Protective effect of silymarin against ethanol-induced gastritis in rats: Role of sulfhydryls, nitric oxide and gastric sensory afferents

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Abstract
Silymarin has been known to exert antioxidant, anti-carcinogenic and anti-inflammatory effects. In this study, we examined the effect of silymarin on gastritis in rats. Oral administration of silymarin dose-dependently decreased gastric lesions in ethanol-induced gastritis model. Silymarin also significantly suppressed the development of gastric lesions in aspirin- or water immersion-restraint stress-induced gastritis models. Further study demonstrated that the gastroprotective effect of silymarin was blocked by nitric oxide (NO) synthase inhibitor L-NAME, SH blocker N-ethylmaleimide or TRPV1 antagonist capsazepine in ethanol-induced gastritis model. In addition, ex vivo analysis revealed that ethanol-induced decrease in gastric mucus and non-protein sulfhydryl (NPSH) groups was significantly reversed by silymarin treatment and lipid peroxidation was also suppressed by silymarin in ethanol-induced gastritis model. Taken together, these results suggest that silymarin exerts gastroprotective effects and the gastroprotective effects of silymarin might be related to the protection of gastric mucosal NO and NP-SH and the modulation of capsaicin-sensitive gastric sensory afferents.

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1. Introduction
Gastritis refers to an inflammation of the epithelial lining of the stomach. The most common symptom of gastritis is an upper central abdominal pain and other symptoms, such as nausea, vomiting, belching, bloating, and weight loss, are also reported (Glickman and Antonioli, 2001). Chronic gastritis is associated with Helicobacter pylori infection, stress or autoimmune disorders and part of gastritis with H. pylori infection is known to advance to gastric ulcer and gastric neoplasias (Kandulski et al., 2008). In contrast, acute gastritis is commonly caused by excessive alcohol consumption, prolonged use of aspirin/non-steroidal anti-inflammatory drugs (NSAIDs) or stress (Srivastava and Lauwers, 2007; Franke et al., 2005). It has been reported that acute gastritis arises when there is an acute imbalance between mucosal injury and repair mechanisms (Srivastava and Lauwers, 2007). A variety of factors, including nitric oxide (NO), prostaglandins, non-protein sulfhydryls (NP-SH) and capsaicin-sensitive gastric sensory afferents, has been reported to be implicated in mucosal protection and gastritis development.
Silymarin, a polyphenolic flavonoid antioxidant, is extracted from the fruits and seeds of milk thistle, Silybum marianum. Silymarin has been clinically used for a long time to treat liver diseases due to its hepatoprotective effects (Mereish et al., 1991; Carini et al., 1992; Kang et al., 2004). In addition, a variety of studies demonstrated that silymarin exhibits anti-carcinogenic, anti-inflammatory and anti-atherosclerotic activities (Zi et al., 1998; Kang et al., 2002, 2003). Protective effects of silymarin against gastric ulcers induced by cold-resistant stress or ischemia–reperfusion were also reported previously (Alarcon de la Lastra et al., 1992, 1995). It was reported that the inhibition of lipoxigenase and neutrophil function might be involved in the gastroprotective effects of silymarin in these models. However, there were no reports describing the effect of silymarin against acute gastritis induced by other common factors, such as alcohol or NSAIDs. In this study, we examined the effect of silymarin against gastritis induced by ethanol, aspirin or water immersion-restraint stress. We also investigated the mechanisms responsible for the gastroprotective effects of silymarin.

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2. Materials and methods

2.1. Reagents and animals

All reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise noted. Silymarin was suspended in 0.5% carboxymethyl cellulose (CMC) for in vivo experiments. Male SD (Sprague Dawley) rats (200–220 g, 6 weeks old) were purchased from Koatech (Pyungtaek, Gyeonggi, Korea) and cared for as described previously (Kang et al., 2004). Animals were allowed to acclimate to the local environment for at least 1 week before use. All animal experiments were conducted using protocols approved by Institutional Animal Care and Use Committee at Korea Research Institute of Bioscience and Biotechnology.

2.2. Gastritis model induced by ethanol, aspirin or water immersion-restraint

SD rats (n = 5) were pretreated with vehicle (0.5% CMC) or indicated concentrations of silymarin (25, 50, 100 or 200 mg/kg, p.o.) 30 min before the induction of gastric damage by oral administration of absolute ethanol (1 ml/animal) or aspirin (200 mg/kg). The animals were sacrificed 1 h after ethanol administration and 3 h after aspirin administration, respectively. In water immersion-restraint stress model, rats were put into stress cage and immersed in water (23 ± 1°C) for 7 h and sacrificed. The stomachs were removed, opened along the greater curvature, rinsed with saline (0.9%) and photographed using digital camera. The gastric lesion area was analyzed using Image-Pro Plus 4.5.1 (Media Cybernetics, Inc., Rockville, MD, USA) and expressed as % of total area.

2.3. Determination of NP-SH

NP-SH was determined according to the method described by Sedlak and Lindsay (1968). 100 mg of gastric tissue samples were homogenated in 1 ml of 0.02 M EDTA, 400 µl of tissue homogenates were mixed with 320 µl of distilled water and 80 µl of 50% (w/v) trichloroacetic acid and centrifuged at 3000 g for 15 min. The supernatants (400 µl) were then mixed with 800 ml of 40 mM Tris buffer (pH 8.9) and 20 ml of 10 mM 5,5′-dinitrosalicylic acid (2-nitrobenzoic acid) (DTNB) was added. The absorbance was measured within 5 min after DTNB addition at 412 nm. The absorbance values were extrapolated to a glutathione standard curve and expressed as µg/g of stomach tissue.

2.4. Determination of total thiobarbituric acid-reactive substances (TBARS)

Total TBARS were determined according to the method of Okhawa and coworkers with minor modifications (Okhawa et al., 1979). 100 mg of gastric tissue samples were homogenated in 1 ml of ice-cold phosphate buffer (50 mM, pH 7.4) and incubated at 37°C for 60 min. After adding 50 µl of 0.2 M sucrose solution, the mucous–dye complex was extracted by placing the tissue in 0.5 M MgCl2 for 10 min and 0.6% thiobarbituric acid was added to the upper layer. The mixture was then submitted to a water bath at 95°C for 10 min and 0.6% thiobarbituric acid was added to the upper layer. The mixture was then added to a water bath at 95°C for 10 min and the absorbance was measured at 532 nm. The standard curve was obtained using several concentrations of MDA solutions and TBARS content was expressed in nmol/g of wet tissue.

2.5. Determination of gastric mucus

Gastric wall mucus was determined according to the method of Corne et al. (1974) with minor modifications. The gastric tissues were collected and transferred to 1% Alcian blue solution (0.16 M sucrose in 0.05 M sodium acetate, pH 5.8). Excess dye was removed by washing the tissue with 0.25 M sucrose solution. The mucus–dye complex was extracted by placing the tissue in 0.5 M MgCl2 for 2 h. The extract was mixed with equal volume of diethyl ether, centrifuged at 3500 g for 10 min, and the absorbance was measured at 598 nm. The quantity of mucus–dye complex (µg/g of tissue) was then calculated using a standard curve of Alcian blue.

2.6. Statistical analysis

Results are expressed as mean ± S.D. One-way ANOVA and Bonferroni’s multiple comparison test was used for statistical analysis using GraphPad Prism (GraphPad Software; La Jolla, CA, USA). The criterion for statistical significance was set at p < 0.05.

3. Results

3.1. Silymarin suppresses gastric lesions induced by ethanol, aspirin or water immersion-restraint stress in SD rats

To investigate the antigastritic effect of silymarin, we examined the effect of silymarin on gastric lesion development induced by various stimuli. As shown in Fig. 1, oral administration of ethanol induced gastric lesions in SD rats. However, pretreatment of silymarin suppressed ethanol-induced gastric lesions in a concentration-dependent manner. The inhibitory effect of silymarin on ethanol-induced gastric lesion was comparable to that of Stillen®, a drug developed from natural products and prescribed for the gastric ulcers in South Korea and China (Choi et al., 2011). We also examined the effect of silymarin on aspirin- or water immersion-restraint stress-induced gastric lesions in SD rats. As shown in Table 1, oral administration of silymarin significantly suppressed gastric lesions induced by aspirin or water immersion-restraint stress in SD rats.

3.2. Effect of N-ethylmaleimide, L-NAME, indomethacin and capsazepine on the antigastritic effect of silymarin in ethanol-treated SD rats

To investigate the possible involvement of NP-SH, NO, prosta-glandins and capsaicin-sensitive gastric sensory afferents, we pre-treated SD rats with N-ethylmaleimide (NEM, an SH blocker), L-NAME (an inhibitor of NO synthase), indomethacin (an inhibitor of prostaglandins) or capsazepine (a transient receptor potential cation channel subfamily V member 1 (TRPV1) antagonist) before the treatment with silymarin and ethanol. In rats treated with NEM, L-NAME or capsazepine, the gastroprotective effects of silymarin, carbenoxolone (against NEM), L-arginine (against L-NAME) and capsaicin (against capsazepine) were reduced significantly (Fig. 2A, B and D). In contrast, indomethacin pretreatment had no significant effect on the gastroprotective effect of silymarin, whereas the effect of misoprostol, a synthetic prostaglandin E2, analogue and a drug used for the prevention of NSAID-induced gastric ulcer, was significantly reduced by indomethacin treatment (Fig. 2C).

3.3. Effect of silymarin on ethanol-induced changes in gastric NP-SH, TBARS and mucus

To further confirm the gastroprotective effect of silymarin, we examined the effect of silymarin on the gastric levels of NP-SH and TBARS and the gastric mucus in rats treated with ethanol. As shown in Fig. 3A, the level of NP-SH in the gastric mucosal tissue was markedly decreased by ethanol treatment. However, ethanol-induced decrease in gastric mucosal NP-SH was recovered significantly by silymarin treatment (Fig. 3A). The administration of ethanol substantially elevated the gastric mucosal TBARS relative to untreated group and this was also blocked by silymarin treatment in a

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![Fig. 1. Effect of silymarin on gastric lesions induced by ethanol in SD rats. SD rats were pretreated orally with vehicle (0.5% CMC) or the indicated concentrations of silymarin (25, 50, 100 or 200 mg/kg). After 30 min, all animals received 1 ml of absolute ethanol (p.o.) to induce gastric lesions. Animals were killed after 1 h and the stomachs were excised for analysis of gastric lesions as described in Section 2. Each column shows the mean ± S.D. of five determinations.](image-url)