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Research Paper

The role of erythropoietin in the protection of gastric mucosa from indometacin-induced gastric injury and its relationship with oxidant and antioxidant parameters in rats

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Abstract

Objectives Erythropoietin has anti-oxidative and anti-inflammatory activity. We wanted to evaluate its activity in preventing damage to the gastric mucosa.

Methods We examined the protective effect of erythropoietin on indometacin-induced gastric mucosa damage in the rat stomach and compared its potency with that of famotidine. We also measured effects on oxidant and antioxidant parameters in the rat stomach.

Key findings Famotidine and erythropoietin 2500 and 5000 IU/kg reduced the ulcer area by 98%, 31% and 58%, respectively, compared with the indometacin group. Superoxide dismutase activity and glutathione level were decreased and myeloperoxidase activity increased in the indometacin group compared with healthy rats. Famotidine and erythropoietin at all doses increased superoxide dismutase and glutathione levels significantly compared with the indometacin group. Myeloperoxidase activity was decreased by erythropoietin and famotidine.

Conclusions These results support the view that erythropoietin counteracts the effects of indometacin in inducing gastric ulcer and could be used as a an antiulcer compound. Its antiulcer effect is less potent than that of famotidine. The antiulcerogenic effects of erythropoietin may be related to its intrinsic ability to sustain the activities of free-radical scavenging enzymes and the bioavailability of glutathione.

Keywords antioxidants; erythropoietin; indometacin; rat; ulcer

Introduction

Peptic ulcer is a common clinical problem and is associated with significant patient morbidity and management costs. Peptic ulcers contribute to the majority of complications in the elderly and are accounted for by the increasing use of non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs such as indometacin have anti-inflammatory, antipyretic and analgesic effects, and are widely used to treat arthritic diseases; however, they also have side-effects, including gastrointestinal irritation and erosion, mediated through the inhibition of prostaglandin biosynthesis.[1] Indometacin is well known to activate polymorphonuclear granulocytes, and a decreased prostaglandin level impairs almost all aspects of gastroprotection and results in gastrointestinal damage,[2] including bleeding, ulceration and perforation in both animal and human models.[3] Other processes responsible for the gastric damage induced by indometacin include the infiltration of polymorphonuclear leucocytes,[4] the induction of apoptosis and expression of pro-inflammatory tumour necrosis factor-α[5–7] and nitric oxide (NO) imbalance.[7,8] However, recent studies have suggested that reactive oxygen species (ROS) also play a vital role in the gastric damage induced by indometacin.[9] ROS levels are increased during the reduction of oxygen and can subsequently lead to cell injury and also play a role in gastric injury. Indometacin has a pro-oxidant activity by increasing lipid peroxidation and decreasing glutathione peroxidase activity, thereby interfering with the endogenous antioxidant systems of mucosal cells.[10] Cells have developed several endogenous

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antioxidant systems to control the destructive potential of ROS, including enzyme systems such as superoxide dismutase (SOD), catalase, glutathione (GSH) peroxidase and gastric peroxidases.[11] Treatment with SOD and catalase inhibits the gastric lesions caused by ROS.[10,12,13] Alternative non-toxic antioxidants with potent anti-ulcer activity were therefore sought and numerous products have been evaluated as therapeutics for the treatment of gastric damage.

The haematopoietic cytokine erythropoietin is produced by the kidneys in response to hypoxia and stimulates erythroid progenitor cells to increase the number of mature red blood cells, thereby increasing their oxygen-carrying capacity.[14] Clinical studies have suggested that erythropoietin has important roles in the renal system[15] and the nervous system.[16] More recent studies have shown that treatment of animals with erythropoietin can protect the ischaemic and infarcted heart by inhibiting apoptosis.[17] Erythropoietin plays roles in the modulation of intracellular calcium metabolism[18] and the attenuation of NO production[19] and is also reported to exert anti-oxidant[20] and anti-inflammatory activities,[21] but its gastroprotective activity has not been elucidated.

In the present study, we examined the protective effect of erythropoietin on indometacin-induced gastric mucosal damage in the rat stomach and compared its potency with that of famotidine, a histamine H_2-receptor antagonist. We also investigated the effect of erythropoietin on oxidant and antioxidant parameters in the rat stomach to determine the mechanism of its protective effect.

Material and Methods

Animals

Male Wistar rats (180–200 g) were obtained from the Medical Experimental Research Centre of Ataturk University. The animals were housed under normal conditions (22°C) in separate groups. The animal experiments were performed in accordance with national guidelines for the use and care of laboratory animals and were approved by the local animal care committee of Ataturk University.

Chemicals

All of the chemicals used in the laboratory experimentation were purchased from Sigma Chemical Co. (Munich, Germany). Indometacin was from Deva Holding, erythropoietin from Santa Farma İlaç Sanayi A.Ş. and thiopental sodium from Organon & Schering Plough (all Istanbul, Turkey).

Effects of erythropoietin on indometacin-induced gastric ulcers

In this experiment, the gastroprotective effects of erythropoietin on indometacin-induced gastric damage were compared with those of the H_2-receptor blocker, famotidine.[22] The rats were divided into six groups of six and were fasted for 24 h before the experiments. Erythropoietin, 2500, 5000 and 10 000 IU/kg was administered by intraperitoneal injection; famotidine 20 mg/kg was administered orally. Control rats received the same volume of drug vehicle (distilled water). Five minutes after the drug was administered, all rats received indometacin 25 mg/kg by oral gavage in order to induce gastric damage. One group of rats were given indometacin only, to determine the extent of ulcer formation. Rats were killed 6 h after administration of indometacin by an overdose of thiopental sodium (50 mg/kg).

The rats' stomachs were removed and opened along the line of greatest curvature, and were then washed with 0.9% sodium chloride. The ulcerated areas on the surface of the stomachs were examined macroscopically and measured on millimeter square paper. The results obtained from erythropoietin groups were compared with those from the control and famotidine groups. The percentage inhibition in the ulcer index (UI) in relation to the indometacin group was estimated from the following formula: % inhibition = $(1 - [UI_T/UI_{IC}]) \times 100$, where UI_T and UI_{IC} are the ulcer index in the treatment and indometacin control groups, respectively.

Biochemical analyses

After the macroscopic analyses, the SOD and myeloperoxidase (MPO) activities and GSH levels in the rats' entire stomach tissues were determined. To prepare the tissue homogenates, stomach tissues were ground with liquid nitrogen in a mortar. The ground tissues (0.5 g each) were then treated with 4.5 ml of appropriate buffer. The mixtures were homogenized on ice for 15 min using an Ultra-Turrax homogenizer. Homogenates were filtered and centrifuged at 4°C. The supernatants were used to determine the enzymatic activities. All assays were carried out in triplicate at room temperature.

Superoxide dismutase activity

Measurements were made using the method of Sun *et al*.[23] Estimation of SOD was based on the generation of the superoxide radicals produced by xanthine and xanthine oxidase, which reacts with nitro blue tetrazolium to form formazan dye. SOD activity was measured at 560 nm based on the degree of inhibition in this reaction, and expressed as mmol/min per mg tissue.

Myeloperoxidase activity

MPO activity was measured according to the method of Bradley *et al*.[24] with some modification. The homogenized samples were frozen and thawed three times, and centrifuged at 1500*g* for 10 min at 4°C. MPO activity in the supernatant was determined by adding 100 ml supernatant to 1.9 ml 10 mmol/l phosphate buffer (pH 6.0) and 1 ml 1.5 mmol/l O-dianisidine hydrochloride containing 0.0005% (w/v) hydrogen peroxide. The absorbance changes of each sample were recorded on a UV-vis spectrophotometer at 450 nm. MPO activity in the gastric tissues was expressed as μmol/min per mg tissue.

Total glutathione

The amount of GSH in the gastric mucosa was measured based on the method of Sedlak & Lindsay.[25] The mucosal surface of the stomach was collected by scraping, and then weighed and homogenized in 2 ml 50 mol/l Tris-HCl buffer containing 20 mol/l EDTA and 0.2 mol/l sucrose, pH 7.5. The homogenate was immediately precipitated with 0.1 ml

25% trichloroacetic acid and centrifugation at 4200 rpm for 40 min at 4°C. The supernatant was used to determine GSH using 5,5′-dithiobis(2-nitrobenzoic acid). Absorbance was measured at 412 nm using a spectrophotometer. GSH levels were expressed as nmol/mg tissue.

Serum erythropoietin concentration

Erythropoietin concentration was determined using a commercially available ELISA (Quantikine MEP00 erythropoietin ELISA kit, R&D Systems Europe, Abingdon, UK). The calibration curves for erythropoietin was $y = 0.0006x - 0.0442$ and the correlation coefficient was $R = 0.9995$. Erythropoietin concentrations are given in pg/ml.

Statistical analyses

UI and serum erythropoietin levels are given as means ± SD; levels of oxidant and antioxidant are given as means ± SE. Differences in ulcer area and erythropoietin concentrations were analysed by one-way analysis of variance followed by the Scheffe option. Levels of GSH and enzyme activities were compared by one-way ANOVA followed by Duncan's multiple range test. Differences between the groups were considered significant at $P < 0.05$. Statistical calculations were performed using SPSS 12.0 software.

Results

Effects of erythropoietin on indometacin-induced gastric ulcers

The gastroprotective effect of erythropoietin on indometacin-induced gastric damage was determined macroscopically in rats. The percentage inhibition of ulcer formation is shown in Figure 1. There was remarkable hyperaemia in the stomachs of indometacin-treated rats. In the groups treated with erythropoietin and famotidine, hyperaemia was slight compared with indometacin-treated rats. The UI in rats treated with erythropoietin at doses of 2500, 5000 and 10 000 IU/kg was 25.33 ± 5.16, 15.00 ± 3.89 and 40.83 ± 9.64, respectively, compared with 36.33 ± 6.71 and 0.83 ± 1.16, in the indometacin and famotidine groups, respectively (Figure 1). Famotidine reduced the ulcer area by 98%; 2500 and 5000 IU/kg doses of erythropoietin reduced the ulcer area by 31% and 58%, respectively, compared with the indometacin group. Thus, famotidine and the 2500 and 5000 IU/kg doses of erythropoietin had a significant protective effect against the gastric damage caused by indometacin. The 10 000 IU/kg dose of erythropoietin did not have a protective effect against the gastric damage caused by indometacin. The gastroprotective effect of the 5000 IU/kg dose of erythropoietin was stronger than that of the 2500 IU/kg dose. None of the doses resulted in any mortality.

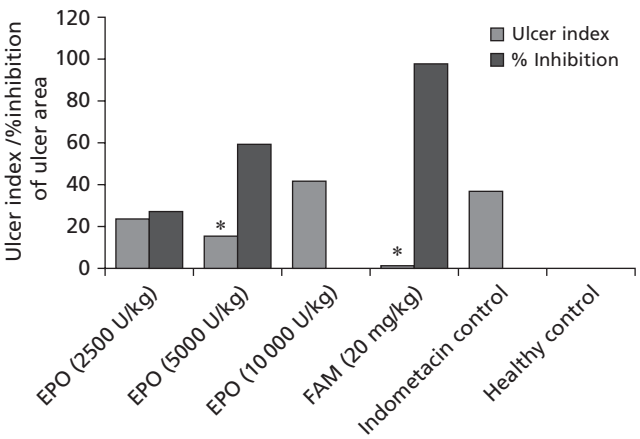

Figure 1 Effects of erythropoietin and famotidine on indometacin-induced gastric damage in rat. Ulcer index is the mean ± SD ($n = 6$). Percentage inhibition is relative to the ulcer area in the indometacin control group. EPO, erythropoietin; FAM, famotidine. *$P < 0.01$ vs indometacin control group.

Effects of erythropoietin and famotidine on GSH, SOD and MPO

To explore the effects of antioxidant defences on the ulceration process in gastric tissues, SOD, MPO and GSH were evaluated (Figure 2). MPO activity, as an index of neutrophil infiltration, was significantly elevated in the indometacin-treated group, indicating that there was substantial neutrophil influx into the mucosa in response to the oral administration of indometacin. This observed increase in MPO was significantly attenuated by 2500 IU/kg erythropoietin pretreatment ($P < 0.05$). MPO activity was only slightly decreased by the administration of 10 000 IU/kg erythropoietin. Famotidine significantly decreased MPO activity.

Indometacin administration resulted in marked reductions in SOD activity and GSH level in gastric mucosa ($P < 0.05$). Erythropoietin pretreatment led to a significant increase in gastric GSH level and SOD activity compared with the ulcer control group (Figure 2). The GSH level and SOD activity were markedly reduced in the indometacin control group as a result of ulceration. SOD and GSH levels were lower and the MPO level higher in the indometacin groups compared with the healthy control group. The reverse pattern was seen in tissue from erythropoietin and famotidine-treated rats: SOD and GSH levels were increased and MPO level decreased compared with indometacin-treated tissue. As shown in Figure 2, the levels of GSH were higher in the famotidine-treated group.

Plasma erythropoietin levels

Six hours after the last erythropoietin administration, plasma erythropoietin levels were comparable between the control groups. The plasma erythropoietin levels of rats receiving erythropoietin at doses of 2500, 5000 and 10 000 IU/kg were 3462.08 ± 181.43, 4005.83 ± 332.66 and 4387.58 ± 487.17 pg/ml, respectively ($n = 6$; all $P < 0.01$). No erythropoietin was detected in plasma from rats in the indometacin and famotidine groups.

Discussion

Gastrointestinal toxicity associated with the use of NSAIDs results in erosion, ulceration, bleeding and perforation and is a major clinical problem.[26] The search for new drugs with novel action mechanisms is therefore important.

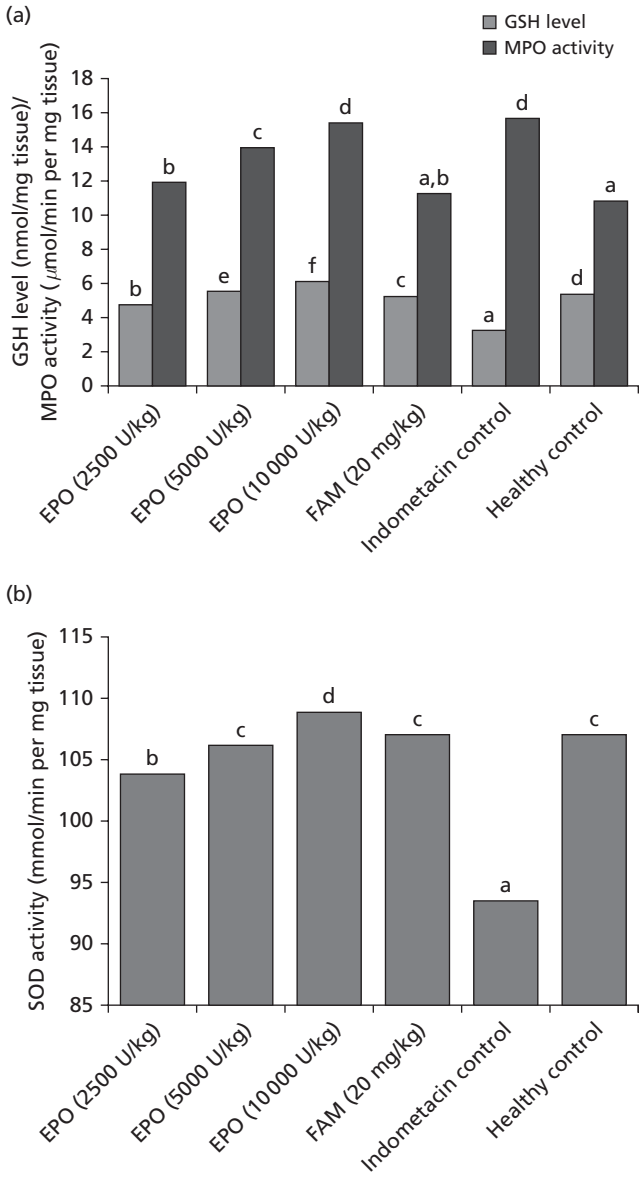

Figure 2 Effects of erythropoietin and famotidine pretreatment on changes in (a) total glutathione levels and myeloperoxidase activity and (b) superoxide dismutase activity induced by indometacin. Bars show means ± SE ($n = 6$). EPO, erythropoietin; FAM, famotidine; GSH, glutathione; MPO, myeloperoxidase; SOD, superoxide dismutase. Means in the same column with the same letter do not differ significantly (one-way ANOVA followed by Duncan test ($\alpha = 0.05$)).

This study indicates that 2500 and 5000 IU/kg doses of erythropoietin offer protective effects in a rat model of indometacin-induced gastric mucosa damage. Two main findings support this suggestion. First, 2500 and 5000 IU/kg doses of erythropoietin significantly decreased the tissue levels of MPO and increased SOD and GSH. Second, erythropoietin significantly attenuated macroscopic mucosal damage. The 2500 and 5000 IU/kg doses of erythropoietin reduced the ulcer area by 31% and 58%, respectively, compared with the indometacin group. However, the 10 000 IU/kg dose of erythropoietin had no effect on the gastric damage caused by indometacin. The 5000 IU/kg dose showed the strongest gastroprotective effect.

MPO has been used as an index of neutrophil infiltration and as a marker for acute inflammation in various gastric injuries when polymorphonuclear cell infiltration occurred.[27] Our results showed that 2500 IU/kg erythropoietin significantly reduced gastric mucosal MPO levels compared with the indometacin control group, which may be related to its gastroprotective effect. In the current study, MPO activity was increased by indometacin, agreeing with our previous studies.[28,29] It is documented that MPO is responsible for oxidative tissue damage in addition to ROS. The elevation of MPO can cause depletion of endogenous gastric antioxidant compounds.[30] The administration of 10 000 IU/kg erythropoietin significantly increased the gastric tissue MPO level compared with the healthy group and, as a result, did not attenuate ulcer formation induced by indometacin. Mimic-Oka *et al*.[31] showed that plasma levels of malondialdehyde (MDA) and SOD activity were significantly higher in haemodialysed patients than in healthy subjects. The increased plasma and red blood cell MDA levels after erythropoietin treatment suggest that, despite improvement in erythrocyte and plasma antioxidant capacity, the susceptibility of intracellular and extracellular lipids to oxidation remains significant.[31] An increased MDA level during the initial phase of erythropoietin therapy had also been reported in children.[32] In our study, the dose that was dependent on increased lipid peroxidation levels after erythropoietin administration may have occurred similarly to the previous studies mentioned above in which MDA levels were increased after erythropoietin administration.

GSH is considered to be one of the major defence mechanisms against oxidative cell stress and is particularly abundant in the mucosal cells of the gastrointestinal tract.[33] It maintains the normal redox potential, and works against free radical- or toxic-induced damage.[34,35] GSH participates in many aspects of oxidative metabolism, including the neutralisation of hydroperoxides and maintenance of the physiological sulfhydryl status of proteins. Adequate levels of sulfhydryl compounds are also important for the prevention of NSAID-induced gastropathy.[36] On the contrary, perturbations in the oxidative status of the GSH system were caused by indometacin.[37] Savoye *et al*. also reported that GSH levels were reduced in patients with NSAID-induced gastric-ulcer bleeding.[38] Consistent with these findings in the current study, GSH levels in stomach tissues from indometacin-treated rats were lower that in tissues of healthy rats, as found in our previous studies.[28,29] The present results also revealed that erythropoietin significantly increased the gastric mucosal GSH level. The observed decrease in GSH levels upon erythropoietin administration was similarly reported in previous studies.[39] Furthermore, the present results support other studies demonstrating that the gastroprotective effects of erythropoietin against indometacin-induced acute gastric damage are partly dependent on the restored bioavailability of GSH.

Treatment with SOD and catalase inhibits the lesions, suggesting the involvement of ROS in gastric damage.[10] In the present investigation, indometacin decreased SOD, while all erythropoietin doses increased SOD activity to the

levels found in the control group. It has been reported that erythropoietin has antioxidant activities and improves enzymatic antioxidant parameters like SOD, catalase and GSH peroxidase.[40,41] The gastroprotective effects of erythropoietin may be mediated by several distinct mechanisms, such as the promotion of cell survival signalling cascades,[42] upregulation of the expression of anti-apoptotic proteins,[43] the modulation of intracellular calcium metabolism,[18] the attenuation of NO production,[19] anti-apoptotic processes,[19,44] anti-oxidative processes[45] and anti-inflammatory actions.[21] Thus, the gastroprotective effect of erythropoietin against indometacin-induced gastric injury may be linked with its antioxidant properties.

Conclusions

The present results support the view that erythropoietin protects against indometacin-induced gastric ulcer and could be used as an antiulcer compound. Its antiulcer effect is less than that of famotidine. The gastroprotective effects of erythropoietin may be related to its intrinsic ability to sustain activities of free-radical-scavenging enzymes and to the bioavailability of GSH, which protects mucosa cells against oxidative injury by decreasing MPO. However, other mechanisms may also be involved and these need to be clarified through further investigation.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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