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The role of epidermal growth factor in prevention of oxidative injury and apoptosis induced by intestinal ischemia/reperfusion in rats

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ABSTRACT

Intestinal ischemia/reperfusion is a major problem which may lead to multiorgan failure and death. The aim of the study was to evaluate the effects of epidermal growth factor (EGF) on apoptosis, cell proliferation, oxidative stress and the antioxidant system in intestinal injury induced by ischemia/reperfusion in rats and to determine if EGF can ameliorate these toxic effects. Intestinal ischemia/reperfusion injury was produced by causing complete occlusion of the superior mesenteric artery for 60 min followed by a 60-min reperfusion period. Animals received intraperitoneal injections of 150 μg/kg human recombinant EGF 30 min prior to the mesenteric ischemia/reperfusion. Mesenteric ischemia/reperfusion caused degeneration of the intestinal mucosa, inhibition of cell proliferation, stimulation of apoptosis and oxidative stress in the small intestine of rats. In the ischemia/reperfusion group, lipid peroxidation was stimulated accompanied by increased intestinal catalase and glutathione peroxidase activities, however, glutathione levels and superoxide dismutase activities were markedly decreased. EGF treatment to rats with ischemia/reperfusion prevented the ischemia/reperfusion-induced oxidative injury by reducing apoptosis and lipid peroxidation, and by increasing antioxidant enzyme activities. These results demonstrate that EGF has beneficial antiapoptotic and antioxidant effects on intestinal injury induced by ischemia/reperfusion in rats.

Introduction

Ischemia/reperfusion of the gut in humans is a serious condition occurring after trauma, burns, septic shock, and liver or small intestine transplantation. Ischemia/reperfusion-induced intestinal injury in experimental animals is a practical model commonly used in studies on pathogenesis of systemic inflammation, respiratory failure and multiple organ failure. Experimental intestinal ischemia/reperfusion is one of the important models used to investigate oxidative stress induced by reactive oxygen species especially during reperfusion in small intestine (Horton and Walker, 1993; Nilsson et al., 1994; Kacmaz et al., 1999). Intestinal ischemia/reperfusion damages the mucosa by impairing its barrier function, and by causing bacterial translocation (Biffl and Moore, 1996; Wu et al., 2004). Various mediators play a role in the pathogenesis of ischemia/reperfusion-induced intestinal injury including reactive oxygen/nitrogen species, proinflammatory cytokines, leukocyte adhesion and infiltration (Nilsson et al., 1994; Xia et al., 2002; El Assal and Besner, 2004). Recent evidence has indicated that apoptosis is increased significantly during ischemia/reperfusion of the gut and may play a key role in the pathogenesis of ischemia/reperfusion-induced intestinal injury. The intestinal ischemia/reperfusion model has often been used to study apoptosis (Shah et al., 1997; Ikeda et al., 1998; Noda et al., 1998; Fujise et al., 2006). Although intestinal ischemia/reperfusion does not have a specific treatment, several antioxidants and monoclonal antibodies against adhesion molecules have shown to be protective against ischemia/reperfusion injury (Kacmaz et al., 1999; Kubes, 1999; Kazez et al., 2000).

Epidermal growth factor (EGF) is a mitogenic peptide that is secreted into the lumen of the duodenum by Brunner's glands. EGF is implicated in the regulation of a wide variety of physiological processes, including growth, cell proliferation, regeneration, differentiation, and wound repair. It is suggested that EGF, not only increases mucosal repair, but also behaves as a cytoprotective and trophic agent for gastrointestinal epithelium (Buret et al., 1998; Berlanga et al., 2002). Many in vivo or in vitro studies have demonstrated that members of the EGF family are among the important factors for healing after damage in diverse experimental models (Pillai et al., 1999; Michalsky et al., 2001; Xia et al., 2002; Jahovic et al., 2004; Martin et al., 2005; Clark et al., 2005; El-Assal et al., 2007, 2008). However, there are only a limited number of studies regarding the effects of EGF on apoptosis and on the antioxidant system in ischemia/reperfusion-induced intestinal injury. In this

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study, we aimed to investigate the role of EGF on apoptosis, cell proliferation, oxidative stress and antioxidant system in intestinal injury induced by ischemia/reperfusion in rats.

Materials and methods

Animals

Two- to 2.5-month-old Sprague–Dawley male rats weighing 220–240 g ($n = 39$) obtained from DETAE (Experimental Medical Research Institute of Istanbul University) were used in the study. The experiments were reviewed and approved by the Animal Care and Use Committee of Istanbul University. The animals were fed with Purina Laboratory Rodent Diet 5001 and tap water *ad libitium*, but fasted overnight prior to the experiments.

Induction of ischemia/reperfusion injury

The rats were anesthetized with ketamin (100 mg/kg) (Ketalar, Pfizer, Istanbul, Turkey) and chlorpromazine (0.75 mg/kg) (Largactil, Eczacıbaşı, Istanbul) intraperitoneally. After induction of anesthesia, the abdomen was opened through a midline abdominal incision. Intestinal ischemia/reperfusion (I/R) injury was produced by causing complete occlusion of the superior mesenteric artery followed by a period of reperfusion. The superior mesenteric artery was clamped for 60 min. After 60 min of ischemia, the vascular clamp at the superior mesenteric artery was removed and three drops of 2% lidocaine (Aritmal, Osel, Istanbul, Turkey) were applied directly on the superior mesenteric artery to facilitate reperfusion. Blood circulation was restarted for a 60-min reperfusion period (Noda et al., 1998). At the end of the reperfusion period, the animals were killed by an overdose of anesthesia.

Experimental protocol

Animals were selected randomly and divided into five groups.

Group I (Sham): Sham-operated animals. These animals were subjected to abdominal incision and their organs exposed for 120 min, but without clamping of the mesenteric artery so as to distinguish the differences between the effects of intestinal I/R and the changes because of non-specific surgical stress ($n = 7$); **Group II** (Control): Control rats received i.p. injection of 10 mM acetic acid with 0.1% BSA (vehicle) ($n = 7$); **Group III** (EGF): Animals administered i.p. injection of 150 μg/kg human recombinant EGF (Sigma-Aldrich, St. Louis, MO, USA; E 9644) ($n = 7$); **Group IV** (I/R): Rats received an i.p. injection of 10 mM acetic acid with 0.1% BSA (vehicle) 30 min prior to the intestinal I/R ($n = 9$); **Group V** (I/R + EGF): Animals injected intraperitoneally (i.p.) with 150 μg/kg human recombinant EGF dissolved in 10 mM acetic acid solution with 0.1% BSA 30 min prior to the intestinal I/R ($n = 9$).

The animals belonging to groups II, III, IV and V, to which EGF or vehicle were applied, were killed 2.5 h after the injections. The control rats in group I were killed 2 h after the sham operation in order to supply the same conditions as with group IV. At the end of the experiments, samples from the jejunum were taken from the animals for all examinations.

Histological assessment of intestinal injury

Samples from the jejunum were taken from the animals for histological examination. The tissues were fixed in Bouin's solution for 24 h at room temperature and embedded in paraffin wax. Serial sections 5 μm thick were cut and stained with Masson's trichrome for general morphological evaluation and the detection of connective tissue. Periodic acid-Schiff (PAS) staining was performed to observe the changes in the goblet cells and brush border of villi. The sections obtained from different groups were examined under an Olympus CX 41 light microscope, and photomicrographs were recorded using an Olympus DP71 digital camera.

TUNEL assay

In order to detect apoptotic cells, in situ labeling of the fragmented DNA generated by apoptosis associated endonucleases was performed with terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling (TUNEL) assay in the paraffinized cross-sections of jejunum. Tissue parts taken from the jejunum were fixed in 10% phosphate-buffered formalin. Paraffin sections at 5 μm were dewaxed, rehydrated, and then treated with proteinase K (20 μg/mL), and blocked for endogenous peroxidase activity with 3% hydrogen peroxide (H_2O_2). The labeling procedure was performed with the commercially available ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA, USA; S7101) according to the manufacturer's instructions. The apoptotic cells were made visible with 3,3'-diaminobenzidine (DAB) and the nuclei were counterstained with methyl green. Rat breast tissue taken 4 days after breast-feeding cessation was used as a positive control. For negative control, the TdT enzyme was replaced by phosphate-buffered saline. A dark brown nuclear staining was regarded as positive. Besides, the apoptotic bodies were also positively stained. TUNEL positive epithelial cells in villi and crypts were evaluated for each sample using an Olympus CX41 light microscope using a 40× objective. A minimum of 20 full crypts or 10 full-length villi were randomly selected for apoptotic index analysis in the sections of jejunum. The apoptotic index values were expressed as a percentage of apoptotic cells in the total cells counted in villi or crypts.

Active caspase-3 staining

The major executioner caspase involved in apoptosis is caspase-3, and activation of caspase-3 is one of the hallmarks of apoptosis. We examined apoptosis by using a polyclonal antibody to active caspase-3. Immunohistochemical active caspase-3 staining was performed using the streptavidin-biotin-peroxidase method. Jejunum tissues were fixed in 10% phosphate-buffered formalin. Following dewaxing and rehydration of paraffin-embedded sections, to facilitate antigen retrieval sections were permeabilized with 0.3% Triton X-100 for 10 min and then were heated in 10 mM citrate buffer (pH 6.0) for 15 min in a microwave oven at 700 W. Endogenous peroxidase activity was blocked by incubating with 3% H_2O_2 for 10 min. The labeling procedure was performed with the Histostain Plus Broad Spectrum Kit (Invitrogen, Zymed Laboratories, San Francisco, CA, USA), followed by the application of polyclonal rabbit anti-active caspase-3 (1:50 in PBS, Millipore, Billerica, MA, USA; AB-3623) for 60 min at room temperature. Slides were then incubated with 3-amino-9-ethylcarbazole, and counterstained with Mayer's hematoxylin. For negative control, phosphate-buffered saline was used instead of antibody. Cytoplasm with dark red staining was assessed as positive. Quantitative analysis of caspase-3 immunoreactive epithelial cells was made by using an Olympus CX41 light microscope with a 40× objective. For the labeling index, 20 full crypts or 10 full-length villi were randomly selected in the jejunum sections of each sample and caspase-3 positive cells were counted. The caspase-3 labeling index was calculated as a percentage of caspase-3 positive epithelial cell in the total cells counted in the crypts for each animal.

Proliferating cell nuclear antigen assay

The proliferating cells in intestine sections were detected by proliferating cell nuclear antigen (PCNA) immunohistochemistry.