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Gastroprotective effect of Astragaloside IV: role of prostaglandins, sulfhydryls and nitric oxide

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Abstract

This investigation evaluated the gastroprotective activity of Astragaloside IV, a cycloartane-type triterpene glycoside isolated from *Astragalus zahlbruckneri*. Gastric mucosal damage was induced in rats by intragastric ethanol (1 mL/rat). Rats treated orally with Astragaloside IV suspended in Tween 80 at 3, 10 and 30 mg kg^{-1}, showed 15, 37 and 52% gastroprotection, respectively. The gastroprotection observed at 30 mg kg^{-1} for this compound was attenuated in rats pretreated with N^G-nitro-L-arginine methyl ester (70 mg kg^{-1}, i.p), a nitric oxide (NO)-synthase inhibitor, suggesting that the gastroprotective mechanism of this glycoside involves, at least in part, the participation of NO. The gastroprotective effect of Astragaloside IV was not affected by the inhibition of prostaglandin synthesis with indometacin (10 mg kg^{-1}, s.c.) nor by the block of endogenous sulfhydryls with *N*-ethylmaleimide (NEM, 10 mg kg^{-1}, s.c.). Carbenoxolone was used as a gastroprotective model drug and showed a dose-dependent gastroprotective effect (25, 43 and 88% of gastroprotection, at 3, 10 and 30 mg kg^{-1}, respectively). The partial participation of prostaglandins, sulfhydryls and NO was observed in the gastroprotective mechanism of carbenoxolone.

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Introduction

Ulceration occurs when there is a disturbance of the normal equilibrium caused by either enhanced aggression or diminished mucosal resistance. The defence mechanisms of the gastrointestinal mucosa mainly consist of functional, humoral and neuronal factors. Mucus alkaline secretion, mucosal microcirculation and motility act as functional factors, while prostaglandins and nitric oxide (NO) act as humoral factors, and capsaicin-sensitive sensory neurons (CPSN) act as neuronal factors (Calatayud et al 2001). Several triterpenoids, including carbenoxolone, and sterols have been shown to possess anti-ulcer activity (Lewis & Hanson 1991; Borrelli & Izzo 2000; Oliveira et al 2004). We have proposed previously that a hydroxyl group (free or derivative) at position C-3 is necessary for sterols and triterpenoids to have anti-ulcer activity (Navarrete et al 2002; Arrieta et al 2003). This hypothesis is based on the observation that triterpenoids or sterols with this structural feature have shown anti-ulcer activity (Borrelli & Izzo 2000). An additional characteristic for anti-ulcer triterpenoids is that they also have anti-inflammatory activity (Rajic et al 2001). In addition, several plants containing high amounts of saponins have been shown to possess anti-ulcer activity (Borrelli & Izzo 2000). In this sense, and to give additional support to our hypothesis, we were interested in evaluating the gastroprotective effect of Astragaloside IV, a cycloartane-type triterpene glycoside (3-*O*-ß-D-xylopyranosyl-6-*O*-ß-D-glucopyranosylcycloastragenol, Figure 1), regarded as a characteristic and active constituent of *Astragalus* species (Fabaceae) (Gu et al 2004; Yesilada et al 2005). Astragaloside IV possesses those characteristics: it is a saponin with a hydroxyl derivative group at C-3 and it has anti-inflammatory activity (Zhang et al 2003). Astragaloside IV belongs to one group of pharmacologically active saponins described in *Astragalus* species (Rios & Waterman 1997). Antinociceptive (Yang et al 2001), anti-aging (Lei et al 2003) and immunostimulant (Yesilada et al 2005) effects, cytotoxicity on several human cancer lines (Verotta et al 2001), a protective effect against ischaemic brain and myocardial injury (Zhou et al 2000; Luo et al 2004) and antimicrobial (Calis et al 1997) and

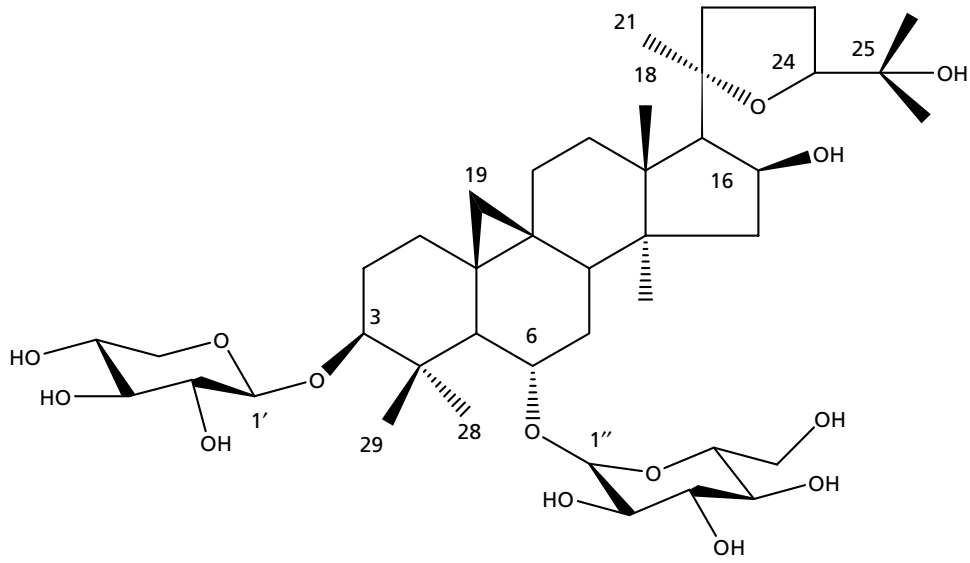

Figure 1 Structure of Astragaloside IV.

antioxidative (Ma & Yang 1999) effects have been described, in addition to in-vitro and in-vivo anti-inflammatory activity for Astragaloside IV (Zhang et al 2003).

This study was undertaken to investigate the gastroprotective effect of Astragaloside IV using as an experimental model the inhibition of ethanol-induced ulcers in rats. We also discuss the role of endogenous NO, sulfhydryls and prostaglandins in the gastroprotection of this glycoside.

Materials and Methods

Animals

All the experiments were performed with male Wistar rats, 55–60 day old, weighing 180–220 g, obtained from Centro UNAM-Harlan (Harlan México, S.A. de C.V.). Procedures involving rats and their care were conducted in conformity with the Mexican Official Norm for Animal Care and Handing (NOM-062-ZOO-1999) and in compliance with international rules on care and use of laboratory animals. Unless otherwise specified, the rats were placed in single cages with wire-net floors and deprived of food 24 h before experimentation but allowed free access to tap water throughout. All experiments were carried out using 6 rats per group.

Astragaloside IV

Astragaloside IV was isolated from roots of *Astragalus zahlbruckneri* (Abou-Gazar & Calis 2000; Calis et al 2001). The roots of *Astragalus zahlbruckneri* Hand.-Mazz (Leguminosae) were collected from Sivrice, 28 km southeast of Elazig, East Anatolia, Turkey, in June 1999. A voucher specimen was deposited in the Herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Hacettepe University, Ankara Turkey (HUEF 99-047). The air-dried powdered roots (450 g) were extracted with 80% aqueous ethanol (2×3 L) under reflux. The ethanol extracts were combined and evaporated to dryness in-vacuo to yield 54 g of crude extract (yield 12%). A sample of ethanol extract (40 g) was fractionated by open-column chromatography by silica gel (600 g) employing gradient CH_2Cl_2–MeOH–H_2O mixtures (90:10:1, 1500 mL; 80:20:2, 1000 mL; 70:30:3, 1000 mL; 60:40:4, 1700 mL; and 50:50:5, 1000 mL), yielding 12 fractions (fractions A–L: A, 500 mg; B, 500 mg; C, 733 mg; D, 1860 mg; E, 1640 mg; F, 737 mg; G, 1300 mg; H, 4730 mg; I, 4700 mg; K, 4700 mg; and L, 5800 mg). Fraction J gave colourless crystals from methanol, which afforded Astragaloside IV (4500 mg). IR (KBr) ν_{max} 3400, 2930,1040 cm^{-1}; FAB^+ MS: m/z = 807 $[M + Na]^+$; $[\alpha]_D^{20}$: + 19.3 (c 0.4, MeOH); ^1H NMR (300 MHz, C_5D_5N): aglycon moiety d 3.22 (1H, m, H-3), 1.63 (1H, d, J = 9.0 Hz, H-5), 4.67 (1H, ddd, J = 7.5, 7.5, 7.0 Hz, H-16), 2.38 (1H, d, J = 7.8Hz, H-17), 1.27 (3H, s, Me-18), 0.29 and 0.62 (each 1H, d, J_{AB} = 4.4 Hz, H-19a and H-19b, respectively), 1.23 (3H, s, Me-21), 3.80 (1H, dd, J = 8.0, 7.0 Hz, H-24), 1.15 (3H, s, Me-26), 1.28 (3H, s, Me-27), 1.31 (3H, s, Me-28), 1.04 (3H, s, Me-29), 1.04 (3H, s, Me-30); sugar moiety d 4.30 (1H, d, J = 7.5 Hz, H-1'), 4.34 (1H, d, J = 7.8 Hz, H-1″). ^{13}C NMR (75.5 MHz, C_5D_5N) aglycon moiety d 32.9 (t, C-1), 30.5 (t, C-2), 89.8 (d, C-3), 43.1 (s, C-4), 53.2 (d, C-5), 80.0 (d, C-6), 35.1 (t, C-7), 46.6 (d, C-8), 22.1 (s, C-9), 29.9 (s, C-10), 27.0 (t, C-11), 34.1 (t, C-12), 45.9 (s, C-13), 47.0 (s, C-14), 46.1 (t, C-15), 74.5 (d, C-16), 58.9 (d, C-17), 21.4 (q, C-18), 29.6 (t, C-19), 88.3 (s, C-20), 28.6 (X2) (each q, C-21 and C-28), 35.5 (t, C-22), 26.8 (t, C-23), 82.4 (d, C-24), 72.4 (s, C-25), 26.8 (q, C-26), 27.8 (q, C-27), 16.7 (q, C-29), 20.3 (q, C-30); sugar moiety d 107.5 (d, C-1′), 75.6 (d, C-2′), 78.0 (d, C-3′), 71.3 (d, C-4′), 66.7 (t, C-5′), 105.0 (d, C-1″), 75.5 (d, C-2″), 78.6 (d, C-3″), 71.7 (d, C-4″), 77.8 (d, C-5″), 62.9 (t C-6″). These data were in agreement with those previously reported (Kitagawa et al 1983; Calis et al 1997).

Drug and dosage

Carbenoxolone (Sigma-Aldrich Co.) was used as a model gastroprotective drug (Wan & Gottfried 1985). The drugs were prepared freshly each time and administered suspended in 0.5% Tween 80 by the intragastric route. Control rats received vehicle (0.5% Tween 80) in the same volume (0.5 mL/100 g) by the same route. N^G-nitro-L-arginine methyl ester (L-NAME), *N*-ethylmaleimide (NEM) and indometacin were purchased from Sigma Chemical Co (USA).

Acute gastric ulcers induced by absolute ethanol

Ulceration was induced according to the method described by Robert (1979), by intragastric instillation of absolute ethanol. Rats were divided into different groups of six each. Each group received either vehicle ($1\,mL\,kg^{-1}$), Astragaloside IV ($3–30\,mg\,kg^{-1}$) or carbenoxolone ($3–30\,mg\,kg^{-1}$) by gastric gavage. Thirty minutes after drug administration, absolute ethanol was given orally to each rat at a dose of 1 mL/rat. Two hours after ethanol administration, the rats were killed by ether inhalation. The stomach and duodenum were dissected out, inflated with 10 mL of 2% formalin and placed in 2% formalin for 15 min to fix both the inner and outer layers. The duodenum was opened along its anti-mesenteric side and the stomach along the greater curvature. The damage area (mm^2) was measured under a dissection microscope ($\times 10$) with an ocular micrometer. The sum of the area of all lesions in the corpus for each rat was calculated and served as the ulcer index. Gastroprotection (%) was calculated according to: % Gastroprotection = (UIC − UIT) × 100/UIC, where UIC is ulcer index in control and UIT is ulcer index in test rats (Navarrete et al 1998).

Ethanol-induced gastric mucosal lesions in indometacin-pretreated rats

To investigate the involvement of endogenous prostaglandins in the gastroprotective effects of Astragaloside IV and carbenoxolone, the rats were divided into groups according to the respective treatment. The control group received a subcutaneous injection of $NaHCO_3$ (5 mM) in saline solution and the others, an injection of indometacin ($10\,mg\,kg^{-1}$, dissolved in $NaHCO_3$, 5 mM) by the same route (Matsuda et al 1999). After 75 min, all the groups received the respective treatment orally (saline solution, $30\,mg\,kg^{-1}$ of Astragaloside IV or $30\,mg\,kg^{-1}$ of carbenoxolone). Absolute ethanol was given to each rat 30 min after drug administration and the rats were killed 2 h later by ether inhalation. The stomachs were subsequently removed to measure the ulcer index, as described earlier.

Ethanol-induced gastric mucosal lesions in L-NAME-pretreated rats

To investigate the involvement of endogenous NO in the protective effects Astragaloside IV and carbenoxolone, L-NAME ($70\,mg\,kg^{-1}$ dissolved in saline solution) was intraperitoneally injected 30 min before the administration of either vehicle, Astragaloside IV ($30\,mg\,kg^{-1}$) or carbenoxolone ($30\,mg\,kg^{-1}$) by the oral route (Matsuda et al 1999). Absolute ethanol was given to each rat 30 min later and rats were killed 2 h after the administration of ethanol to measure the intensity of the gastric ulcers. Two control groups (L-NAME-treated and non-L-NAME-treated) were included in this experiment.

Ethanol-induced gastric mucosal lesions in NEM-pretreated rats

To investigate the involvement of endogenous sulfhydryls in the protective effects of Astragaloside IV and carbenoxolone, NEM ($10\,mg\,kg^{-1}$, dissolved in saline solution) was subcutaneously injected 30 min before the administration of either vehicle, Astragaloside IV ($30\,mg\,kg^{-1}$) or carbenoxolone ($30\,mg\,kg^{-1}$) by the oral route (Matsuda et al 1999). Absolute ethanol was given to each rat 30 min later and rats were killed 2 h after the administration of ethanol to measure the intensity of the gastric ulcers. Two control groups (NEM-treated and non-NEM-treated) were included in this experiment.

Statistics

Data are presented as the mean ± s.e.m. from 6 rats per group. Statistically significant differences between the treatments were tested by Kruskal–Wallis test (non-parametric one-way analysis of variance) followed by Dunn's multiple comparison test. $P < 0.05$ was considered significant.

Results

Astragaloside IV ($3–30\,mg\,kg^{-1}$) administered orally reduced the ethanol-induced gastric haemorrhagic lesions in a dose-dependent manner compared with responses obtained to control group (Figure 2A). The maximum percentage inhibition of ulcers (% gastroprotection) obtained with $30\,mg\,kg^{-1}$ Astragaloside IV following oral administration was 52.3 ± 9%.

Similarly, carbenoxolone ($3–30\,mg\,kg^{-1}$), given to rats orally 30 min before ethanol treatment, produced a dose-dependent inhibition of haemorrhagic lesions (Figure 2B). The maximum % gastroprotection obtained for the highest dose of carbenoxolone ($30\,mg\,kg^{-1}$) was 88.4 ± 13.6%, showing carbenoxolone to be more potent than Astragaloside IV.

The ulcer indexes of the rats treated with $70\,mg\,kg^{-1}$ of L-NAME ($121.94 \pm 11.4\,mm^2$, Figure 3A), $10\,mg\,kg^{-1}$ of indometacin ($100.96 \pm 11.52\,mm^2$, Figure 3B) and $10\,mg\,kg^{-1}$ of NEM ($111.5 \pm 11.50\,mm^2$, Figure 3C) were not significantly ($P > 0.05$) different compared with their respective controls treated only with saline solution ($120.57 \pm 10.79\,mm^2$, $132.17 \pm 11.4\,mm^2$ and $120.57 \pm 10.79\,mm^2$). It is very well recognized that these doses inhibit NO synthase (NOS), inhibit prostaglandin synthesis and blockade the endogenous sulfhydryls, respectively, but do not cause ulcers (Matsuda et al 1999; Arrieta et al 2003).

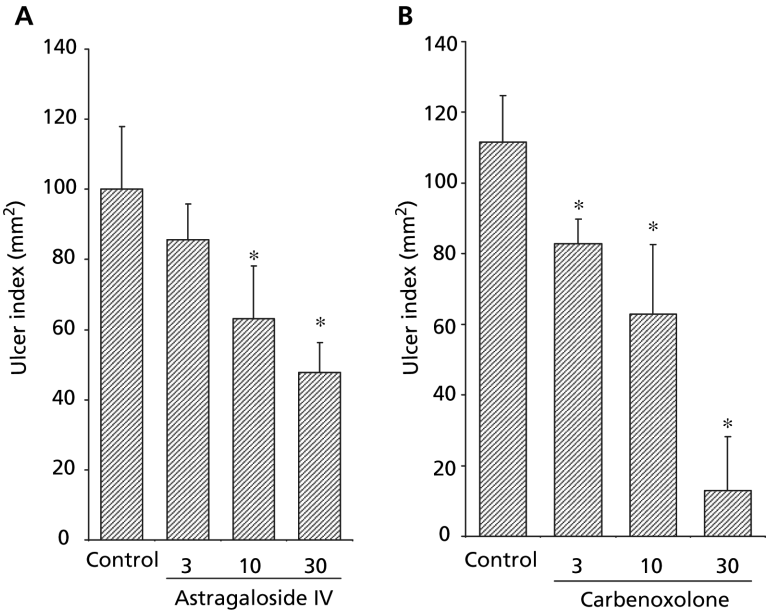

Figure 2 Effect of different doses of Astragaloside IV (3–30 mg kg^{-1}) (A) and carbenoxolone (3–30 mg kg^{-1}) (B) administered orally, on gastric haemorrhagic lesions induced in rats by absolute ethanol (1 mL/rat). Bars represent the mean ± s.e.m., n = 6. *$P < 0.05$, vs respective control; Dunn's multiple comparison test after Kruskal–Wallis test.

Pretreatment with L-NAME (70 mg kg^{-1}, s.c.) attenuated the gastroprotective effect of both Astragaloside IV (30 mg kg^{-1}) and carbenoxolone (30 mg kg^{-1}) (Figure 3A). The ulcer index obtained in the rats treated with Astragaloside IV (87.95 ± 16.25 mm^2) and carbenoxolone (85.9 ± 18.0 mm^2) were not significantly ($P > 0.05$) different compared with the L-NAME-pretreated controls (121.94 ± 11.4 mm^2), whereas the values for Astragaloside IV and carbenoxolone in L-NAME-pretreated rats were significantly ($P < 0.05$) higher than the ulcer index values obtained for the same drugs in non-L-NAME-treated rats (47.7 ± 8.69 mm^2 and 12.94 ± 5.40 mm^2, respectively) (Figure 3A).

Astragaloside IV (30 mg kg^{-1}), administered orally, produced inhibition of ethanol-induced haemorrhagic lesions in indometacin (10 mg kg^{-1})-pretreated rats. The ulcer index obtained for Astragaloside IV-treated rats was 47.6 ± 16.3 mm^2. This value was significantly ($P < 0.05$) different to that of the indometacin-pretreated controls (100.96 ± 11.52 mm^2), whereas the value for 30 mg kg^{-1}

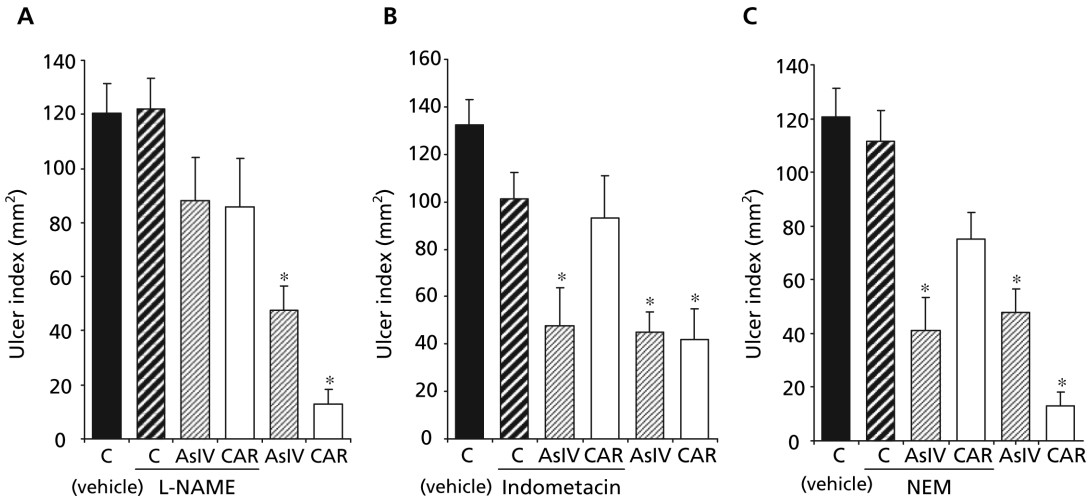

Figure 3 Effect of orally administered Astragaloside IV (AsIV, 30 mg kg^{-1}) and carbenoxolone (CAR, 30 mg kg^{-1}) on gastric haemorrhagic lesions induced by absolute ethanol (1 mL/rat) in rats pretreated with L-NAME (70 mg kg^{-1}, i.p.) (A), indometacin (10 mg kg^{-1}, s.c.) (B) or NEM (10 mg kg^{-1}, s.c.) (C) before the test drug. Bars represent the mean ± s.e.m., n = 6. *$P < 0.05$, vs the respective control; Dunn's multiple comparison test after Kruskal–Wallis test. No differences were observed between vehicle-treated control and control pretreated with the inhibitor or blocker.

carbenoxolone ($93.0\ 6 \pm 17.9\,mm^2$) was not significantly different ($P > 0.05$) from the control value (Fig 3B). There was significant difference ($P < 0.05$) between indometacin-treated and non-indometacin-treated rats for carbenoxolone ($93.06 \pm 17.9\,mm^2$ vs $42.0 \pm 17.9\,mm^2$, respectively), but not for Astragaloside IV ($47.6 \pm 16.3\,mm^2$ vs $45.0 \pm 8.6\,mm^2$, respectively) (Figure 3B).

Oral administration of $30\,mg\,kg^{-1}$ Astragaloside IV to NEM ($10\,mg\,kg^{-1}$)-pretreated rats produced a reduction in the ethanol-induced gastric haemorrhagic lesions. The ulcer index obtained following the administration of Astragaloside IV was $41.3 \pm 11.9\,mm^2$, which was significantly ($P < 0.05$) different to the NEM-pretreated control value of $111.5 \pm 11.5\,mm^2$ (Figure 3C), whereas the value for $30\,mg\,kg^{-1}$ carbenoxolone ($75 \pm 9.9\,mm^2$) was not significantly different ($P > 0.05$) to the control value (Figure 3C).

Discussion

According our hypothesis, Astragaloside IV, with a hydroxyl derivate with ß-D-xylopyranosyl at position C-3, should show anti-ulcer activity, as it was observed. This gastroprotective effect was dose dependent. Therefore, Astragaloside IV is one more triterpenoid with a hydroxyl derivate at C-3 and with anti-ulcer activity. Several plants containing high amounts of saponins have been shown to possess anti-ulcer activity in several experimental ulcer models (Borelli & Izzo 2000). Glycyrrhizic acid, aescine (a mixture of saponins) and momordin Ic are some examples of saponins with anti-ulcer activity (Marhuenda et al 1993; Matsuda et al 1999). The gastroprotective activity of those saponins are not due to inhibition of gastric acid secretion but probably due to activation of mucous membrane protective factors (Borrelli & Izzo 2000).

It is well known that ethanol produces gastric mucosal damage within 1–3 min of its instillation into the gut and lasts for more than 2 h by causing areas of focal hyperaemia and haemorrhage (Chandranath et al 2002). Moreover, intragastric administration of ethanol increases vascular permeability and vascular damage occurring in capillaries near the luminal surface and not in the deeper muscularis mucosa, which indicates a role for impaired blood flow in the genesis of ethanol-induced gastric lesions (Chandranath et al 2002). It is known that authentic NO or NO donors markedly reduce the severity of damage to the gastric mucosa induced by topical application of ethanol (Elliot & Wallace 1998). NO, interactively with prostanoids and sensory neuropeptides, regulates gastric mucosal integrity in rats (Whittle et al 1990). NO participates in the gastric defence mechanisms by regulating the gastric mucosal blood flow, acid and alkaline secretion and gastric mucus secretion (Whittle & Lopez-Belmonte 1993). The previous administration of L-NAME, an NO-synthase inhibitor, reduced the anti-ulcerogenic activity of Astragaloside IV, suggesting that NO participates in the gastroprotection of this saponin. Astragaloside IV shares several activities with NO, such as the inhibition of leucocyte adhesion to endothelial cells (Zhang et al 2003), potent free radical scavenging capable of reducing both superoxide and hydroxyl radicals (Ma & Yang 1999) and inhibition of the production and release of the inflammatory mediators interleukin-1 and tumour necrosis factor (TNFα) (Elliot & Wallace 1998; Luo et al 2004). It has been suggested that NO acts as an antioxidant in mucosal dysfunction associated with ischaemia–reperfusion or induced by ethanol or indometacin, or in other inflammatory conditions of the gastrointestinal tract (Denizbasi et al 2000). Recently, protective activity against ischaemic brain injury has been described for Astragaloside IV (Luo et al 2004). The results obtained here provide the first experimental evidence that relates the activity of Astragaloside IV with NO. It may be possible that other activity described for Astragaloside IV occurs also through participation of NO.

As it was demonstrated here, the inhibition of NO synthesis by L-NAME reversed the gastroprotective activity of Astragaloside IV, suggesting that the anti-ulcer activity of this glycoside is through the participation of NO. The role of NO in gastroprotection has been widely accepted (Elliot & Wallace 1998; Cui et al 2002). An increase of NO levels by L-arginine (a substrate for NOS), but not D-arginine, has been shown to reduce gastric lesions induced by absolute ethanol (Brzozowski et al 1997). The mechanism underlying the L-arginine-induced gastric protection has been described as being due to a significant increase in gastric mucosal blood flow (GMBF) and angiogenesis, without reduced secretion of mucus and bicarbonates. In contrast, treatment with N^G-nitro-L-arginine (L-NNA, an inhibitor of NO synthase) reverses the effects of L-arginine (Brzozowski et al 1997). The opposing effects of L-arginine and L-NAME have also been observed in other ulcer models (Elliot & Wallace 1998; Cui et al 2002).

Prostaglandins and sulfhydryls seem to have little participation in the mechanism of gastroprotection of Astragaloside IV, because the gastroprotection of this compound was not affected by pretreatment with indometacin, a cyclooxygenase inhibitor, or NEM, a sulfhydryl blocker (Szabo et al 1981; Wan & Gottfried 1985). NO appeared to be the major gastroprotective factor associated with the mechanism of gastroprotection of Astragaloside IV. However, other protective mechanisms involving the activation of capsaicin-sensitive afferent neurons, not explored here, may be stimulated, as shown by Oliveira et al (2004) with other triterpenoids.

The results obtained here for carbenoxolone are in agreement with those previously reported (Wan & Gottfried 1985; Arrieta et al 2003). As it is known, prostaglandins and, partially, NO and sulfhydryls, are intensively involved in the gastroprotective mechanism of carbenoxolone (Arrieta et al 2003).

Conclusion

In this work the gastroprotective activity of Astragaloside IV was demonstrated. Endogenous NO plays an important role in the gastroprotective mechanism of Astragaloside IV on ethanol-induced gastric lesions.

These results give additional support to our proposal that triterpenoids with free or derivate hydroxyl at C-3 have anti-ulcer activity.

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