Gastroprotection Induced by Silymarin, the Hepatoprotective Principle of *Silybum marianum* in Ischemia-Reperfusion Mucosal Injury: Role of Neutrophils

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Abstract

Investigations were carried out to determine the antiulcer effects of silymarin, the hepatoprotective principle of *Silybum marianum* L. Gaertn., in gastric injury induced by ischemia-reperfusion and its effects on mucosal myeloperoxidase activity, an index of polymorphonuclear leukocyte infiltration, after injury in rats. These results were compared with those from rats that received allopurinol, an inhibitor of xanthine oxidase and with those from rats made neutropenic by prior administration of dexamethasone and methotrexate. Pretreatment with silymarin prevented post-ischemic mucosal injury. The mean ulcer indexes (U.I.) of rats treated with 25, 50 mg, and 100 mg silymarin/kg body weight (4.79 ± 0.75, 4.50 ± 0.81, and 3.63 ± 0.74, respectively) were significantly lower ($p < 0.05$, 0.05, and $p < 0.005$) than that of control rats. Allopurinol was considerably more potent in reducing the U.I. than silymarin, with a calculated U.I. of 2.33 ± 0.45, $p < 0.001$. These protective effects were specifically related to a reduction in the number of neutrophils in the gastric mucosa. Reduction in the numbers of circulating neutrophils by treating rats with methotrexate (MPO level of $7.2 \times 10^{-2} \pm 0.56 \times 10^{-2}$ U/mg wt) and dexamethasone (MPO level of $6.97 \times 10^{-2} \pm 0.68 \times 10^{-2}$ U/mg wt) also resulted in a significant reduction in the susceptibility to gastric damage induced by ischemia-reperfusion. These results suggest that neutrophils play an important role in the gastric mucosal dysfunction associated with ischemia-reperfusion. These findings also indicate that the inhibitory effects of silymarin on neutrophil function may contribute significantly to its gastroprotective actions.

Key words

Silybum marianum, Asteraceae, silymarin, ischemia-reperfusion gastric ulcers, neutrophils.

Introduction

Experimental studies have demonstrated that oxygen free radicals and lipid peroxidation play important roles in the pathogenesis of acute gastric lesion induced by ischemia-reperfusion (1). Xanthine oxidase has been proposed as the primary source of these radicals. Another potential source of oxygen radicals are inflammatory neutrophilic polymorphonuclear leukocytes (neutrophils). Stimulation of neutrophilic oxidative metabolism results in the release of large amounts of O_2^-, H_2O_2 and $H^\cdot$. In addition, neutrophils secrete the enzyme myeloperoxidase (MPO) into the extracellular medium where it catalyses the two-electron oxidation of Cl^- by H_2O_2 to yield the potent cytotoxic oxidants hypochlorous acid (HOCl) and *N*-chloramines (2, 3).

Bioflavonoids and related natural compounds exert significant scavenging properties on oxygen radicals *in vitro* and *in vivo* thus affecting various steps in the arachidonate cascade via cyclooxygenase or lipoxygenase (4). Silymarin has been known for a long time to exert marked anti-hepatotoxic activity especially in hepatitis of various origins (5). Silymarin has also been found to be effective in the prevention of gastric ulceration induced by cold-restraint stress and in pylorus ligated rats (6).

The aim of this study has been to examine the antiulcer activity of silymarin on gastric injury induced by ischemia-reperfusion and its effects on mucosal myeloperoxidase activity; the latter is an index of polymorphonuclear leukocyte infiltration after injury in rats.

Materials and Methods

Animal model

Male Wistar rats, 180–200 g were used with the Animal Ethics Committee (University of Seville) approval. Rats were placed in single cages with wire-mesh floors in a room in which the temperature was maintained at 22–24 °C and the humidity at 70–75 %. All animals were fed a normal laboratory diet. The animals were deprived of food for 24 h before experimentation but allowed free access to tap water throughout.

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital at a dose of 50 mg/kg body weight. The left side of the abdomen was shaved and a 3 cm incision was made from the midline to below the ribcage using a diathermy.

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The celiac artery was dissected free of excess fat and clamped for 30 min approximately 0.5 cm from its origin out of the aorta, using an atraumatic microvascular clamp. Reoxygenation was allowed by removal of the clamp for 60 min (1). At the end of the experimental period, stomachs were removed and opened along their line of greater curvature to examine the lesions macroscopically. The sum of the areas of damage was calculated. Results were expressed as % of surface area with inhibition of mucosal injury. The severity of mucosal ulceration was also determined according to the score of Cioli (7). Mean ulcer scores for each animal were calculated and expressed as the ulcer index (U.I.).

Treatment groups

In a separate experiment, groups of 6 rats were treated with silymarin (10, 25, 50, and 100 mg/body weight) which were dissolved in tween 80 (0.5 %), by orogastric gavage once daily for two consecutive days prior to the experiments and 1 h before ischemia. Control rats were pretreated identically with the same vehicle.

Allopurinol, an inhibitor of the xanthine-oxidase system, was administered *p.o.* at a daily dose of 100 mg/kg body weight on two consecutive days prior to experiments to one group of 6 rats. The control group received vehicle (distilled water) by the same route. On day 3, the rats were anesthetized and subjected to ischemia as described Itoh et al. (8).

Because the ability of glucocorticoids to interfere with neutrophil adherence to the vascular endothelium is well documented (9), the effects of pretreatment with dexamethasone on the susceptibility of the gastric mucosa to ischemia-reperfusion injury were assessed. Groups of 6 rats were treated with 1 mg/kg body weight *i.p.*, or the vehicle, 2 h before ischemia.

To study the effects of a reduction in the numbers of circulating neutrophils on mucosal injury, further groups of 6 rats were treated with methotrexate as described by Wallace et al. (10). The rats received methotrexate (2.5 mg/body weight/day *i.p.*) or the vehicle (normal saline) daily on 3 consecutive days. On the fifth day after the final methotrexate administration, the susceptibility to ischemia-reperfusion damage was assessed.

Assessment of leukocyte involvement

Neutrophil infiltration *in vivo* has been studied biochemically by measuring granulocyte specific enzymes such as myeloperoxidase (MPO) in tissue.

Tissue preparation: In all animals two mucosal biopsy specimens corresponding to gastric lesions were excised and rapidly rinsed with ice-cold saline, blotted dry, and frozen at −70 °C. The assay for the MPO activity was always performed within 2 weeks of an experiment. The tissue was thawed, weighed, and homogenized in 10 volumes of 50 mM PBS (pH 7.4). The homogenate was centrifugated at 2000 *g*, 20 min, 4 °C and the pellet homogenized again in 10 volumes of 50 mM PBS (pH 6.0) containing 0.5 % HETAB and 10 nM EDTA. The HETAB containing homogenate was subjected to one cycle of freezing/thawing and a brief period of sonication.

Myeloperoxidase assay: MPO activity was assayed spectrophotometrically using a minor modification of the method, which utilizes 3,3',5,5'-tetramethylbenzidine (TMB) as substrate (11). In this method 0.5 µg of homogenate was added to a 0.5 ml reaction volume containing 80 mM PBS (pH 5.4), 0.5 % HETAB, and 1.6 mM TMB. The mixture was incubated at 37 °C. The reaction was terminated by the sequential addition of catalase (20 µg/ml) and 2 ml of 0.2 M Na acetate (pH 3.0). The changes in absorbance at 655 nm were measured with a spectrophotometer (Perkin-Elmer Lambda 3). One unit of MPO activity was defined as the amount of enzyme present that produced a change in absorbance of 1.0 Unit/min at 37 °C in the final reaction volume containing the acetate.

Drugs

The following drugs were used: silymarin (70 % silybin, 16.5 % silydianin, 13.5 % silychristin) was kindly supplied by Laboratories Madaus Cerafarm S.A., Spain; methotrexate was obtained from Laboratories Almirall S.A., Spain; dexamethasone was purchased from Merck. All other reagents were obtained from Sigma Chemical Company.

Statistical analysis

All data are expressed as means ± S.E.M. Groups of data were compared using Student's t test.

Results and Discussion

Our results show that gastric injury is significantly increased during reperfusion following 30 min of ischemia induced by clamping the celiac artery. The injury assessed macroscopically appeared to reflect the extent of hemorrhage within or on the surface of mucosal vascular injury.

Silymarin (25, 50, and 100 mg/kg body weight) produced a significant reduction in the number and severity of ulcers, reaching a percentage inhibition of ulceration of 89.45 % (Fig. **1**). In addition, pretreatment with silymarin at doses of 50 and 100 mg/kg body weight reduced the mean I.U. from 6.9 ± 0.27 seen in controls to 4.50 ± 0.81 ($p < 0.05$) and 3.63 ± 0.74 ($p < 0.005$), respectively (Fig. **2**). With allopurinol at the dose used in our experiment, the gastric mucosal lesions were prevented to a significant degree, and this compound was considerably more potent in reducing the mean U.I. than silymarin. The mean U.I. in allopurinol treated animals was 2.33 ± 0.45 ($p < 0.001$) (Fig. **3**).

As shown in Fig. **3**, the highest protective effects were observed in rats receiving dexamethasone. These rats showed a mean U.I. of 1.7 ± 0.48 ($p < 0.001$).

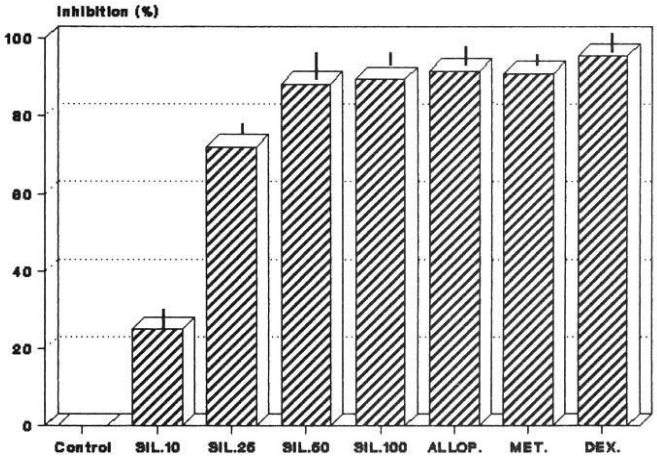

Fig. 1 Inhibition (%) of ischemia-reperfusion induced gastric mucosal lesions by silymarin (SIL., 10, 25, 50, and 100 mg/kg *p.o.*), allopurinol (ALLOP., 100 mg/kg, *p.o.*), methotrexate (MET., 2.5 mg/kg, *i.p.*), and dexamethasone (DEX., 1 mg/kg *i.p.*).

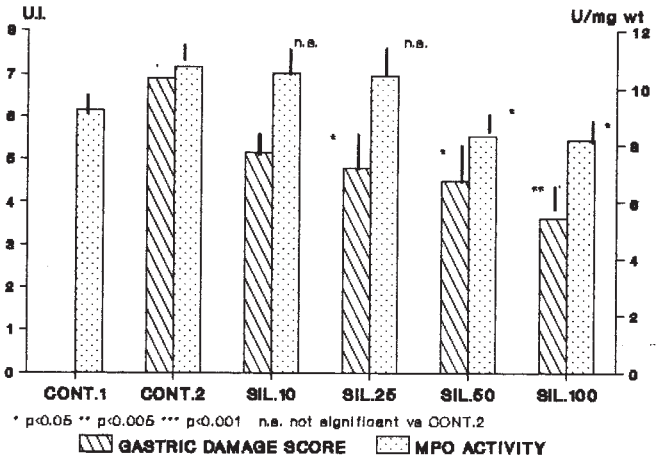

Fig. 2 Effects of pretreatment with silymarin (10, 25, 50, and 100 mg/kg p.o.) on susceptibility to ischemia-reperfusion induced gastric damage and myeloperoxidase (MPO) activity. CONT. 1 (non-ischemia control group), CONT. 2 (ischemia control group).

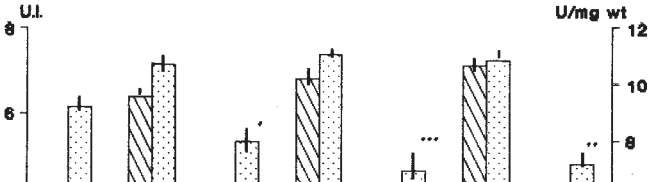

Fig. 3 Effects of pretreatment with allopurinol (ALL, 100 mg/kg *p.o.*), dexamethasone (DEX, 1 mg/ *i.p.*), and methotrexate (MET, 2.5 mg/kg *i.p.*) on susceptibility to ischemia-reperfusion induced gastric damage and myeloperoxidase (MPO) activity. CONT. 1 (non-ischemia control group), CONT. 2 (ischemia control group).

Methotrexate significantly reduced the severity of ischemia reperfusion damage by approximately the same extent as allopurinol.

MPO levels in mucosal samples were lower ($9.2 \times 10^{-2} \pm 0.46 \times 10^{-2}$ U/mg wt) in non-ischemic rats than in rats from the ulcerated-control dexamethasone group ($11.05 \times 10^{-2} \pm 0.87 \times 10^{-2}$ U/mg wt).

Oral administration of silymarin (50 and 100 mg/kg) resulted in marked decreases in MPO levels which were significantly reduced compared with the control group ($8.30 \times 10^{-2} \pm 0.67 \times 10^{-2}$, $8.02 \times 10^{-2} \pm 0.60 \times 10^{-2}$, $p < 0.05$), although no dose-dependency was observed.

Pretreatment with allopurinol effectively attenuated the increase in MPO activity, as was also found by Grisham et al. (2). This attenuation of MPO activity represents a decrease in the amount of enzyme and no inhibition of MPO catalytic activity by allopurinol and this effect is significant when compared with control ulcerated rats. In addition, the MPO activity was similar to those seen in silymarin groups. As shown in Fig. 3, dexamethasone and methotrexate treatments were effective in neutrophil-reduction, described by various authors (10). We observed a great reduction in MPO values which were statistically significantly lower than for respective control groups. These results were lower than those obtained with silymarin.

Flavonoids constitute a group of naturally occurring benzo-γ-pyrone derivatives which have been found to possess several biological properties e.g. antithrombotic (12), anti-inflammatory (13), antiviral (14), and antiulcerogenic (15, 16) activities. Many of these actions have been correlated with their ability to scavenge oxygen-generated free radicals (ROM) and to inhibit lipid peroxidation *in vitro* (4). Silymarin has been found to be effective in reducing ischemia and reperfusion gastric injury. Oral administration of silymarin also results in marked decreases in MPO levels, an index of PMN leukocyte infiltration.

Prolonged ischemia alone will result in injury due to oxygen depuration. However, cellular changes occurring during shorter periods of ischemia initiate the production of toxic reactive metabolites when the tissue is reoxygenated. Sources of ROM in reperfusion tissues include the xanthine oxidase system, which is modified during ischemia, such that it produces O_2^- and H_2O_2 during reperfusion. These ROM may then be converted to the highly cytotoxic hydroxyl radical by the iron-catalyzed Haber-Weiss reaction (3). This initiates the process of lipid peroxidation which, in turn, results in the production and release of substances that recruit and activate PMNs (3). Activated neutrophils produce reactive oxygen metabolites and release a variety of cytotoxic proteins, e.g. proteases, lactoferrin and MPO, an enzyme that catalyzes the formation of such potent cytotoxic oxidants as hypochlorous acid and *N*-chloramines (2, 3).

It has been reported that beneficial effects of silymarin are partly due to its membrane stabilization activity, possibly related to an interference in calcium influx (17) as well as to its ability to scavenge oxygen-generated free radicals and to inhibit peroxidation of biological membranes by way of the 5-lipoxygenase inhibition of biosynthesis of leucotrienes (18, 19). Of these agents in particular, LTB_4 is a potent chemoattractant which is frequently implicated as a mediator of reperfusion-induced neutrophil infiltration. Recently, Zimmermann et al. (20) showed that in animals pretreated with either a lipoxygenase inhibitor (L-663,536) or the LTB_4 antagonist SC-41930 the magnitude of the reperfusion-induced granulocyte infiltration decreased, providing the first direct evidence that LTB_4 accumulation is a cause rather than an effect of reperfusion-induced granulocyte infiltration.

We have demonstrated that pretreatment with allopurinol (an XO inhibitor) reduces the extent of injury and neutrophil infiltration into the post-ischemic gastric mucosa. These results suggest that XO-derived

radicals at the time of reperfusion can act either directly or indirectly on neutrophils to promote vascular adherence and chemotaxis into the gastric wall. Alternatively XO-generated radicals may cause the release and metabolism of arachidonic acid to yield neutrophilic chemoattractants/activators as LTB_4.

Depletion of number of PMNs and inhibition of its adherence by treatment with methotrexate and dexamethasone, respectively, diminished significantly the exacerbation of injury after ischemia and reperfusion. These results are in agreement with other authors (2, 10) and suggest that the I/R induced impairment of mucosal function may be mediated by the products of neutrophil activation, e.g. reactive oxygen metabolites and cytotoxic proteins.

In conclusion, the data presented here confirm the impression that silymarin exerts its protective effect during ischemia and reperfusion by interfering with the oxidative metabolism of the neutrophil thus decreasing neutrophil extravasation and neutrophil-mediated cytotoxicity.

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