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Ischemic preconditioning increased the intestinal stem cell activities in the intestinal crypts in mice

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ARTICLE INFO

Article history:
Received 12 May 2013
Received in revised form
7 September 2013
Accepted 2 October 2013
Available online 10 October 2013

Keywords:
Intestinal stem cells
Ischemic preconditioning
Small intestine
Stem cell markers

ABSTRACT

Background: Ischemic preconditioning (IPC) can protect against ischemia–reperfusion injury in the small intestine. Because intestinal stem cells (ISCs) control the recovery and growth of intestinal villi, this study investigated whether IPC had any effects on the activity of ISCs.

Materials and methods: The small intestines of mice were treated with IPC, laparotomy only (sham), or no surgery. The crypt fractions were isolated and the characteristics of ISCs among various groups were compared. The regenerative ability and the number of organoids grown from various crypt fractions were compared. The expression of hypoxia-inducible factor-1α (HIF-1α) and the related proteins of the Wnt-/β-catenin pathway in the crypt fractions were studied.

Results: The IPC group had higher messenger RNA levels of various stem cell markers than the sham group at days 1 and 2 after surgery. The IPC group exhibited greater regenerative activity and more crypt organoids than the sham group ($P < 0.05$). The expression of HIF-1α, β-catenin, and phosphoglycogen synthase kinase 3β was increased in the IPC-treated crypt fractions *in vivo* and cultured crypt organoid cells with deferoxamine-mimicked hypoxia *in vitro*.

Conclusions: IPC significantly upregulated the activity of ISCs, possibly through the HIF-1α response and Wnt-/β-catenin signaling pathway.

1. Introduction

The small intestine is sensitive to ischemia–reperfusion (I/R) injury [1]. Intestinal I/R injury may damage the mucosa, impairing its barrier function and leading to bacterial translocation [1]. The I/R injury in small bowel transplantation can even induce sepsis, and acute and chronic rejections [2]. Ischemic preconditioning (IPC) is defined as one or more brief periods of ischemia with intermittent reperfusion, and can protect against sustained I/R injury in the heart, brain, kidney,

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http://dx.doi.org/10.1016/j.jss.2013.10.001

and intestinal tissues [3–7]. Mechanistic studies in the heart, brain, and kidney have demonstrated that this protective effect is correlated with the increased cell proliferation and survival of stem cells [3,4,6]. However, there is no research on the direct linkage between the function of intestinal stem cells (ISCs) and the protective benefits of IPC treatment.

The intestinal epithelium, ordered into crypts and villi, is lined by simple columnar epithelial cells that undergo complete regeneration every 3–7 d in murine or human [8,9]. The ISC population lying in the crypt region is responsible for this rapid rate of cell production [8,9]. These ISCs can differentiate into Paneth cells, absorptive enterocytes, goblet cells, and enteroendocrine lineages [8,9]. The regeneration activity of ISCs also plays an important role in the recovery of intestinal mucosa after physical or pathologic injury, including the I/R injury [8,9]. The ISCs may present various kinds of markers, such as leucine-rich repeat-containing G protein–coupled receptor 5 (Lgr5), B lymphoma Moloney murine leukemia virus insertion region homolog-1 (Bmi1), Musashi-1 (Msi1), EphB3, olfactomedin 4, mouse telomerase reverse transcriptase, and so forth [10–16]. The *Lgr5* gene marks the crypt base columnar cells, which are actively cycling cells, interspersed between Paneth cells [11]. The isolated Lgr5-expressing cells are able to grow into crypt organoids *in vitro* and generate all epithelial lineages [13,15,17]. Bmi1 is another marker of distinctive stem cell population, predominantly located at position +4 directly above the Paneth cells and is restricted to the proximal small intestine [11,13,14,18]. Msi1 helps maintain neural stem cells and is expressed in the crypt base columnar cells located between the Paneth cells [12]. EphB3 is a key regulator of epithelial cell positioning, restricts cell intermingling, and allocates cell populations within the intestinal epithelium [10]. Demonstration of these markers in the intestines is helpful for the identification of the ISCs.

Exposure to low oxygen levels has been found to stabilize hypoxia-inducible factor-1α (HIF-1α) and initiate a "HIF response" ranging from rapid changes at the cellular level, such as altered carbohydrate metabolism, to systemic changes including erythropoiesis and angiogenesis [19]. Because IPC of small intestine includes several brief periods of ischemia with intermittent reperfusion, it might activate the HIF response in the ISCs. Furthermore, the proliferation and differentiation activities of stem cells are under the combinatorial control from several pathways governing the epithelium–mesenchymal interactions (such as hedgehog and bone morphogenetic protein signals), cell-to-cell communication (such as notch signals), and intrinsic proliferative behavior (such as Wnt-/β-catenin signals) [9,20]. Among these pathways, the Wnt-/β-catenin pathway has been emphasized for its effect in the proliferation of ISCs [21]. In addition, HIF-1α can activate the Wnt-/β-catenin signaling in stem cells during hypoxia [22] and the ISC marker *Lgr5* is a target gene of Wnt pathway [19], which re-enforce the significant role of HIF-1α and Wnt-/β-catenin signaling in ISC activation. Thus, IPC may affect the HIF response and Wnt-/β-catenin signaling pathway in the ISCs.

This study investigated the possibility that IPC exhibited its protective effect through the regulation of ISCs. The crypt fractions from mice receiving IPC or laparotomy only (sham control) were compared for their expression levels of stem cell markers and *in vitro* growth of crypt organoids. The expression of HIF-1α and activity of Wnt-/β-catenin pathway in the crypt fractions after IPC *in vivo* and the cultured crypt organoid cells with deferoxamine (DFX)-mimicked hypoxia *in vitro* were studied. Our results demonstrated that IPC significantly induced the HIF response and Wnt signaling, and increased the activity of ISCs in the intestinal crypts.

2. Materials and methods

2.1. Animal experiments

The animal experiments in this study were approved by the Committee on Laboratory Animal Research of the Far Eastern Memorial Hospital, Taiwan, and conducted according to the guidelines of the Laboratory Animal Center of the Far Eastern Memorial Hospital. Five-week-old C57BL/6JNarl mice weighing 15–20 g were used for the experiments. The mice were provided food and water *ad libitum* on a 12:12 h day–night cycle (lights on from 6 AM to 6 PM) with room temperature maintained at around 20°C.

2.2. IPC of intestine in C57BL/6JNarl mice

The mice were weighed and anesthetized with zolazepam hydrochloride and tiletamine hydrochloride (3 mg/kg) (Virbac Laboratories, Carros, France). The procedure for IPC of the small intestine was modified from the method described previously [1,23]. The mice were placed in a supine position and a lower midline laparotomy was performed. The superior mesenteric artery was identified and clamped with a bulldog microclamp (Vascu-Statt; Scanlan, St Paul, MN) providing 10–25 g/cm^2 of pressure. After 10-min ischemia, the reperfusion was performed by removing the vascular clamps for 10 min. The procedure was repeated three times, and the laparotomy wound was then closed. Tissue samples from the small intestines were harvested after 1, 2, and 3 d and subjected to isolation from the ISC fractions. The mice in the sham group received laparotomy only. The control mice received no surgery.

2.3. Isolation of crypt fractions from the intestinal tissues

The protocol of isolating crypt fractions in this study followed the method described previously [11]. Intestinal tissues from the mice were sliced into 10-mm fragments and cut longitudinally. The samples were then soaked in ice-cold phosphate-buffered saline (PBS) containing gentamicin (0.5 mg/mL) with gentle shaking at 4°C for 10 min to remove feces and contaminants. The tissue fragments were then washed again in ice-cold PBS (Mg^{2+}/Ca^{2+}) with gentle shaking at 4°C for 20 min. The fragments were incubated in PBS0 (PBS containing 1 mmol/L of ethylene diamine tetraacetic acid and 1 mmol/L of ethylene glycol tetraacetic acid) with gentle shaking at 4°C for 10 min. After discarding the supernatant PBS0 with suspended small tissue pieces, fresh PBS was immediately added and mixed with the fragments by vortex. The cells suspended in PBS0 were collected. The PBS0 incubation and PBS0 vortex steps were