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Original article

Sustained delivery of insulin-loaded block copolymers: Potential implications on renal ischemia/reperfusion injury in diabetes mellitus

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ABSTRACT

The purpose of this research was to evaluate the protective effects of insulin-loaded poly(ethylene glycol)-*b*-poly((2-aminoethyl-L-glutamate)-*g*-poly(L-lysine)) (PEG-*b*-P(ELG-*g*-PLL)) on renal ischemia/reperfusion (I/R) injury in rats with diabetes mellitus. Rats were preconditioned with free insulin or insulin/PEG-*b*-P(ELG-*g*-PLL) polyplexes, then subjected to renal I/R. The blood and kidneys were then harvested, Glucose uptake rate, glucose transporter 4 (GULT4) mRNA level, cell membrane GULT4 content and GULT4 expression were measured, the level of serum creatinine and blood urea nitrogen were determined, the activity of superoxide dismutase and inducible nitric oxide synthase, the content of malondialdehyde and nitric oxide, reactive oxygen species (ROS) production and nuclear factor κB (NF-κB) mRNA level, Bcl-2 assaciated x protein (Bax) mRNA and B cell lymphoma/lewkmia-2 (Bcl-2) mRNA level, and the expression of protein 47 kDa phagocyte oxidase (p47phox) in renal tissues were measured. Insulin preconditioning improved the recovery of renal function, reduced oxidative stress injury, restored nitroso-redox balance and downregulated the expression of p47phox induced by renal I/R injury, while the application of block copolymer PEG-*b*-P(ELG-*g*-PLL) as an insulin nanocarrier significantly enhanced the protective effect of insulin. Block copolymer PEG-*b*-P(ELG-*g*-PLL) could be used as a potential nanocarrier for insulin with sustained release and enhanced bioavailability.

1. Introduction

Kidney is a vital adjuster of body homeostasis and is involved in excreting the toxic products of metabolism and exogenous protein drugs [1]. Renal ischemia/reperfusion (I/R) injury (RI/RI) is a common pathophysiologic phenomenon, and it is a complex and important pathological process with many factors involved. I/R damage is one of the underlying causes of acute renal failure and reactive oxygen species (ROS) play important parts in mediating tissue damage during I/R damage [2,3]. Oxidative stress is generated via an imbalance between ROS and antioxidant production. Renal I/R leads to tissue damage by the way of oxygen radicals and disturbs balance between oxidants and antioxidants in renal tissue [4].

The hyperglycemia of diabetes mellitus (DM) can increase renal sensitivity to I/R injury and cause organ dysfunction [5–8]. The I/R damage is one of the dangerous complications in DM [9]. It has been revealed that the renal injury on RI/RI in DM is more serious than that in nondiabetic rats [9–11]. Reduction of RI/RI improves the survival and prognosis of diabetic patients, which is urgently required in clinical settings. Insulin preconditioning is commonly recognized as a promising method for renal protection in DM [12]. It has been demonstrated that insulin can protect the isolated rat kidney from I/R injury in DM and non-DM, and the underlying mechanisms are implicated in attenuation of renal cell apoptosis, quenching renal oxidative stress and restoration of nitroso-redox balance [12–16]. However, the half-life of insulin is very short in the blood circulation. It is also very difficult to control the insulin dose clinically because of the frequent occurrence of hypoglycemia [17].

In our previous work, we reported a brush-like poly(L-lysine) based block copolymer poly(ethylene glycol)-*b*-poly((2-aminoethyl-L-glutamate)-*g*-poly(L-lysine)) (PEG-*b*-P(ELG-*g*-PLL)) as a potential insulin nanocarrier to prolong the *in vivo* half-life of insulin and to protect lung against renal ischemia/reperfusion-induced injury in rats [18]. Previous studies showed that insulin nanocarrier improved insulin bioactivity [19,20]. In the present study, we aim to evaluate the sustained pharmacological effect of

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insulin-loaded PEG-*b*-P(ELG-*g*-PLL) on protecting kidney against I/R injury in diabetic rats using free insulin as the control.

2. Materials and methods

2.1. Materials

Bovine pancreas insulin, fluorescein isothiocyanate-insulin (FITC-insulin), and streptozotocin (STZ) were from Sigma (St. Louis, MO). Serum creatinine (Scr) kit, blood urea nitrogen (BUN) kit, superoxide dismutase (SOD) kit, malondialdehyde (MDA) kit, nitric oxide (NO) kit, inducible nitric oxide synthase (iNOS) kit, glucose transporter 4 (GULT4) kit, reactive oxygen species (ROS) kit and nuclear factor κB (NF-κB) kit, Bcl-2 assaciated x protein (Bax) kit and B cell lymphoma/lewkmia-2 (Bcl-2) kit were from Nanjing Jiancheng Bioengineering (Nanjing, China). Protein 47 kDa phagocyte oxidase (p47phox) rabbit IgG antibody was from Santa Cruz Biotechnology (Shanghai) (Shanghai, China). Block copolymer PEG-*b*-P(ELG-*g*-PLL), where the molecular weight of PEG was 5 kDa, the degree of polymerization for PELG was 50, and the degree of polymerization for PLL was 3 (structure shown in Fig. 1), was synthesized and characterized following the reported method [18].

2.2. Synthesis and cytotoxicity of PEG-b-P(ELG-g-PLL)

Detailed courses of the synthesis and cytotoxicity measurement of PEG-*b*-P(ELG-*g*-PLL) refer to our previous work [18].

2.3. Encapsulation of insulin in and release of insulin from PEG-b-P(ELG-g-PLL)

The FITC-insulin/PEG-*b*-P(ELG-*g*-PLL) polyplexes were prepared according to the methods described in the previous report [18]. Briefly, a given volume of FITC-insulin (0.2 mg mL^{-1}) in a PB buffer of 0.01 mol L^{-1} (pH 7.4) was added to PEG-*b*-P(ELG-*g*-PLL) in a PB buffer. After 30 min, the mixed solution was transferred to a dialysis tubing (MWCO, 100 kDa) and dialyzed against a 0.01 mol L^{-1} PB buffer under sink conditions. The dialysis of free FITC-insulin in a PB buffer was also conducted under the same conditions to serve as controls. When FITC-insulin in the control experiment was completely dialyzed, the amount of insulin in the outer dialysate of the polymer solution was measured using a RF-5301PC spectrofluorophotometer (Shimadzu) and then subtracted from the total amount of added insulin. All of the experiments were carried out in triplicate.

The release of insulin from PEG-*b*-(PELG-*g*-PLL) was investigated using a dialysis method (MWCO, 100 kDa) at room temperature with a polymer/FITC-insulin polyplex solution (5 mL) against a PB buffer (100 mL, pH 7.4, 0.1 mol L^{-1}). At desired time intervals, a given volume of release media was withdrawn and replenished with an equal volume of fresh release media. The amounts of released insulin were determined by the spectrofluorophotometer.

2.4. Diabetic animal model

Sprague-Dawley (SD) male rats (180–220 g) were obtained from the Animal Center of Shantou University, and all animal experiments were performed according to the Guiding Principles for the Care and Use of Experimental Animals in Shantou University. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee, Shantou University. Diabetes in SD rats was induced by a single intraperitoneal injection to the left abdomen of STZ (50 mg/kg) dissolved in citrate buffer (pH 4.5). Blood glucose levels were monitored using a commercial JPS-6 glucose meter (Beijing Yicheng Bioelectronics Technology, Beijing, China). The rats were considered diabetic if fasting blood glucose level was higher than 16.7 mmol/L one week after STZ injection. Because of the short duration of diabetes mellitus, proteinuria, renal impairment or other vascular complications were not expected.

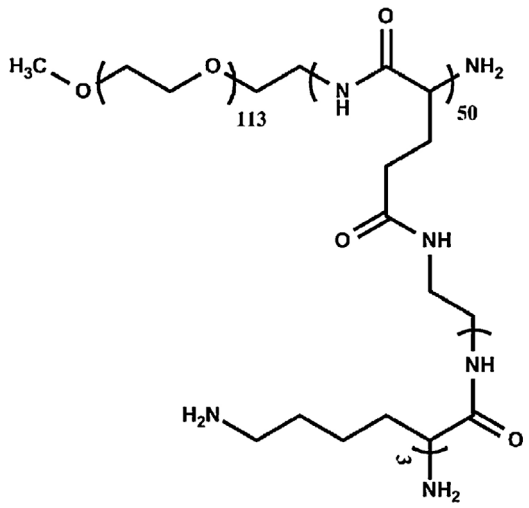

Fig. 1. The structure of block copolymer PEG-*b*-P(ELG-*g*-PLL). Abbreviations: PEG-*b*-P(ELG-*g*-PLL), poly(ethylene glycol)-*b*-poly((2-aminoethyl-L-glutamate)-*g*-poly(L-lysine)).

2.5. In vivo evaluation of hypoglycemic effect of insulin/polymer polyplexes

Four diabetic rats were used for each group of rats without any injection, rats with insulin only, and rats with insulin/PEG-*b*-P(ELG-*g*-PLL) polyplexes. Insulin or insulin/PEG-*b*-P(ELG-*g*-PLL) solution (0.5 mL, 30 IU/kg) in 0.01 mol L^{-1} PB was injected subcutaneously into the abdomen of diabetic rats that had been anesthetized with ethyl ether. At the predetermined time intervals, blood glucose levels were measured using the glucose meter.

2.6. Animal experimental design and surgical procedures of I/R

40 normal SD male rats and 40 diabetic male rats were assigned to eight groups (10 rats in each group): 1) Non-DM sham-operated group (Non-DM); 2) DM sham-operated group (DM); 3) Non-DM + I/R group: I/R was achieved by clamping renal artery and vein of non-DM rats for 45 min followed by 24 h of reperfusion according to the previous report [18]; 4) DM + I/R group: I/R was achieved by clamping renal artery and vein of DM rats for 45 min followed by 24 h of reperfusion; 5) Non-DM + INS + I/R group: insulin (30 U/kg) was administrated once by abdominal subcutaneous injection to non-DM rats 6 h prior to the I/R procedure; 6) DM + INS + I/R group: insulin (30 U/kg) was administrated once by abdominal subcutaneous injection to DM rats 6 h prior to the I/R procedure; 7) Non-DM + P + I/R group: insulin/PEG-*b*-P(ELG-*g*-PLL) polyplexes with the same dose of insulin were administrated once by abdominal subcutaneous injection to non-DM rats 6 h prior to the I/R procedure; 8) DM + P + I/R group: insulin/PEG-*b*-P(ELG-*g*-PLL) polyplexes with the same dose of insulin were administrated once by abdominal subcutaneous injection to DM rats 6 h prior to the I/R procedure.

After I/R procedure, the blood samples were collected via abdominal aorta, and centrifuged at 3600 rpm for 15 min to harvest the sera. The left kidneys of rats were immediately removed. After the blood was rinsed thoroughly, the kidneys were stored at −80 °C for the following analyses.