. The perfusion medium (pH 7.4) consisted of Krebs–Ringer solution enriched with (mmol/l) glucose (10), sodium pyruvate (5) and sodium lactate (5) and contained creatinine (3-5) for measurement of glomerular filtration rate (GFR). The medium was bubbled with O₂ + CO₂ (19:1, v/v). The whole system operated in a chamber thermostatically controlled at 37°C. Perfusion flow through the isolated kidney in situ was performed with the use of a peristaltic pump (American Instrument Co., U.S.A.; Cat. no. 5-8954), at constant pressure of 100–110 mmHg (rate of infusion oscillated between 9 and 12 ml/min).

Multiple-indicator-dilution technique

The technique consisted of a single injection into the right renal artery of a solution containing appropriate indicators. Serial collections of samples from the venous effluents were made and the concentration of the indicators was determined in each sample. Percentage recoveries were plotted on a logarithmic scale against the time of collection (linear scale) and corrected by extrapolation to the abscissa [23]. Mean transit times and cumulative recoveries were calculated as described [23, 24].

Theoretical considerations

After the injection into the renal artery of a solution containing unconjugated bilirubin and inulin, a fraction of the injected solution will be filtered by the glomeruli. The remainder of the injected compounds will circulate through the peritubular capillary network without interfering with the steady state. Conversely, according to previous data [16, 17], it is possible that some of the filtered unconjugated bilirubin might be recovered in the venous effluent after reabsorption by the proximal tubular cells (assuming that unconjugated bilirubin may be reabsorbed fast enough within the time of the experiment). Since a similar volume of distribution of unconjugated bilirubin and inulin in the postglomerular circulation is likely, we can assume that the fraction of unconjugated bilirubin recovered in the venous effluent at each time interval will be equal to or higher than that of inulin. Conversely, if the recovery of unconjugated bilirubin in the venous sample is lower than that of inulin this implies that some interaction between unconjugated bilirubin and the peritubular cell membrane has occurred, which will delay the circulation of unconjugated bilirubin in the postglomerular circulation [25, 26]. With this technique it is not possible to distinguish between the following processes: (a) unidirectional binding to the antiluminal surface of the nephron; (b) unidirectional binding to a constituent of the interstitium; (c) antiluminal uptake and subsequent sequestration within the tubular cell followed by unconjugated bilirubin metabolic degradation [10, 11] or unconjugated bilirubin secretion into the lumen resembling secretion of conjugated bilirubin [27].
Experimental procedure

The isolated kidney was perfused (15–20 min) until the pressure and the flow remained constant [17, 21, 22]. The following first 5 min were used for clearance studies. As inulin was used as an extracellular marker, GFR was measured with creatinine. A small volume of the mixture (0.25–0.50 ml) was then injected through the mesenteric artery. Immediately venous effluent samples were collected every 2 s for 1 min and urine samples every 30 s for 4 min. At the end of the experiment the organ was removed, gently blotted on filter paper and weighed.

Analyses

To measure function of the kidney the clearance of creatinine and tubular reabsorption of glucose and sodium were determined. Determination of creatinine in urine and perfusate samples was carried out by the Jaffé reaction, but without the use of fuller’s earth [28]. Glucose was determined by the glucose oxidase method [29] and sodium flame photometry. Urine volume was estimated by gravimetry. Samples of the injected solution and of the venous effluent were used for determination of the respective concentrations of the indicators added to the system. Inulin was determined by the method of Roe et al. [30], albumin by the bromocresol green method [31], unconjugated bilirubin by direct spectrophotometry at 450 nm and p-aminohippuric acid by Brun’s technique modified as described [32]. Volumes of the injected solution and of catheter dead-space were determined by gravimetry.

Experimental groups

Three experimental groups were used. (a) Kidneys with a single injection of a mixture containing unconjugated bilirubin (1.0–4.7 mmol/l), albumin (0.05–0.2 mmol/l) and inulin (1.0 mmol/l). (b) Kidneys with a single injection of a mixture containing inulin (1.0 mmol/l), albumin (0.05–0.2 mmol/l) and p-aminohippuric acid (20.0 mmol/l). (c) Kidneys with a single injection of a mixture containing inulin (1.0 mmol/l), albumin (0.08 mmol/l), p-aminohippuric acid (20.0 mmol/l) and unconjugated bilirubin (1.5 mmol/l). This last group was designed to detect any influence of unconjugated bilirubin on the interaction of p-aminohippuric acid with the peritubular membranes. The three different mixtures were prepared in Krebs–Ringer solution. The doses of inulin, unconjugated bilirubin and p-aminohippuric acid were similar to those used in previous investigations [17, 33, 34]. As stated above, some albumin in the amount necessary to prepare unconjugated bilirubin in solution was present in all experiments. Unconjugated bilirubin was dissolved in 0.2–0.3 ml of NaOH (0.1 mol/l) and bovine albumin solution (pH 7.4). The unconjugated bilirubin/albumin molar ratio was 20:1; this ensured stability of the unconjugated bilirubin in solution [35], although it was mostly in a diffusible form [35].

Results

Functional criteria of the preparation

Data showing functional characteristics of the isolated organ are shown in Table 1. As expected, owing to the protein-free perfusate composition, urine flow value was higher than that reported when antidiuretic hormone and albumin were used in physiological concentrations [36] and the percentage of sodium reabsorption reached only 58%, a value similar to that noted previously when a protein-free perfusion medium was used [36–38]. The percentage of glucose reabsorbed was close to 80%.

Experiments with single injection of a mixture containing inulin and unconjugated bilirubin or inulin and p-aminohippuric acid

The single injection of the mixture containing inulin, unconjugated bilirubin and albumin was followed by the appearance of the three compounds in the venous effluent. In every case the concentrations of each substance reached a peak and then fell off in an exponential manner. Fractional recoveries of injection mass were plotted on a logarithmic scale against the time of venous effluent collection on a linear scale. To provide a basis for comparison of the curves within each experiment, the total amount of each solute injected was defined as one unit, and the respective concentration in the venous effluent.

<table>
<thead>
<tr>
<th>TABLE 1. Functional criteria of the isolated rat kidney preparation</th>
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<tbody>
<tr>
<td>Results are means ± SEM (n = 15).</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
</tr>
<tr>
<td>Urine flow (μl min⁻¹ g⁻¹)</td>
</tr>
<tr>
<td>Perfusion flow (ml/min)</td>
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<tr>
<td>GFR (μl min⁻¹ g⁻¹)</td>
</tr>
<tr>
<td>Glucose tubular reabsorption (% filtered load)</td>
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<tr>
<td>Sodium tubular reabsorption (% filtered load)</td>
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FIG. 1. Renal artery-to-vein transit curves, for two separate experiments. Percentage of fractional recovery of amount injected per millilitre of venous effluent vs time of collection (s). (a) Recovery of: ○, albumin (ALB); ▲, inulin; ○, p-aminohippuric acid (PAH). (b) Recovery of: ○, albumin; ○, inulin; ▲, unconjugated bilirubin (UB).

TABLE 2. Relative renal artery-to-vein transit times
All transit times were corrected for the dead-space delays; respective mean ratios of \( n \) determinations are shown in each case with range in parentheses.

<table>
<thead>
<tr>
<th>Solute</th>
<th>( t_s/t_n )</th>
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<tbody>
<tr>
<td>Unconjugated bilirubin ( (n = 8) )</td>
<td>0.86 (0.80-0.93)</td>
</tr>
<tr>
<td>p-Aminohippuric acid ( (n = 10) )</td>
<td>0.89 (0.73-0.99)</td>
</tr>
<tr>
<td>Albumin ( (n = 6) )</td>
<td>0.78 (0.67-0.86)</td>
</tr>
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</table>

TABLE 3. Cumulative venous recoveries of indicators added to the system
Results are means ± SEM. \( n \), Number of determinations.

<table>
<thead>
<tr>
<th>Solute</th>
<th>Inulin + unconjugated bilirubin or p-aminohippuric acid</th>
<th>Inulin + unconjugated bilirubin or p-aminohippuric acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin ( (n = 6) )</td>
<td>104 ± 3</td>
<td>Undetermined</td>
</tr>
<tr>
<td>Inulin ( (n = 23) )</td>
<td>88 ± 3</td>
<td>86 ± 3</td>
</tr>
<tr>
<td>p-Aminohippuric acid ( (n = 16) )</td>
<td>62 ± 2</td>
<td>75 ± 1*</td>
</tr>
<tr>
<td>Unconjugated bilirubin ( (n = 9) )</td>
<td>41 ± 2</td>
<td>36 ± 4</td>
</tr>
</tbody>
</table>

* Significantly higher \( (P < 0.01) \) as compared with data of the first column.

was expressed as a fraction of the injected amount per millilitre of venous effluent. The unconjugated bilirubin curve was always under the curves of inulin or albumin. A similar pattern was observed for p-aminohippuric acid in the absence of unconjugated bilirubin. Typical curves may be seen in Fig. 1.

Mean transit time for unconjugated bilirubin, calculated in the usual manner [23] and corrected for catheter transit time by simple subtraction, was always less than that for inulin. Values for mean transit times relative to the simultaneously measured transit time for inulin are shown in Table 2.

The uptake of unconjugated bilirubin \( (\mu mol \text{ per } 30 \text{ s}^{-1} \text{ g}^{-1} \text{ of kidney}) \) was calculated on the basis of its fractional recovery in the venous effluent, as shown below.

Unconjugated bilirubin uptake

\[
\text{Uptake} = \frac{100 - (\% \text{ recovery/30 s})}{100} \cdot \frac{\text{dose (\mu mol)}}{\text{kidney wt. (g)}}
\]

When unconjugated bilirubin uptake was plotted against the injected dose of unconjugated bilirubin, the relationship suggested a saturation phenomenon (Fig. 2).
Renal peritubular uptake of bilirubin

Experiments with a single injection of a mixture containing inulin, unconjugated bilirubin and p-aminohippuric acid

The curve shapes for inulin, p-aminohippuric acid and unconjugated bilirubin were similar to those described previously, but the venous p-aminohippuric acid recovery was significantly increased when bilirubin was added to the injection ($P < 0.01$) (Table 3).

Discussion

The purpose of these investigations was to investigate a possible interaction between unconjugated bilirubin and the peritubular side of renal tubular cells. Since bilirubin is normally very tightly bound to albumin [20, 39], and bilirubin transferred from blood into tissues is a free form of pigment (as described in unconjugated hyperbilirubinaemia [40]), the study is only possible in a protein-free medium.

The use of unconjugated bilirubin/albumin molar ratio of 20:1 ensured that a major portion of unconjugated bilirubin was diffusible, which facilitates its uptake as observed in other tissues [41].

The indicator-dilution technique has been widely used [24, 26, 33, 34, 42, 43] and its application to the kidney provides information concerning the fate of unconjugated bilirubin injected into the renal artery. The shape of renal artery-to-vein transit curves favoured the conclusion that unconjugated bilirubin was taken up by the rat kidney at peritubular membrane sites. Because unconjugated bilirubin was not restricted to the vascular space, unlike albumin, and the concentration wave was retarded compared with both inulin and albumin, it seems likely that diffusion out of the vascular space occurred, followed by uptake by peritubular cell membranes.

Nevertheless, binding of unconjugated bilirubin to interstitial macromolecules might well occur. The conclusion that unconjugated bilirubin is specifically taken up by peritubular membranes is supported by the impairment of p-aminohippuric acid uptake when both compounds were injected simultaneously, and by the increased recovery of p-aminohippuric acid in the venous effluent (Table 3). Furthermore, the uptake of p-aminohippuric acid and unconjugated bilirubin (calculated as percentage of injected dose) was similar, despite the fact the dose of p-aminohippuric acid was very much higher. This suggests a lower affinity of unconjugated bilirubin for possible common sites of interaction. The observation of a possible saturation for the uptake of unconjugated bilirubin at peritubular level might indicate that a protein or lipid component of the membrane may be involved in the phenomenon as described in the liver plasma membrane [44]. Previous studies in our laboratory [16-18] indicated that diffusible
unconjugated bilirubin may be filtered by the glomeruli and reabsorbed by the proximal tubules by an efficient mechanism not completely explained by diffusion. However, the results described in this paper support the view that unconjugated bilirubin is taken up by the kidney both at cell membranes facing tubular lumina and across cell borders in contact with interstitium as represented in Fig. 3. This is in accordance with previous data which indicated that the percentage of sodium reabsorption by the isolated rat kidney reached only 48% in the presence of unconjugated bilirubin. This may represent a disturbance of sodium reabsorption from the tubular fluid due to the pigment [17], in agreement with data reported by other authors in Gunn rats [2]. In this connection a reversible inhibition by unconjugated bilirubin of (Na+, K+-activated)-ATPase activity from rat cerebrum has been reported [45]. Moreover we observed in preliminary experiments (E. J. Comin, M. M. Elias, M. Grossman, E. A. Rodriguez Garay, unpublished work) that the excretion of p-aminohippuric acid in the urine formed by the perfused organ was decreased by the addition of unconjugated bilirubin to the perfusate.

The uptake described at both sites might be explained by: (a) unconjugated bilirubin binding to polar components of cell membranes and further precipitation of pigment with the formation of an unconjugated bilirubin-phosphatidylcholine complex [39, 46]; (b) unconjugated bilirubin transfer to the cells, where it may be metabolized [10, 11] or exert a toxic effect over some subcellular particles [47], or even be translocated to the lumen.

Acknowledgments

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References

Renal peritubular uptake of bilirubin


