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Original article

Hypoxia-induced extracellular vesicles mediate protection of remote ischemic preconditioning for renal ischemia-reperfusion injury

Guangyuan Zhang[a], Yu Yang[a], Yeqing Huang[a], Lei Zhang[a], Zhixin Ling[a], Yingjian Zhu[b], Feng Wang[c], Xiangyu Zou[b,**], Ming Chen[a,*]

[a] Department of Urology, Zhongda Hospital, Southeast University, Nanjing, China
[b] Department of Urology, Xinhua Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China
[c] Department of Nephrology and Rheumatology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China

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ABSTRACT

Remote ischemic preconditioning (rIPC) is a reliable strategy for prevention of injury to various organs. However the mechanism by which it does so is still unclear. In the present study, serum and EVs isolated from ischemic preconditioned right renal venous perfusates were injected into rats with ischemia-reperfusion-injured kidneys immediately after reperfusion. The animals were killed 24 h later. Tubular scores and renal function were tested to evaluate the therapeutic effects. To further explore the underlying mechanism, HK-2 cells derived EVs under hypoxia were also administrated to rats with left kidney IRI. Results showed that transient ischemia of the right kidney induced renal tubular epithelial cells to release functional extracellular vesicles (EVs), which were found to alleviate left kidney ischemic reperfusion injury (IRI) by circulation and the EV-depleted serum lost this property. Further, human kidney cells (HK2) were cultured under hypoxic conditions to generate EVs *in vitro*. These EVs also showed obvious therapeutic effects for renal IRI. Our results suggested that remote ischemic preconditioning plays a therapeutic role in renal IRI through EVs induced by hypoxia.

1. Introduction

Renal ischemic reperfusion injury is commonly seen in patients with renal transplantation and surgery [1]. Severe ischemia could induce tubular cell death, which leads to dysfunction of the kidney and proceeds to acute kidney injury [2]. In this case, it would affect the renal functions and evolve into chronic kidney disease [3]. In this way, it is imperative to develop a potent intervention to prevent ischemic induced acute kidney injury.

In early 1986, ischemic preconditioning (IPC) was regarded as a reliable method of delaying the onset of necrosis during lethal ischemic insult. IPC has been used for the prevention of myocardial injury due to reduced ATP deprivation and catabolite accumulation [4]. Recently, various studies have focused on the function of IPC in this process and multiple mechanisms were found to be involved. These include the modulation of reactive oxygen species (ROS), nitric oxides (NOS), and MAPK pathways [5–7]. For the alleviation of renal ischemic injury, it has been reported that different miRNAs and pathways may be involved in IPC [8,9]. The ischemic preconditioning of situ organs could also have therapeutic effects for delayed ischemic injury, and remote ischemic preconditioning (rIPC) shows similar effects. Hussein et al. reported that rIPC could activate antioxidant and anti-apoptotic genes to alleviate renal ischemic/reperfusion injury [10]. Further, the treatment of rIPC has been reported to reduce renal injury in clinical patients undergoing major vascular surgery, cardiac surgery, and coronary angiography [11–13]. This means that remote ischemic preconditioning may induce bioactive molecules to target remote organs through circulatory systems. However the true mechanisms underlying rIPC are still unclear.

Extracellular vesicles (EVs) are small vesicles which are released by various cells under different conditions. The amount and concentration of EVs was found to vary when cells were under stress conditions, such as hypoxia and radiation [14]. EVs contain many bioactive molecules contained in EVs, such as miRNAs, mRNAs, and proteins, and EVs could be delivered to target cells to transport these functional molecules through circulatory systems.

* Corresponding author at: Department of Urology, Zhongda Hospital, Southeast University, No.87 Dingjia Qiao Road, Nanjing 210009, China.
** Corresponding author at: Department of Urology, Xinhua Hospital, School of Medicine, Shanghai Jiao Tong University, No. 1665 Kongjiang Road, Shanghai 200092, China.
E-mail addresses: chenmingseu@126.com (M. Chen), zouxiangyudy@126.com (X. Zou)..

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Recently, Giricz et al. showed that ischemia for myocardium-induced EVs mediated myocardial preservation of rIPC [15], which could be regarded as a novel explaining for rIPC. However, whether EVs could also participate in renoprotection of rIPC is still unknown.

In present study, we established a new animal model of rIPC, which is transient ischemia for right kidney protecting against left kidney IRI. More importantly, we focused on the ischemia-induced release of EV and investigated the role of these EVs in alleviating renal ischemic reperfusion injury.

2. Materials and methods

2.1. Ethics statement

This study was conducted in strict accordance with the recommendations from the Guide for the Care and Use of Laboratory Animals of Southeast University. The protocol was approved by the Committee on the Ethics of Animal Experiments of Southeast University. All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

2.2. Animal study

All animals used were male SD rats (180–200 g) housed at a constant temperature, with a 12:12 h light-dark cycle. All surgeries were performed under the induction of isoflurane anesthesia.

The animal operation protocol is shown in Fig. 1A:

a: Ischemia preconditioning was performed on the right kidneys after ischemia-reperfusion of the left kidneys. After performing a midline laparotomy, right renal artery underwent 3-cycles of 5 min of ischemia and 5 min of reperfusion using non-traumatic microvascular clamps. After 10 min, the right kidney was removed and the left kidney was subjected to ischemia for 45 min. Animals were killed 24 h after reperfusion; and their left kidneys and blood were collected for examination.

b: Right kidney vein perfusate was collected. After performing a midline laparotomy, each right kidney artery and vein were separated. The paracentesis for right kidney vein was conducted and perfusate was collected continuously. After puncture, right renal artery underwent 3-cycles of 5 min of ischemia and 5 min of reperfusion. Animals were killed 10 min after IPC. Each right kidney was collected.

c: Treatment of left kidney ischemia-reperfusion injury. Midline laparotomy was conducted and then the right kidney was removed. The left kidney was subjected to ischemia for 45 min. Right after reperfusion, EVs (or vesicle, or serum, or EVs depleted serum) was injected intravenously. Animals were killed 24 h after reperfusion; the left kidney and blood were collected for examination.

Animals were divided into groups: sham-operated group (Sham) (n = 6), unilateral kidney ischemia-reperfusion injury group (IRI) (n = 6), treatments (100 μg EVs, 1 mL serum or EV-depleted serum) for IRI groups (n = 6 respectively). All animals were kept in separate groups until they were killed.

To trace EVs in injured kidney, a PKH-26 dye (Sigma, Saint Louis, MO, U.S.) kit was used to label the EVs. Then PKH-26 labeled EVs were injected intravenously into AKI rats, and the unlabeled EVs were used as a control. Rats were killed after 24 h and organs were acquired for frozen sectioning. Hoechst 33258 dye was added for nuclear staining (Sigma, Saint Louis, MO, U.S.).

2.3. Isolation of EVs from serum and EV depletion

EVs were isolated from collected right renal vein perfusates by filtration and differential centrifugation as described previously [15]. Briefly, perfusates were dialyzed against 0.45% saline containing 5 mM EDTA for 4 h at room temperature then vacuum-distilled to 40 mL (for approximately 40 min) at room

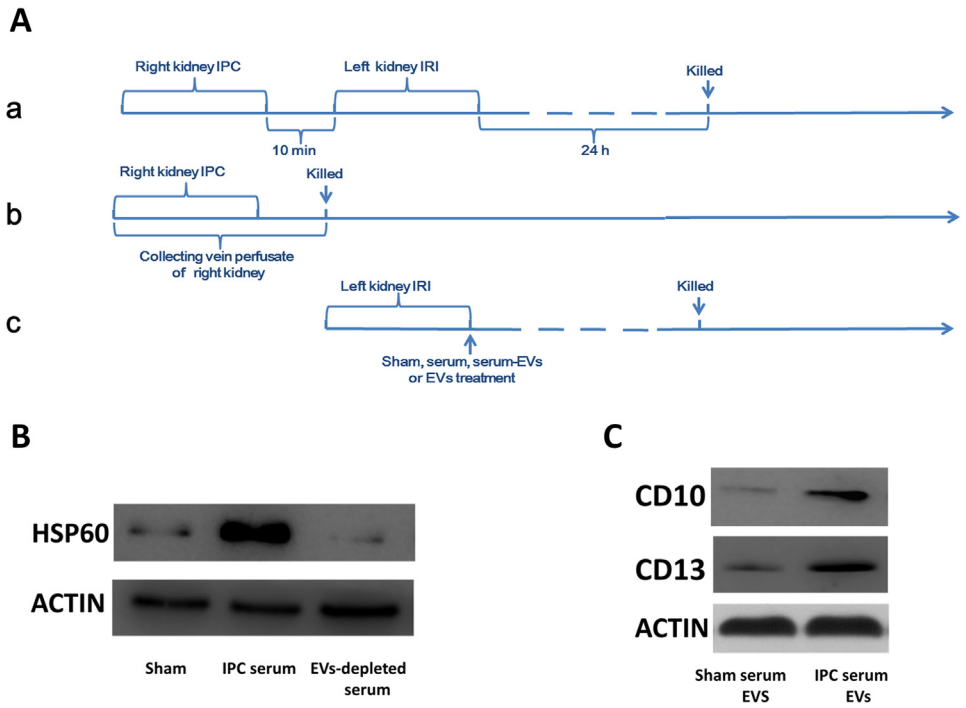

Fig. 1. Schema for the animal experiment protocols and increased numbers of EVs from renal tubular epithelial cells after IPC. A. Animal study protocol: a. rIPC followed by ischemia/reperfusion protocol; b. Right kidney vein perfusate collection; c. Treatment *via* renal IRI protocol; B. HSP60 expression in right renal vein serum from sham-operated animals (Sham serum), ischemic preconditioned animals (IPC serum) and IPC serum containing no EVs (EV-depleted serum); C. Renal tubular cell marker (CD10 and CD13) expression in EVs from serum of right renal vein serum from sham-operated animals (Sham serum EVs) and EVs from IPC serum (IPC serum).