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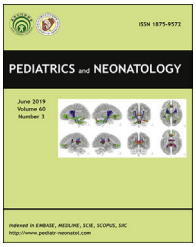

Original Article

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) protected intestinal ischemia-reperfusion injury through JNK and p38/MAPK-dependent pathway for anti-apoptosis

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anti-apoptotic effects;
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Background: Heparin-Binding Epidermal Growth Factor-Like Growth Factor (HB-EGF) is a potent cytoprotective factor in various body systems, including gastrointestinal tract. In this study, we intended to examine whether HB-EGF exerts its protective effects through MAPK dependent anti-apoptosis after intestinal I/R injury.
Methods: We randomly divided 30 laboratory 30 rats into 5 groups: (A) normal control group, (B) ischemia group with normal saline, (C) I/R group with normal saline, (D) ischemia group with HB-EGF (400 ug/kg), and (E) I/R group with HB-EGF (400 ug/kg). With Western blotting study, we determined JNK and p38/MAPK pathway and caspase-3 activity protein levels using Western analyses.
Results: The JNK phosphorylation protein levels increased after intestinal ischemia or intestinal reperfusion phase, and HB-EGF pre-treatment was significantly decreased in JNK

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phosphorylation protein levels ($p < 0.01$). We found that p38 protein levels was increased after intestinal reperfusion phase, and that HB-EGF pre-treatment significantly decreased p38 protein levels ($p < 0.01$). The expression protein level of caspase 3 was increased after intestinal ischemia or intestinal reperfusion phase. HB-EGF pre-treatment significantly decreased Caspase 3 proteins. ($p < 0.01$).

Conclusion: Our study revealed that pre-treatment of HB-EGF decreased the amount of activity of JNK and p38/MAPK pathway and caspase-3 protein after intestinal I/R injury. These results may further support that the cytoprotective of HB-EGF after I/R injury could be through anti-apoptotic effect of activity of JNK and p38/MAPK pathway.

1. Introduction

Intestinal ischemic and reperfusion (I/R) injury is known to be a key factor to cause various clinical conditions, such as midgut volvulus, neonatal necrotizing enterocolitis, intestinal transplantation, and hemorrhagic shock in resuscitation.[1] When the organs are exposed to I/R stress, various responses such as the proliferation or apoptosis of the cells can occur.[2] The molecular events in intestinal mucosal—repair processes after I/R injury are complex, involving many transcriptional factors to activate and/or inactivate relevant genes. The members of the mitogen-activated protein kinase (MAPK) families are stress-associated with a broad spectrum of cellular behaviors in response to extracellular signals.[3] In mammals, MAPKs are divided into three major groups—ERK, c-Jun N-terminal kinase (JNK), and p38—depending on their degree of homology, biological activities, and phosphorylation motifs.[4] ERK signaling involves cell survival properties that are initiated by such stimuli as hypoxia and growth factor withdrawal, while JNK and p38 MAPK have distinct apoptotic properties through various cellular stress.[5]

The regulation of phosphorylated JNK is associated with promoting apoptosis, whereas p38 phosphorylation is linked to inducing apoptosis.[6] Heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) has been identified initially as a product of cultured human macrophages. It promotes cell survival and protects cell hypoxia and intestinal ischemia/reperfusion injury.[7,8] Recent studies revealed that HB-EGF can reduce the incidence of NEC in a neonatal rat model in part through decreasing apoptosis.[9] Our previous study revealed that the protective role of an anti-apoptotic effect of HB-EGF was through down-regulating AP-1 factor.[10] In this study, we intended to examine whether the protective role of HB-EGF was through the mechanism of JNK and p38/MAPK-dependent anti-apoptosis.

2. Methods

2.1. Experimental protocol

The study protocol was approved by the animal research and ethics committee at Chang Gung Memorial Hospital. Sprague—Dawley adult rats (200—300 g), aged 8—12 weeks, were anesthetized with ketamine hydrochloride at a dose of 40 mg/kg intraperitoneally. The midline laparotomy was done, the small intestine was exposed to the left, and a 10-cm segment of the distal isolated ileal loop was created. We divided the marginal vessels for complete separation of the vascular distribution. The mesentery to isolated loop was occluded for 30 min in experimental animals using a micro-bulldog clamp. At the end of the ischemic period, we released the clamp and the bowel became pink in color. Animal preparations and methods of surgery have been described in detail where.[11]

We randomly divided 30 rats into 5 groups: (A) the normal control group (n = 6) with sham operation; (B) the disease group including ischemia group (n = 6), receiving vascular occlusion for 30 min with normal saline; (C) the I/R group (n = 6), receiving vascular occlusion for 30 min and reperfusion for 30 min with normal saline; (D) the disease experimental group including ischemia group with HB-EGF (400 μg/kg, n = 6); and (E) I/R group with HG-EGF (400 μg/kg, n = 6).

2.2. Collection of samples

We used cotton swabs and flushed them with cold (4 °C) normal saline to remove luminal contents. The specimens were blotted dry, weighed, frozen in liquid nitrogen, and stored at −80 °C till subsequent protein extraction.

2.3. Western blotting

Frozen rat intestinal tissues were ground up using a mortar and pestle with liquid nitrogen (or homogenized) and lysed in PRO-PREPTM Protein Extraction Solution (iNtRON Biotechnology, Krikland, Washington, USA) according to the manufacturer's instructions. We quantified proteins with Bradford analysis. Tissue lysates (total protein 50 ug) were separated by 10% SDS-PAGE and transferred to PVDF membrane (Millipore Corporation, Billerica, Massachusetts, USA).

Membranes were blocked with 5% non-fat milk in TBS-T buffer (150 mM NaCl, 10 mM Tris/pH 8.0, and 0.05% Tween 20) at room temperature for 1 h. Then the membranes were immunoblotted with primary antibodies (JNK phosphorylation p38 protein and caspase protein 1: 1,000, GAPDH 1:

50,000, Chemicon, Millipore Corporation) overnight at 4 °C, followed by incubation with secondary antibodies for 1 h at room temperature (AP124P, Chemicon, Tokyo, Japan). Blots were visualized using chemi-luminescence ECL system (Millipore Corporation) and the Western blot data were quantified using BIO-PROFIL BIO-1D++ software. We measured the intensity of each band standardized with internal control gene (GAPDH) to show the sequential patterns of relative expression.

2.4. Statistical analysis

Results were expressed as mean ± standard deviation (n ± SD). The results of the protein levels of the JNK phosphorylation, p38 and Caspase 3 protein in different groups were analyzed using repeated measures of analysis of variance (ANOVA) with post hoc comparison (Dunn's test).

All statistical analyses were done using the Statistical Analytic System Version 9.3 (SAS Institute, Cary, North Carolina, USA). The differences between groups were considered significant if p values were smaller than 0.05.

3. Results

Fig. 1 shows the protein levels of JNK in the ischemia and I/R groups in Western blotting study. HB-EGF pretreatment resulted in significantly decreased JNK phosphorylation in ischemia group compared to that in the I/R group ($p < 0.01$).

Fig. 2 depicts the protein level of p38 in the ischemia and the I/R group in Western blotting study. The expression of p38 protein level was not significantly increased in the

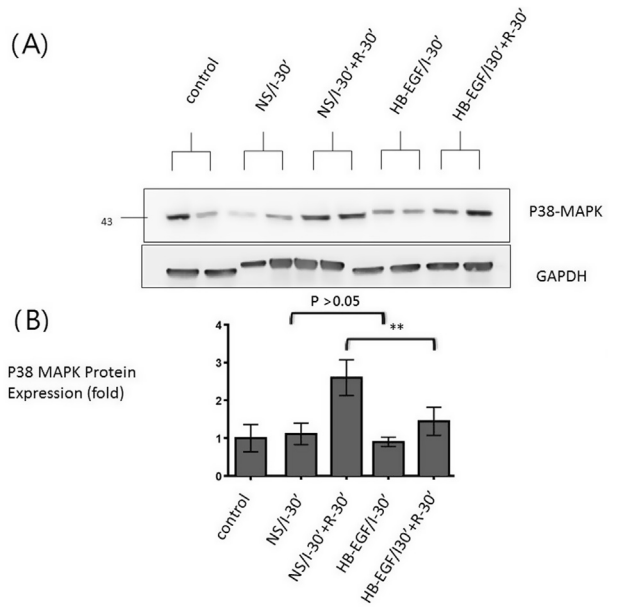

Figure 2 A: Representative Western blots for the effect of I/R injury and pretreatment of HB-EGF on the protein expression of p38 phosphorylation. The protein level of p38 was strongly detected in I/R group and mildly detected in ischemia group. HB-ECF administration decreased p38 protein level in ischemia and I/R group. **B:** Bar graph shows mean data of protein densitometries computed as the ratio of protein to GADPH. Significant difference existed between I/R group with and without HB-EGF pretreatment (**$p < 0.01$). Significant difference was seen between ischemia group without and with HB-EGF pretreatment ($p > 0.05$).

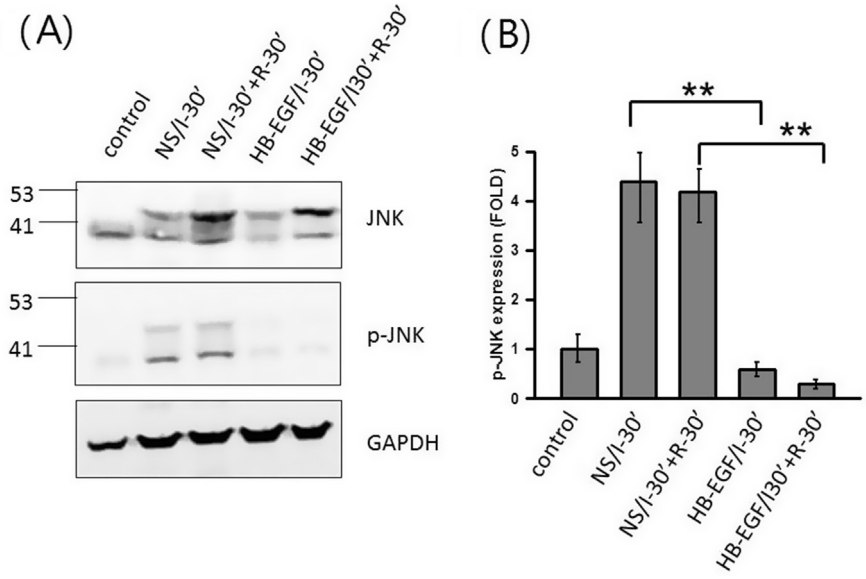

Figure 1 A: Representative Western blots for the effect of I/R injury and pretreatment of HB-EGF on the protein expression of JNK phosphorylation. The protein level of JNK phosphorylation was strongly detected in ischemia and I/R group. HB-ECF administration decreased JNK phosphorylation protein levels in ischemia and I/R group. **B:** Bar graph shows mean data of protein densitometries computed as the ratio of protein to GADPH. **$p < 0.01$, relative to the comparison between ischemia and I/R group without and with HB-EGF pretreatment.

ischemia group. HB-EGF pretreatment showed that p38 protein level was significantly decreased compared with the ischemia group ($p > 0.01$).

Fig. 3 demonstrates the expression of caspase 3 protein levels were both strongly detected in the ischemia and I/R groups in Western blotting study. HB-EGF pretreatment revealed that caspase-3 protein levels were significantly decreased compared with the ischemia and I/R group ($p < 0.01$).

4. Discussion

The exact mechanism of hypoxia-induced intestinal damage is not clear. It has been reported that damage in ischemia-induced tissue is milder compared to that after reperfusion. Parks and Granger[1] suggested that a greater part of intestinal mucosal damage was caused by cytotoxic oxidants formed after reperfusion. Intestinal I/R injury may cause barrier failure and bacterial translocation, resulting in developing multiple organ failure, allowing bacteria or endotoxin of the gastrointestinal tract to reach the systemic and portal circulations. The molecular events underlying intestinal mucosal—repair processes after I/R injury are complex, involving multiple transcriptional factors to activate and inactivate relevant genes. Recent studies reported that cellular responses of proliferation or apoptosis are correlated to the expression profiles of c-Fos and c-Jun in the rat liver I/R injury model.[12] Our previous study revealed that post-ischemic and early reperfusion injury of intestinal tissue is closely related to the expression patterns of c-Fos, whereas late reperfusion injury is closely to that of c-Jun.[2]

The principal of the signal transduction molecules is thought to involve in cell death from reactive oxygen species through MAPK pathway. The MAPK signaling cascades regulate various cellular activities, including cell growth, differentiation, survival, and death.[6,13] In mammals, MAPKs are divided into three major groups: ERK, c-Jun N-terminal kinase (JNK) and p38, depending on their degrees of homology, biological activities, and phosphorylation motifs.[3] The biological effects of MAPK signaling are through phosphorylation of downstream substrates, most notably many signal-responsive transcription factors.

A previous report mentioned that ERK signaling is involved in cell survival properties that are activated through hypoxia and growth factor withdrawal, while JNK and p38 MAPK have distinct apoptotic properties through various cellular stress.[5] Because apoptotic properties were used in our study, we studied only JNK ($p < 0.01$, Fig. 1) and p38 ($p < 0.01$, Fig. 2), but not ERK which was not studied.

Heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) has been initially identified as a product of cultured human macrophages,[14] which has been subsequently found to be a member of the EGF family. HB-EGF is synthesized as a membrane-anchored precursor protein composed of 208 amino acids, which is enzymatically cleaved to yield a 14—20 kDa soluble growth factor. HB-EGF plays an important role in maintaining gastrointestinal integrity.[8] Recent data showes that HB-EGF decreases reactive oxygen species production in the intestine,[15] reduces intestinal I/R-induced inflammation,[16] and decreases apoptosis in intestinal epithelial cells.[8]

HB-EGF has a protective role through mitogenic, chemoattractant, and anti-apoptotic function. The exact mechanism of the protective effect remains to be elucidated. Recent reports also showed that the mechanism of HB-EGF induces proliferation in intestinal cells was mediated through MAPK pathway and cyclin D1.[17] HB-EGF can excite the MAPK pathway, resulting in inactivating caspase-3

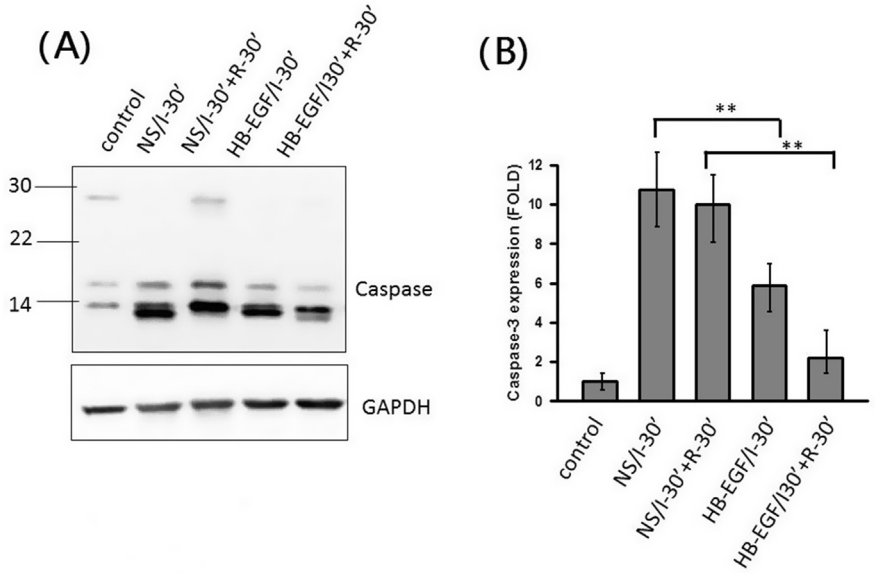

Figure 3 A: Representative Western blots for the effect of I/R injury and pretreatment of HB-EGF on the protein expression of caspase-3. The protein level of caspase 3 was strongly detected in ischemia and I/R group. HB-EGF administration decreased Caspase 3 protein levels in ischemia and I/R group. **B:** Bar graph shows mean data of protein densitometries computed as the ratio of protein to GADPH. Significantly difference was seen between ischemia and I/R group without and with HB-EGF pretreatment (**$p < 0.01$).

to prevent apoptosis induced by ethanol in human embryonic stem cells.[18] In our study, we found that pretreatment of HB-EGF significantly decreased JNK phosphorylation level ($p < 0.01$, Fig. 1), significantly decreased p38 level ($p < 0.01$, Fig. 2) and significantly decreased caspase-3 protein level ($p < 0.01$, Fig. 3).

5. Study limitations

The results of the study show contradictory findings, the data of active phosphorylation of JNK and caspase-3 strongly support our experiment, but p38 has against by ischemia data. We were not able to ascertain the reasons for the different findings of p38 MAPK from those of JNK phosphorylation and caspase-3. The authors suggest that JNK and p38 MAPK have different apoptotic properties to face various of cellular stresses including ischemia. NK phosphorylation could promote apoptosis while p38 MAPK is associated with inducing apoptosis.

6. Summary

The data presented in this report revealed that pretreatment of HB-EGF decreased the protein levels of JNK phosphorylation, p38 and caspase −3 that had been induced by intestinal I/R injury. These results may further support that the HB-EGF can protect intestinal I/R injury through JNK and p38/MAPK pathway-dependent anti-apoptosis.

Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgments

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