Rapid mitogen-activated protein kinase by basic fibroblast growth factor in rat intestine after ischemia/reperfusion injury

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INTRODUCTION
Previous studies have shown that intestinal ischemia/reperfusion (I/R) injury reduce the expression of endogenous basic fibroblast growth factor (bFGF) in rats, and the intravenous administration of exogenous bFGF could induce the expression of endogenous bFGF and improve the physiological functions of the intestine, lung, kidney, and other internal organs after I/R injury[1-6]. However, the protective mechanisms of bFGF on intestinal I/R injury remain unknown.

Mitogen-activated protein kinase (MAPK) cascade, a cytoplasmic protein kinase that requires dual phosphorylation on specific threonine and tyrosine residues for their activation, can transmit mitogen or differentiation signals from the cell surface into the nucleus, thus regulating the gene expression[7-10]. P42/p44 MAPK, p38 MAPK and stress activated protein kinase/C-Jun N-terminal kinase (SAPK/JNK) are three important members of the MAPK family. The purpose of the present study was to detect the activities of mitogen-activated protein kinase (MAPK) signaling pathway in rat intestine after I/R injury, and to investigate the protective mechanisms of bFGF on intestinal ischemia injury.

METHODS: Rat intestinal I/R injury was produced by clamping the superior mesenteric artery (SMA) for 45 minutes and followed by reperfusion for 48 hours. Seventy-eight Wistar rats were used and divided randomly into sham-operated group (A), normal saline control group (B), bFGF antibody pre-treated group (C), and bFGF treated group (D). In group A, SMA was separated without occlusion. In groups B, C and D, SMA was separated and occluded for 45 minutes, then, released for reperfusion for 48 hours. After the animals were sacrificed, blood and tissue samples were taken from the intestine 45 minutes after ischemia in group A and 2, 6, 24, and 48 hours after reperfusion in the other groups. Phosphorylated forms of p42/p44 MAPK, p38 MAPK and stress activated protein kinase/C-Jun N-terminal kinase (SAPK/JNK) were measured by immunohistochemistry. Plasma levels of D-lactate were examined and histological changes were observed under the light microscope.

RESULTS: Intestinal I/R injury induced the expression of p42/p44 MAPK, p38 MAPK, and SAPK/JNK pathways and exogenous bFGF stimulated the early activation of p42/p44 MAPK and p38 MAPK pathways. The expression of phosphorylated forms of p42/p44 MAPK was primarily localized in the nuclei of crypt cells and in the cytoplasm and nuclei of villus cells. The positive expression of p38 MAPK was localized mainly in the nuclei of crypt cells, very few in villus cells. The activities of p42/p44 MAPK and p38 MAPK peaked 6 hours after reperfusion in groups B and C, while SAPK/J NK peaked 24 hours after reperfusion. The activities of p42/p44 MAPK and p38 MAPK peaked 2 hours after reperfusion in group D and those of SAPK/J NK were not changed in group B. D-lactate levels and HE staining showed that the intestinal barrier was damaged severely 6 hours after reperfusion; however, histological structures were much improved 48 hours after reperfusion in group D than in the other groups.

CONCLUSION: The results indicate that intestinal I/R injury stimulates the activities of MAPK pathways, and that p42/p44 MAPK and p38MAPK activities are necessary for the protective effect of exogenous bFGF on intestinal I/R injury. The protective effect of bFGF on intestinal dysfunction may be mediated by the early activation of p42/p44 MAPK and p38 MAPK signaling pathways.

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MATERIALS AND METHODS

Animal model

Seventy-eight healthy Wistar rats weighing 220±20 g (Animal Center, Academy of Military Medical Sciences, Beijing) were used. All animals were housed in the laboratory and given free access to food and water for 1 week before being used. The animal was under anesthesia by 3 % sodium pentobarbital (40 mg/kg), a middle incision was made. The superior mesenteric artery (SMA) was identified and freed by blunt dissection. A microvascular clamp was placed at the root of SMA to cause complete cessation of blood flow for 45 minutes, and thereafter the clamp was loosened to form reperfusion injury [1, 11]. After 2, 6, 24 and 48 hours reperfusion, the animals were sacrificed and blood samples and intestinal tissue biopsies were taken. Blood samples were centrifuged and serum was frozen to measure plasma D-lactate. Tissue biopsies were fixed with 4 % paraformaldehyde.

In this study, all operations were performed under aseptic conditions. The animal experiments were approved by the local animal management committee.

Experimental design

The animals were randomly divided into four groups: sham-operated (A), normal saline control (B), bFGF monoclonal antibody (Sigma, St. Louis, MO, USA) pre-treated (C) and bFGF (Sigma, St. Louis, MO, USA) treated groups (D). In group A, SMA was freed but without occlusion and blood samples and tissue biopsies were taken 45 minutes after exposure of the SMA. In groups B and D, 0.15 ml saline or 0.15 ml saline plus bFGF (2 g/rat) was injected immediately 45 minutes after SMA occlusion from the tail vein. In group C, 0.15 ml saline plus bFGF monoclonal antibody (25 g/rat) was injected right before SMA occlusion from tail vein for pre-treatment.

Measurement of phosphorylated forms of p42/p44 MAPK, p38 MAPK and SAPK/JNK

Formalin-fixed, paraffin-embedded small intestinal tissues were used to measure the expression of phosphorylated forms of p42/p44 MAPK, p38 MAPK, and SAPK/JNK by immunohistochemistry. Immunohistochemical staining was performed according to the instructions of the PowerVision™ kit (Santa Cruz, USA). Briefly, sections (5 µm) were dewaxed and rehydrated in graded alcohols. Endogenous peroxidase activity was quenched, and antigen retrieval was performed by heating for 20 minutes at 100 °C in 0.01 mol/L sodium citrate. The primary monoclonal antibodies for p42/p44 MAPK, p38 MAPK and SAPK/JNK (Cell Signaling Technology, Inc., USA) were diluted 1:100 in buffer and incubated for 40 minutes at 37 °C. The sections were then incubated with HRP-conjugated secondary antibodies (Santa Cruz, USA) for 20 minutes at 37 °C. Positive expression was detected with diaminobenzidine (DAB) (Sigma, St. Louis, MO, USA). The sections were lightly counterstained with hematoxylin, dehydrated in graded alcohol, and mounted. For negative control, the sections were processed similarly but PBS was used as primary antibodies instead of the MAPKs monoclonal antibodies.

The result of positive staining was semi-quantitatively defined as -, +, ++ and ++++. This was observed under microscope with 10 times eyepiece and 40 times objective. “-” represents no visible positive staining, “+” less than 10 stained cells and “++” 10-30 stained cells, while “+++” represents more than 30 positively stained cells within one high power field.

Measurement of plasma D-lactate

The levels of plasma D-lactate were measured with modified Brandt’s method [12, 13]. Briefly, heparinized blood was centrifuged at 3 200 rpm for 10 min and 2 ml of the plasma was deproteinized with 0.2 ml perchloric acid (PCA) (1/10 vol), mixed and kept in an ice bath for 10 min. The denatured protein solution was centrifuged at 3 200 rpm for 10 min and the supernatant solution was removed. To 1.4 ml of supernatant solution, 0.12 ml KON was added and they were mixed for 20 s. Precipitant KClO₃ was removed by centrifugation at 3 200 rpm for 10 min. The supernatant solution and neutralized-protein-free plasma were used to measure the absorbency at 340 nm. Plasma D-lactate concentration was expressed as mmol/L.

RESULTS

Activities of p42/p44 MAPK and p38 MAPK

Quantitative immunohistochemical results for phosphorylated forms of p42/p44 MAPK and p38 MAPK were evaluated (Tables 1 and 2). The expression of activated p42/p44 MAPK was localized in the cytoplasm and nuclei of villus cells and in the nuclei of crypt cells, mainly in the epithelium and villus cells (Figure 1). Activated p38 MAPK was localized primarily in the nuclei of crypt cells, very few in villus cells (Figure 2). There was a consistent correlation between positive expression levels and the intensity of p42/p44 MAPK and p38 MAPK. The positive expression of p42/p44 MAPK and p38 MAPK was weak in the sham-operated intestinal tissues and ischemic tissues. However, the number of positive staining cells increased with high staining intensity after reperfusion injury. In the normal saline and bFGF antibody pre-treated groups, the number of positive staining cells of p42/p44 MAPK (Figures 1B and C) and p38 MAPK (Figures 2B and C) increased 2 hours after reperfusion, peaked at 6th hours, and decreased from 24 to 48 hours. In the bFGF treated group, however, the number of positive staining cells and the intensity of p42/p44 MAPK and p38 MAPK peaked 2 hours after reperfusion (Figures 1D and 2D) and decreased afterwards, but they were still higher than those in the sham-operated control at 48 hours. Compared with the normal saline and bFGF treated groups, the intensity of p42/p44 MAPK and p38 MAPK positive staining in the bFGF antibody pretreated group was weaker from 2 hours to 48 hours after reperfusion.

Activities of SAPK/JNK

Weak staining of SAPK/JNK was observed in small intestine after I/R injury. Positive staining was localized in the nuclei and cytoplasm of villus and crypt cells. The staining, however, was weak without much difference among the groups (Table 3). The positive staining of SAPK/JNK in bFGF treated group was slightly higher only at 24 hours after reperfusion. Among all the groups, the positive staining of SAPK/JNK was weaker than that of p42/p44 MAPK and p38 MAPK.

Changes of plasma D-lactate levels

Plasma D-lactate levels were measured 2, 6, 24, and 48 hours after reperfusion in all groups. They were elevated 2 hours after reperfusion in all groups, peaked at 6th hour, and decreased to nearly normal 48 hours later (Table 4). The levels at 45 min after ischemia in the sham-operated group were served as controls.
Figure 1 Immunohistochemical staining of phosphorylated p42/p44 MAPK in intestinal biopsies in rats after ischemia/reperfusion injury (SP×200). A: Negative control of p42/p44 MAPK staining. There was no positive expression signal in this group. B: The expression of phosphorylated p42/p44 in intestinal biopsies in the saline control group 2 hours after reperfusion. The activated p42/p44 MAPK expression was localized in the cytoplasm and nuclei of villus cells and in the nuclei of crypt cells, mainly in the epithelium and villus cells. The number of positive cells in this group was more than that in the bFGF antibody pre-treated group. ISH×400.

Figure 2 Immunohistochemical staining of phosphorylated p38 MAPK in intestinal biopsies in rats after ischemia/reperfusion injury (SP×200). A: Negative control of p38 MAPK staining. There was no positive expression signal in this group. B: Phosphorylated p38 MAPK staining in the saline control group 2 hours after reperfusion. Few p38 MAPK positive expression were localized in the cytoplasm and nuclei of villus cells and in the nuclei of crypt cells, mainly in the...
Table 1  Semi-quantitative results of immunohistochemical staining for phosphorylated forms of p42/p44 MAPK in different groups

| Groups   | Pre-injury | 2 hrs post-injury | 6 hrs post-injury | 24 hrs post-injury | 48 hrs post-injury |
|----------|------------|-------------------|-------------------|--------------------|-------------------|
| Group B  | -          | ++                | ++                | +                  | ++                |
| Group C  | -          | +                 | ++                | +                  | +                 |
| Group D  | -          | +++               | +++               | +                  | +                 |

"-" represents no visible positive staining, "+" less than 10 stained cells and "++" 10-30 stained cells, while "+++" represents more than 30 positively stained cells within one high power field.

Table 2  Semi-quantitative results of immunohistochemical staining for phosphorylated forms of p38 MAPK in different groups

| Groups   | Pre-injury | 2 hrs post-injury | 6 hrs post-injury | 24 hrs post-injury | 48 hrs post-injury |
|----------|------------|-------------------|-------------------|--------------------|-------------------|
| Group B  | -          | ++                | ++                | +                  | ++                |
| Group C  | -          | +                 | ++                | +                  | +                 |
| Group D  | -          | +++               | ++                | +                  | +                 |

"-" represents no visible positive staining, "+" less than 10 stained cells and "++" 10-30 stained cells, while "+++" represents more than 30 positively stained cells within one high power field.

Table 3  Semi-quantitative results of immunohistochemical staining for phosphorylated forms of SAPK/JNK in different groups

| Groups   | Pre-injury | 2 hrs post-injury | 6 hrs post-injury | 24 hrs post-injury | 48 hrs post-injury |
|----------|------------|-------------------|-------------------|--------------------|-------------------|
| Group B  | -          | +                 | +                 | ++                 | +                 |
| Group C  | -          | +                 | +                 | +                  | +                 |
| Group D  | -          | +                 | +                 | +                  | +                 |

"-" represents no visible positive staining, "+" less than 10 stained cells and "++" 10-30 stained cells, while "+++" represents more than 30 positively stained cells within one high power field.

Table 4  The changes of plasma D-lactate levels at different time points in three groups (mmol/L) (x±s)

| Groups   | Animal numbers | Control   | 2 hours     | 6 hours     | 24 hours    | 48 hours     |
|----------|----------------|-----------|-------------|-------------|-------------|-------------|
| Group B  | 24             | 0.332±0.132| 0.372±0.090| 0.397±0.096| 0.463±0.147| 0.511±0.179|
| Group C  | 24             | 0.332±0.132| 0.309±0.079| 0.327±0.098| 0.415±0.177| 0.425±0.208a|
| Group D  | 24             | 0.332±0.132| 0.369±0.124| 0.407±0.089| 0.475±0.128| 0.537±0.098|

*P<0.05 vs compared with control.

epithelium and villus cells. C: P38 MAPK staining in the bFGF antibody pre-treated group. The number of positive cells and localization of p38 MAPK positive cells were similar with those in the saline group. D: Phosphorylated p38 staining in the bFGF treated group 2 hours after reperfusion. Activated p38 MAPK was localized primarily in the nuclei of crypt cells, few in villus cells. The number of positive cells was more than that in the saline control and bFGF antibody pre-treated groups. In the bFGF treated group, the number of positive expression cells of p38 MAPK as well as its intensity peaked 2 hours after reperfusion.

**Histological evaluation**

Intestinal I/R injury resulted in the damage of intestinal barrier and the increase of mucosal permeability. HE staining showed partial loss of the mucosa 2 hours after reperfusion. 6 hours after reperfusion, however, the damage of intestinal epithelial cells, hemorrhage and necrosis were observed and accompanied by inflammatory cell infiltration into the intestinal wall. Histological structure of the intestinal mucosa was markedly improved after administration of bFGF.

**DISCUSSION**

Intestinal I/R injury causes release of bacteria and toxin from the gut into the host blood circulation and changes of inflammatory factors, cytokines and growth factors, resulting in damage to the intestinal barrier and other internal organs[1-3,13-17]. We found that administration of exogenous basic fibroblast growth factor (bFGF) could reduce the intestinal injury caused by I/R insult. However, the mechanisms of this protective effect of bFGF are not elucidated. BFGF is expressed in many normal adult tissues and has mitogenic activity in a wide variety of cells of mesenchymal, neuronal, and epithelial origins, and regulates events in normal embryonic development, angiogenesis, wound repair, and neoplasia[18-20]. Also, it can regulate migration and replication of intestinal epithelial cells in culture[21]. Recent studies have shown that L-glutamine, tumor necrosis factor-α, and epidermal growth factor (EGF) stimulate proliferation of intestinal crypt cells by activating the MAPK pathway, and that p42/p44 MAPK activities are necessary for both cell cycle progression and differentiation of the intestinal cells[22-25]. In many other cell types, growth factor controls proliferation and differentiation.
via the MAPK pathway. MAPK is a common signal pathway to transmit the mitogen or the differentiating signals from the cell surface to the nucleus, and thus ultimately regulates different gene expression.[26-30] Hence, we hypothesized that MAPK activation might be involved in the regulation of bFGF signals in the process of intestinal barrier repair.

To investigate this hypothesis, we evaluated changes of the activated MAPK signal pathway after administration of bFGF and bFGF antibodies. We found that intestinal I/R injury stimulated the activities of phosphorylated forms of the p42/p44 MAPK and p38MAPK pathways, and increased the SAPK/JNK activity slightly. p42/p44 MAPK and p38MAPK activities were increased 2 hours after reperfusion, and peaked at 6 hours. At the same time, the levels of SAPK/JNK increased slightly 24 hours after reperfusion compared with those of the normal control. Phosphorylated forms of p42/p44 MAPK were mainly localized in the nuclei of crypt cells and in the cytoplasm and nuclei of villus cells, whereas those of p38MAPK were primarily localized in the nuclei of crypt cells, few in villus cells. After administration of bFGF, the expression of both p42/p44 MAPK and p38MAPK was quickly stimulated, and the activation of both p42/p44 MAPK and p38MAPK peaked 2 hours after reperfusion, declined gradually to normal at 48 hours. A coherence was noted between the changes of p42/p44 MAPK and p38MAPK and histological findings. These results indicate that intestinal I/R injury induces the activities of the MAPK pathways, and p42/p44 MAPK and p38MAPK activities are necessary for the protective effects of exogenous bFGF on intestinal I/R injury. The early stimulation of the p42/p44 MAPK and p38MAPK signal pathways may mediate the protective effects of bFGF on intestinal dysfunction.

MAPK family is composed of “extracellular signal regulated” p42/p44 MAPK, “stress-regulated” MAPK (SR-MAPKs), stress-activated protein kinases (SAPKs), c-Jun N-terminal kinases (JNKs) and p38-MAPKs. On stimulation, MAPKs are translated into the nucleus where they may phosphorylate nuclear transcription factors and thus regulate gene expression. The four principal differentiated cell lineages of intestinal epithelium are derived from common multipotent stem cells located near the base of each crypt. These crypt stem cells divide to produce daughter stem cells as well as more rapidly replicating transit cells, which in turn undergo 4–6 rapid cell divisions in the proliferative zone located in the lower half of each crypt.[31,32] Factors determining whether cells continue to proliferate, cease dividing, and begin to differentiate, appear to operate during the first gap phase (G1) of the cell cycle. P42/p44 is activated during G0 to G1 transition, and the activity remains elevated up to S phase entry, implicating this family of protein quinches in the control of G1 progression.[33,34] Activation of p42/p44 MAPK is also necessary for growth factor-dependent proliferation of some cell lines.

We propose the possible mechanisms of the protective effects of bFGF on intestinal I/R injury be involved in the activation of MAPK pathway. First, to protect the survival of intestinal stem cells within crypt and mediate the proliferation and differentiation of these cells. Intestinal epithelium is maintained by continuous and rapid replacement of differentiated epithelial cells by replication of undifferentiated epithelial cells. Exogenous bFGF markedly enhances the survival of crypt stem cells before and after irradiation injury.[35,36] Microvascular endothelial apoptosis is the primary lesion leading to stem cell dysfunction, while endothelial apoptosis could be inhibited by intravenous bFGF.[37,38] Second, to regulate the inflammation reactions after I/R injury. The TNF translation by IL-10 is inhibited mainly by inhibiting the activation of the p38 MAPK pathway.[39] This is necessary for maintenance of immune homeostasis in the gut.

In the perfused heart, ischemia/reperfusion activates stress-regulated MAPKs, direct pharmacological activation of p38 triggers delayed preconditioning of the heart, and there is minimal activation of the p42/p44 MAPK subfamily by heart I/R injury.[35-37] Yet phosphorylation of p42/p44 MAPK occurs consistently in the grey matter penumbra of brain tissue after ischemic stroke, and may be associated with neuronal survival and/or angiogenic activity in the recovering brain tissue.[38] The results indicate that the MAPK pathways respond differently to ischemic injury in different sites.

The changes of serum D(-)-lactate were used as a predictor of intestinal I/R injury in this study. D(-)-lactate is the stereosomer of mammalian L(+)-lactate. Mammalian tissue does not produce D(-)-lactate and only slowly metabolizes it. It is a strict product of bacterial fermentation. Since mammals do not possess the enzyme systems to rapidly metabolize D(-)-lactate[39,40], the released D(-)-lactate will pass through the gut barrier and liver in an unchanged form and appear in the peripheral blood. As intestinal ischemia injury causes mucosal injury and subsequent bacterial proliferation, D(-)-lactate is released from gut into the circulation. In this study, the serum D(-)-lactate level was increased after injury, but in the bFGF treated group, it was not significantly increased as in the control group, indicating that bFGF exerts a positive protective effect on the mucosal barrier and decreases the intestinal permeability.

In summary, intestinal I/R injury induces the activities of the MAPK pathways, and p42/p44 MAPK and p38MAPK activities are necessary for the protective effect of exogenous bFGF on intestinal I/R injury. The protective effect of bFGF on intestinal dysfunction may be mediated by the early stimulation of the p42/p44 MAPK and p38 MAPK signaling pathways.

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