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Full Length Research Paper

Effects of intestinal trefoil factor on PI3K and caspase-3/9 in newborn rats with necrotizing enterocolitis

Cai-Xia Yan[1], He-Sheng Luo[2], Bing-Hong Zhang[1]*, Ri-Hong Zhao[1] and Hai-Xia Zhang[1]

[1]Department of Pediatrics, Renmin Hospital of Wuhan University, Jiefang Road 238, Wuhan 430060, PR China.
[2]Department of Gastroenterology, Renmin Hospital of Wuhan University, Wuhan 430060, PR China.

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PI3K/Akt signal pathway was blocked by Wortmannin, a specific inhibitor of phosphoinositide-3 kinase (PI3K), and the effects of intestinal trefoil factor (ITF) on PI3K and caspase-3/9 in newborn rats with necrotizing enterocolitis (NEC) were investigated to explore the protective mechanism of ITF against NEC. Experimental NEC was induced by exposure to hypoxia for 60 sec followed by cold stress at 4°C for 10 min. A total of 50 One-day-old Wistar rats were randomly divided into five groups : group A, NEC+NS; group B, NEC+ITF; group C,NEC+ wortmannin; group D ,NEC+ wortmannin + ITF; group E , Normal control. The animals were euthanized at development of NEC, and at 96 h the intestinal tissue was harvested and examined for histological changes of NEC, and then the PI3K content and Caspase-3/9 activity were detected using ELISA and spectrophotometry, respectively. The PI3K content (pg/ml) in group A was slightly higher than group E (P<0.05), and there was no significant difference between group A and D (P>0.05), but the PI3K content (pg/ml) in group B was significantly higher than the remaining groups (P<0.01), and the PI3K content (pg/ml) in group C was significantly lower compared with group E (P<0.01). Compared with group B and E, Caspase- 3/9 activity was significantly higher in group A than in group B and E (P<0.01), but all lower than that in group C. So we concluded that ITF could activate the PI3K/Akt pathway to down-regulate Caspase-3/9 activity, and to protect against intestinal damage of NEC rats.

Key words: Intestinal trefoil factor, necrotizing enterocolitis, PI3K/Akt signal transduction, Caspase-9, Caspase-3.

INTRODUCTION

Necrotizing enterocolitis (NEC) is a severe intestinal disease in neonatal period, but its pathogenesis remains unknown. At present, it is recognized that NEC results from interaction of multiple factors including premature birth, hypoxia, enteral feeding, bacterial infection, intestinal ischemia and so on (Barclay et al., 2007). Intestinal trefoil factor (ITF), a member of trefoil peptide family, has strong cytoprotection, and can significantly reduce multiple injury factors -mediated intestinal damage, and plays important roles in intestinal self-protection and -repair following injury (Marchbank et al., 2001; Mashimo et al., 1996). The PI3K/Akt signal transduction pathway is one of the important intracellular signal transduction pathways and can be activated by multiple stress factors such as polypeptide growth factors and hypoxia, and played important biological functional roles in apoptosis, cell survival, proliferation and cytoskeleton changes (Baregamian et al., 2007).

ITF plays an important role in the self- protection and post-injury repair of gastrointestinal tract and PI3K/Akt signaling pathway can combat with cell apoptosis and promote cell survival. Previous studies have demonstrated, in a hypoxia induced neonatal rat NEC model, ITF can decrease the contents of numerous inflamematory cytokines including TNF-α, IL-8 and IL-1 and enhance expression of anti-inflammatory cytokine such as IL- 10, which alleviate the neonatal necrotizing enterocolitis and exert protective effects (Yan et al., 2005; Shi et al., 2007). However, the protective effects of ITF are

*Corresponding author. E-mail: lucyok@mspil.edu.cn. Tel: 027-88041911-82206.

still poorly understood, especially the molecular mechanism involving signal transmission. In the present study, hypoxia and hypothermia were employed to establish a NEC model in neonatal Wistar rats and the effects of ITF on the PI3K and its downstream molecules (caspase-3/9) were investigated aiming to explore the role of PI3K/Akt signaling pathway in the protective effects of ITF. We for the first time detected the activity and expression of PI3K and caspase- 3/9 in a neonatal rat NEC model and results showed ITF could activate PI3K/Akt signaling pathway eliciting protective effects. This study may provide theoretical bases for the studies on the pathogenesis and treatment of NEC.

MATERIALS AND METHODS

Animals and NEC modeling

The study was approved by the animal care and use committee of Wuhan University. Fifty 1-day- old Wistar rat pups (5 -10 g) were used in the present experiment. All experiment protocols were performed according to the guidelines for the ethical treatment of experiment animals. Local institutional approval for research was obtained before initiation of the study. The NEC model in rats was established as follows (Clark et al., 2006): hypoxia was accomplished by stressing each rat pup with 1000 ml/l N_2 for 60s, followed by exposure to cold (4°C) for 10 min twice daily. Finally, the newborn rats were orally administrated with newborn infant formula milk powder (Dumex Company, Shanghai) from a initial dose of 200 kcal/kg.d per 3 h, and then milk volume increased for 0.1 ml every time. During the whole experiments, no death was observed in Group B and E. One died in Group A and Group D, respectively and 2 died in Group C. All three neonatal rats died on day 4 (4 a.m.). Autopsy showed intestinal pneumatosis. The intestinal wall had no luster and was dark purple or black. The lesion of intestinal wall was graded 4. These findings supported the diagnosis of NEC. The dead rats were not excluded because they died of NEC and the time of death was very close to the designed time point (scarification at 7 a.m. on the same day) . So we assumed the inclusion of these dead rats for analysis would not affect our results.

Experimental design

50 NEC newborn Wistar rats were randomly divided into five groups with 10 rats in each group. The modeling rats were subcutaneously injected with 0.2 ml normal saline in group A, while 0.2 mg (0.2 ml) ITF in group B, 0.1 mg/kg wortmannin (Majumdar and Du, 2006) in group C, 0.1 mg/kg wortmannin and 0.2 mg ITF in group D. and normal rats in group E served as the controls. On the fourth day, all rats were decapitated and the intestinal tissues were harvested. Recombinant human intestinal trefoil factor (hITF) was purchased from Shanghai BoFu Biotechnology Co., Ltd., China. Bradford protein concentration assay kit, caspase-3, Caspase-9 and PI3K inhibitor Wortmannin were purchased from Jiangsu Province Institute of Biotechnology, China. Rat PI3K ELISA kit was purchased from Shanghai Lengton Biotechnology Company, China.

Histopathological examination

A representative 1 to 2 cm long specimen was taken for histology from each of the following anatomic areas: duodenum, proximal, mid, and distal small intestine, proximal and distal colon. After formalin fixation and parattin embedding, hematoxylin-eosin (HE) staining for histopathological examination was performed. Pathological scoring of intestinal tissues were performed in a double blind method and its scoring standard was as follows (Lu et al., 2006; Hammerman et al., 2002): 0 point: intestinal chorioepithelium was integrated and normal; 1 point: villi were slightly hydropic, and epithelial collapse confined in tips; 2 points: slight necrosis of villi at the middle; 3 points: moderate necrosis of villi at the middle, with recessus; 4 points: severe necrosis of villi was observed and epithelial structure disappeared.

Determination of PI3K, caspase-3 and caspase-9 contents

The remaining intestinal tissues were rinsed with ice-cold normal saline, and then weighted after drying with filter papers. Subsequently, the intestinal tissues were prepared into 2 ml tissue homogenate with ice-cold normal saline with a homogenizer, and then centrifuged at 3500 r/min for 15 min at 4°. Then, the supernatant was transferred to an EP tube and preserved in a -20°C refrigerator. PI3K content in intestinal tissue homogenate was determined with Rat PI3K ELISA double antibody kit in sandwich assay, and caspase-3 and caspase-9 enzyme activity was determined with caspase-3 and caspase-9 activity assay kits in spectrophotometry. Meanwhile, the protein concentration was determined with the Bradford protein concentration assay kit. All assays were performed according to manufactures' instructions.

Statistical analyses

Measurement data were expressed as mean ± standard deviation (x ± s) and compared by analysis of variance and $P<0.05$ was considered significantly different. All statistical analyses were performed with version SPSS 13.0 statistical software.

RESULTS

PI3K, caspase-3 and caspase-9 contents in supernatant

The PI3K content (pg/ml) of tissue homogenate in group A was slightly higher than group E ($P<0.05$), and there was no significant difference between group A and D ($P>0.05$), but the PI3K content (pg/ml) in group B was significantly higher than the remaining groups ($P<0.01$), and the PI3K content (pg/ml) in group C was significantly lower compared with group E ($P<0.01$), indicating that ITF could increase the PI3K content in intestinal epithelial cells and PI3K-specific inhibitor could significantly blocked PI3K. Compared with group B and E, Caspase-3/9 activity of tissue homogenate was significantly higher ($P<0.01$) in group A and was more significantly higher ($P<0.01$) in group C. There were no significant differences between group A and D, or group B and E ($P>0.05$), but there were significant differences among other groups ($P<0.01$) (Table 1).

Clinical manifestations and intestinal histopathological changes after modeling

After modeling, newborn rats in group A, B, C and D suffered from different degrees of yellow-green mucus

Table 1. PI3K, caspase-3 and Caspase-9 content in supernatant (x ± s).

Group	Number	PI3K (pg/ml)	Capase-3 (uM)	Capase-9 (uM)
NEC+NS	10	16.97 ± 2.27a	22.60 ± 2.93 a	23.37 ± 2.360 a
NEC+ITF	10	26.12 ± 4.69	7.62 ± 2.16	10.99 ± 2.057
NEC+Wort	10	7.96 ± 2.04 a	27.34 ± 5.28 a	26.71 ± 3.028 a
NEC+I+W	10	16.99 ± 2.87 a	21.62 ± 3.39 a	23.59 ± 2.373 a
Control	10	13.94 ± 2.06a	6.72 ± 1.45 b	9.77 ± 2.214b
F value		39.34	40.23	84.353
P value		0.0001	0.0001	0.0001

Compared with group B, a P<0.01; b P>0.05 (q= 0.90, 1.22).

loose stools, and then gastric retention, abdominal distension, diarrhea, decreased milk intake, lethargy, sleep in curl, activity decrease, slow response and movement. These symptoms gradually aggravated. However, food intake, defecation and activity were normal, without abdominal distention and gastric retention. Intestinal submucosa or lamina propria was severe edema and accompanied with local villi collapse and ischemic necrosis in group A, C and D, and the median pathological score was 3, 3.1 and 2.8 (range from 2 to 4), respectively. In group B, intestinal submucosa and lamina propria were slightly separated, showing small vascular engorgement and a small amount of villus epithelial cell necrosis, and median pathological score was 1.3 (range from 0 to 2), In group E, the morphology of intestinal mucosa was normal (Table 2). In this study, HE staining of newborn NEC model Wistar rats showed diffused edema in intestinal villi submucosa, obvious necrosis, intermittent necrotic areas in muscular layer. But defluxion of only a small amount of epithelial cells were observed in the ITF treatment group, and small vascular engorgement and necrosis of a small amount of villus epithelial cells indicated that ITF played protective roles in intestinal mucosa of NEC rats (Figure 1 A,B,C,D,E).

Table 2. Histopathological changes of intestinal tissues.

Group	Number	Histological median score (range)
A	10	3 (2-4)
B	10	1.3 (0-2)
C	10	3.1 (2-4)
D	10	2.8 (2-4)
E	10	0.3 (0-1)

There were significant differences among group A, C, D and B, E (p <0.05).

DISCUSSION

Necrotizing enterocolitis (NEC) is a severe intestinal disease in neonatal period, and it is an important cause of death in newborn infants especially in premature infants. With the increase of survival rate of low birth weight infants, the incidence of NE significantly rises. However, the pathogenesis of NEC remains unknown. In this study, many causative factors of human NEC were simulated in NEC modeling, including immature gastrointestinal tract, hypoxia, hypothermia and artificial feeding. The above factors could induce the release of a large amount of inflammatory factors, and then a series of changes in cellular level with the activation of local inflammatory cascade reaction, eventually resulting in the damage and necrosis of intestinal walls. Although the pathological manifestations of late NEC showed extensive degeneration and necrosis of intestinal tissues, apoptosis was the main style of intestinal cell death (Taupin and Podolsky, 2003; Shi et al., 2007). At present, the researches on ITF revealed that its main biological effects included protection on gastrointestinal mucosa, repair after injury and anti-apoptosis (Yan et al., 2005). Moreover, ITF is a kind of anti-apoptotic specific peptide endogenous from gastrointestinal tract, and ITF can inhibit from the apoptosis of enterocytes by regulating nuclear factor signaling cascade.

PI3K/AKT signal transduction pathway, an important intracellular signal transduction pathway, can be activated by polypeptide growth factors and cytokines, and it exerts important biological function in cell apoptosis, survival, proliferation and cytoskeleton and participates in anti-apoptosis by regulating the activity of downstream signaling molecule caspase-9 and caspase-3 zymogen. The over expression and activation of cysteinyl aspartic proteinase which is a member of caspases family can induce cell apoptosis. Caspase-9, an important caspase family member in the upstream of apoptotic signal transduction pathway, was widely expressed in human normal tissues. Caspase- 9 is an initiation factor of the mitochondrial apoptosis pathway, and stimulation including hypoxia, ultraviolet light, chemotherapy drugs and the release of mitochondrial cytochrome C could result in apoptosome formation and procaspase-9 activation. Subsequently, procaspase-9 cut and activates caspase- 3 which results in the disintegration of cellular structure and irreversible apoptosis. Caspase-3 is the core

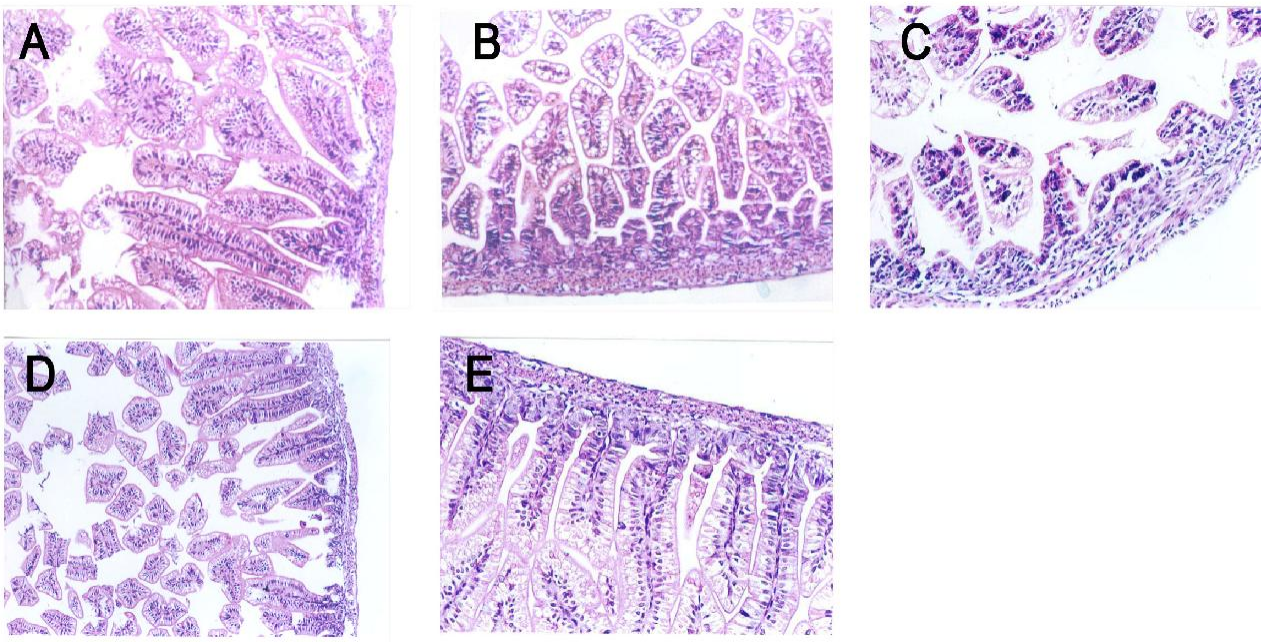

Figure 1. Pathological features under a light microscope (HE staining; ×100). A: Severe necrosis of villi was observed and epithelial structure damage, Grade: 4 (Group A); B: Villi were slightly hydropic, and epithelial collapse was confined to tips, Grade: 1 (Group B); C: Severe necrosis of villi was observed and epithelial structure disappeared, Grade: 4 (Group C); D: Moderate necrosis of villi at the center with recessus, Grade: 3 (Group D); E: Intestinal chorioepithelium was integrated and normal, Grade: 0 (Group D).

protease mediating apoptosis and the most crucial effective protease of caspase cascade reaction, and usually exists in the form of zymogen, and induces cell apoptosis by activating specific substrates (Jiang and Wang, 2000; Mazumder et al., 2008).

An animal study on myocardial ischemical reperfusion injury revealed that the PI3K/Akt pathway was activated in the early stage of ischemia, and its activity was significantly decreased after PI3K-specific inhibitor was added, indicating that the PI3K/Akt pathway was activated after ischemical reperfusion injury in important organs, and then apoptosis was inhibited and cell survival was promoted (Wang et al., 2008). At present, it is recognized that the main causes of NEC intestinal injury include early ischemia after injury, hypoxia, and the release of a large number of cytokines and inflammatory mediators (Chan et al., 2009; Guven et al., 2009). Meanwhile, the excessive release of many cytokines and inflammatory mediators depended on the up-regulated expression of inflammation- related genes and the activation of enzymes. It was found that proinflammatory cytokines could alleviate the apoptosis of colonic epithelial cells including TNF- , IL – and IFN - through the PI3K pathway (Laprise et al., 2002), and thus proinflammatory cytokines played protective roles in intestinal mucosa. The PI3K content of tissue homogenate in the ITF group was significantly higher than the NEC modeling group ($P<0.01$), and the PI3K content in the Wortmannin group was significantly lower than the NEC modeling group and the ITF group ($P<0.01$), but there was no significant difference between the NEC modeling group and the ITF+WT group, indicating that the PI3K pathway is a signaling transduction pathway inhibiting from apoptosis of intestinal epithelial cells and plays important roles in the pathogenesis of NEC. These results suggested ITF could significantly activate the PI3K signaling pathway in the neonatal rat NEC model.

Meanwhile, it has been demonstrated that caspase-9 protease activity and mRNA expression were significantly higher than normal condition, and the expression level rose with time, indicating that hypoxia could up-regulate the gene expression of apoptosis-related protein kinase caspase-9 in rat cadiocytes, and then started apoptosis of rat cadiocytes in a time-effect manner (Wang et al., 2008). It was also found that Caspase-3 protein and Caspase-3 mRNA expression increased 30 min after ischemia, and there were mild cardiocyte apoptosis, but there were no significant differences as compared with the control group (Sasaki et al., 2002). However, the Caspase-3 protein and Caspase-3 mRNA expression were significantly increased compared with the control group and the ischemia group after myocardial ischemical reperfusion injury, and meanwhile the number of apoptotic cells was also significantly elevated, indicating that ischemia could initiate myocardial apoptosis procedure and reperfusion activated Caspase-3 and aggravated cadiocyte apoptosis, leading to more severe apoptosis. In the present study, it was found that Caspase-9 and Caspase-3 activity were

increased in the NEC model group, and further increased in the Wortmannin group, but significantly declined in the ITF group. It was also confirmed that hypoxia could activate apoptosis-related protein kinase Caspase-9 and Caspase-3 inducing the apoptosis of intestinal epithelial cells in the NEC group, and regulate the content of Caspase-9 and Caspase-3 through the PI3K signal transduction pathway.

In conclusion, PI3K and Caspase-9/3 presented opposite changes in intestinal tissues in the NEC modeling groups. However, the PI3K content of intestinal tissues was significantly increased while Caspase-9/3 activity was significantly decreased in the ITF group. PI3K-specific inhibitor wortminin could effectively block the activation of the PI3K/Akt signal transduction pathway, and the Caspase-9/3 activity was significantly elevated in the wortminin group. Thus, ITF might play protective roles in the intestinal mucosa of NEC newborn model rats through the activation of the PI3K/Akt signal transduction pathway, and then the activity of the downstream Caspase-9/3 was inhibited, and the apoptosis pathway with the initiation factor of ITF was inactivated, and the subsequent apoptotic signal transduction was blocked, and enterocyte apoptosis and injury of intestinal mucosa were attenuated in NEC modeling groups.

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