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Original Research Article

A prostacyclin analog prevents the regression of renal microvascular network by inhibiting mitochondria-dependent apoptosis in the kidney of rat progressive glomerulonephritis

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ABSTRACT

We have previously demonstrated that renoprotective effects of a prostacyclin analog, beraprost sodium, on the kidney of anti-glomerular basement membrane glomerulonephritis (GN) rats. The aim of this study is to address the renoprotection mechanism of beraprost sodium, especially in the terminal stage of GN. Beraprost sodium was orally administrated from 2 to 7 weeks after induction of GN, and renal function, morphology, protein and mRNA levels were analyzed. We found the beraprost sodium treatment suppressed the structural regression of renal microvascular network and decline of renal blood flow occurred in the kidney of GN rats. To address the mechanism of the structural maintenance, we focused on apoptosis because the increased number of apoptotic renal microvascular endothelial cells and tubular epithelial cells was observed in the kidneys of GN rats as compared with normal and beraprost sodium treated rats. Protein and mRNA analyses demonstrated that mitochondria-dependent apoptotic pathway was activated in the kidneys of GN rats, and beraprost sodium suppressed the activation by modulating the expression patterns of pro- and anti-apoptotic factors. These results suggest that inhibition of mitochondria-dependent apoptosis of renal cells in GN kidney and consequent maintenance of renal functional structures, including microvascular network might contribute to the renoprotective effect of beraprost sodium in GN.

1. Introduction

Currently immunosuppressant drugs, anti-platelet drugs and anti-hypertensive drugs (angiotensin converting enzyme inhibitors and angiotensin II receptor blockers) are used for the treatment of chronic kidney diseases (CKD), but their efficacies are not sufficient. For example, most patients with advanced glomerulonephritis (GN) do not respond to immnosuppression therapies [1], and although angiotensin converting enzyme inhibitors and angiotensin II receptor blockers can delay development of renal failure, they do not arrest or reverse decline of kidney function. Therefore, a novel therapeutic strategy is needed, especially in the progressed stage of CKD.

In kidney, prostacyclin is mainly produced by endothelial cells and it shows a variety of biological activities [2]. While the half life of endogenous prostacyclin is very short [3], beraprost sodium used in this study is a stable, orally active prostacyclin analog [4]. Beraprost sodium shows vasodilatory, cytoprotective, anti-platelet, and anti-inflammatory effect in various organs as same as endogenous prostacyclin via binding to IP receptor [5–8]. While beraprost sodium is now clinically used in patients with pulmonary arterial hypertension and chronic arterial occlusive disease, its renoprotective effects also demonstrated in several clinical studies. For instance, beraprost sodium prevented the decline of renal function in patients with CKD [9], and reduced the proteinuria of diabetic nephropathy patients [10]. In addition, there are several reports which demonstrated the efficacies of beraprost in experimental renal disease models such as anti-glomerular basement membrane glomerulonephritis (anti-GBM GN) model [11,12], unilateral ureteral obstruction model [13], and cisplatin induced nephropathy model [14]. Anti-GBM GN model in WKY rat is a

Abbreviations: GBM, glomerular basement membrane; GN, glomerulonephritis; CKD, chronic kidney diseases; ICAM-1, intercellular adhesion molecule-1; sCr, serum creatinine; BUN, blood urine nitrogen; MCP-1, monocyte chemotactic protein-1; RPF, renal plasma flow; RBF, renal blood flow.

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good model for human GN [15,16], and progresses to terminal stage renal disease by 7–8 weeks after induction of GN [17,18]. Our and other group previously demonstrated a mechanism that beraprost sodium shows the renoprotective effect by inhibiting pro-inflammatory factors (ICAM-1 and MCP-1) in anti-GBM GN rat [11,12]. These reports focused on early and middle stage of this model (2–4 weeks after induction of GN) [11,12], and there was no information about the effects of beraprost sodium at the terminal stage of CKD model.

Therefore, in the present study, we examined the effects of beraprost sodium on the kidneys of terminal stage anti-GBM rats using histochemical and biochemical analyses.

2. Materials and methods

2.1. Animals

All animals were treated in accordance with procedures approved by the Animal Ethics Committee in Pharmaceutical Research Laboratories, Toray Industries Inc., Japan. Specific pathogen-free male Wistar-Kyoto (WKY) rats, purchased from Charles River Japan (Kanagawa, Japan) were housed in autoclaved metal cages and given a standard cube diet (MF; Oriental Yeast, Tokyo, Japan) and water *ad libitum* in an air-conditioned room ($23 \pm 2\,^{\circ}C$), under controlled lighting conditions (12/12 h light/dark).

2.2. Induction of anti-GBM GN

Anti-rat glomerular basement membrane (GBM) serum was prepared in rabbits as previously described [12,19]. Progressive anti-GBM glomerulonephritis (GN) was induced in 9-week-old, inbred male WKY rats by single intravenous injection of 0.3 ml/100 g body weight of anti-rat GBM serum (10-fold dilution with saline). The concentration of the anti-GBM serum that was sufficient to induce progressive GN was determined by preliminary experiments (data not shown). The normal group rats were injected with normal rabbit serum instead of the anti-rat GBM serum.

2.3. Administration of beraprost sodium

Two weeks after induction of GN, the levels of serum creatinine (sCr) and blood urine nitrogen (BUN) were significantly higher than those of normal rats (sCr; $p < 0.001$, normal group: 0.25 ± 0.01 mg/dl, GN group: 0.45 ± 0.01 mg/dl, and BUN; $p < 0.001$, normal group: 14.8 ± 0.38 mg/dl, GN group: 27 ± 0.78 mg/dl, normal group: $n = 5$, GN group: $n = 18$), which confirmed the presence of established renal failure. The GN-induced rats were randomly divided into two groups, the beraprost sodium-treated group (beraprost group) and the nephritic control group (GN group). Beraprost sodium (Toray, Tokyo, Japan) was dissolved in distilled water and orally administered at 0.3 mg/kg body weight twice a day (which corresponds to a daily dose of 0.6 mg/kg/day). Distilled water was administered to the GN group rats and normal control rats (normal group) instead of beraprost sodium. The dose of beraprost sodium was determined in our previous study [12].

2.4. Analyses of serum biochemical parameters

The biochemical parameters of serum samples were analyzed at 2, 3, 4, 5, 6 and 7 weeks after induction of GN ($n = 9$, respectively). Blood was collected from subclavian veins under ether anesthesia, and serum samples were separated by centrifugation ($1500 \times g$ for 10 min). The levels of serum creatinine were measured by the creatinine sarcosine oxidase-POD method using a commercially available assay kit (N-Assay CRE-L Nittobo, Nittobo medical, Tokyo, Japan). The blood urea nitrogen levels were determined by the urease-GLDH method using a commercially available assay kit (N-Assay BUN-L Nittobo, Nittobo medical, Tokyo, Japan).

2.5. Measurement of renal blood flow and systolic blood pressure

To measure the renal plasma flow (RPF), clearance of PAH (para amino-hippurate) was determined under conditions of pentobarbital anesthesia. In brief, 0.5% of PAH (Daiichi-Sankyo, Tokyo, Japan) was initially administered at a dose of 2 ml/kg body weight as a bolus injection, and followed by a constant infusion at 10 ml/kg/h using a syringe pump (Terumo, Tokyo, Japan). When steady state was reached after a 1 h infusion, urine was collected from the cannulated left and right ureters for 30 min. Blood samples were drawn from the cannulated jugular vein. PAH levels in urine and plasma samples were determined by standard spectrometric assay (Brun's method) [20]. RPF was calculated by the following formula; $\text{RPF (ml/min)} = \text{PAH}_u\ (\mu g/ml) \times V_u\ (\text{ml/min}) / \text{PAH}_p\ (\mu g/ml)$, where PAH_u is PAH concentration in urine; V_u is urine flow rate (urine volume/30 min).

To calculate renal blood flow (RBF), hematocrit was measured using an automated hematology analyzer (SYSMEX, Hyogo, Japan). RBF was calculated as follows: $\text{RBF} = \text{RPF}/(1 - \text{hematocrit}/100)$. Independent experiments were repeated twice ($n = 4$ in each experimental group at each occasion: in the normal, GN and beraprost groups). The renal blood flow was measured 7 weeks after induction of GN in the first experiment. Because the disease progression in the second experiment was unexpectedly more rapid than the anticipated rate (the sCr in the GN group rats had already reached 2.09 ± 0.62 mg/dl 6 weeks after induction of GN in the second experiment, while the sCr at 7 weeks was 1.97 ± 0.65 mg/dl in the first experiment), we decided to measure the renal blood flow 6 weeks after induction of GN, taking into consideration the condition of the animals, in the second experiment.

Systolic blood pressure was measured noninvasively approximately 6 h after drug administration by the tail-cuff method (BP-98A, Softron, Tokyo, Japan). To measure blood pressure correctly, rats were habituated to the tail-cuff from 1 week before measurement. Five times measurements were performed in a rat and the mean of three times excluding the maximum and minimum data was adopted as blood pressure of each animal.

2.6. Preparation of renal vascular corrosion casts and scanning electron microscopy

Under conditions of pentobarbital anesthesia, 6 weeks after induction of GN, the right and left kidneys were transfused using a polyethylene catheter placed in the abdominal aorta, first with 0.1 M phosphate buffer containing 5% sucrose and 5 U/ml heparin), and then the fixative (5% sucrose, 2.5% glutaraldehyde). After 2 min of fixative perfusion, the fixative was washed out with 0.1 M phosphate buffer containing 5% sucrose. Thereafter, 2 ml of acryl resin (Mercox, Dai-Nihon Inki, Tokyo, Japan) was infused with constant pressure by using a syringe pump (Terumo, Tokyo, Japan). After 1 h incubation at room temperature (RT), kidneys were removed and further incubated at $60\,^{\circ}C$ for 1 h. The tissues were then digested in 20% potassium hydroxide at RT for 16 h. The casts were subsequently rinsed three times with distilled water, and air-dried.

Prepared corrosion casts were examined by scanning microscope (JSM-6320F, JOEL, Tokyo, Japan).

2.7. Immunohistochemistry

For preparation of kidney sections, animals were sacrificed under deep ether anesthesia 7 weeks after induction of GN ($n = 4$). Removed kidneys were fixed with 4% (w/v) formaldehyde in 0.5 M