Gastric Cytoprotective Activity of Dehydroleucodine in Rats. Role of Prostaglandins

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Previously, we reported that dehydroleucodine (DhL), a sesquiterpene lactone, protected the gastric mucosa of rats from absolute ethanol-induced lesions in a dose-dependent fashion. The mechanism is not mediated by an antiacid secretory action and DhL stimulated mucous production. In the present study, we report the effect of DhL on the mucosal production of prostaglandin E (PGE) and the mucosal release of PGE₂ in rats stomach. DhL in acute treatment does not modify these values decreased by previous treatment with indomethacin or absolute ethanol. However, DhL in subchronic treatment significantly enhanced the mucosal production of PGE and the mucosal release of PGE₂. Also, indomethacin pretreatment resulted in a significant reduction of the cytoprotective action of DhL. These results indicate the participation of endogenous prostaglandins in DhL protection against ethanol damage. Moreover, we suggest that the gastric protective activity of DhL against ethanol induced gastric mucosal damage is mediated, at least in part, through PGE and PGE₂ in subchronic treatment.

Key words prostaglandin E; prostaglandin E₂; gastric cytoprotection; dehydroleucodine

The aerial part of *Artemisia douglasiana*, used in folk medicine as a cytoprotective agent against the development of peptic ulcer, was studied, and its active principle dehydroleucodine (DhL) was isolated in our laboratory.¹⁾ We have demonstrated that DhL and other related sesquiterpene lactones significantly prevent the formation of gastric lesions induced by various necrotizing agents.²⁾ Furthermore, we attributed this cytoprotective activity to the presence of a non-hindered Michael acceptor in the molecules assayed and suggested that the mechanism of protection would be, at least in part, through prostaglandin (PG) synthesis or mucosal glycoprotein synthesis.²⁾ Later, we clearly demonstrated that gastroprotection is dependent on incremental increases in the synthesis of mucosal glycoproteins produced by DhL,³⁾ and for histological studies we have confirmed the increase of mucous production by treatment with DhL.⁴⁾

It is now well established that PGs can increase the mucosal resistance of the gastrointestinal tract to injury in several experimental models. It is also well established that the suppression of PG synthesis is a key component of the mechanism underlying gastric ulceration caused by nonsteroidal anti-inflammatory drugs (NSAIDs).^{5–8)} Experiments in animals administered exogenous PGs show macroscopic protection from mucosal injury when exposed to various noxious topical agents such as absolute ethanol or when administered NSAIDs.⁹⁾ Robert coined the term cytoprotection for this remarkable phenomenon.⁹⁾ Altered mucin and bicarbonate secretion, along with augmented mucosal blood flow, have been suggested as possible mechanisms to explain this observation^{9–12)} and —through mechanisms which are not yet fully understood— by enhancing the resistance of epithelial cells to injury induced by cytotoxins.¹³⁾

Gastric mucosal damage can be induced experimentally by topical irritants such as ethanol. Since the gastric mucosa has a high capacity to synthesize PGs from an endogenous substrate, it has been suggested that protection against damage

may result from the stimulation of mucosal PG biosynthesis.¹⁴⁾ Alternatively, the increased biological activity of locally generated PGs can be achieved by inhibiting the degradation of PGs to 15-keto-13,14-dihydro-prostaglandins, which are devoid of biological activity.¹⁵⁾ The inhibition of PG-metabolizing enzymes has been demonstrated for the antiulcer drug carbenoxolone.¹⁶⁾

The purpose of this study constitutes a further characterization of the antiulcerogenic activity mechanism of DhL, studying whether this sesquiterpene lactone affects the mucosal production of prostaglandin E (PGE) or the mucosal release of PGE₂ in acute or subchronic treatment.

MATERIALS AND METHODS

General Procedures DhL was isolated and identified as reported in previous works.¹⁾

Animals and Experimental Protocol DhL protected the gastric mucosa of rats from absolute ethanol-induced lesions in a dose-dependent fashion. This effect was significant when DhL at 40 mg/kg was given 1 h before ethanol.¹⁾ Wistar rats (200–220 g) of both sexes were employed. In one experiment for the PGE₂ study, rats were fasted for 24 h and deprived of water for 19 h prior to the experiment. All rats were housed in wire mesh-bottomed cages throughout the study to prevent coprophagy. The animals were grouped in six lots ($n=5$). DhL (40 mg/kg) suspended in 0.4% carboxymethylcellulose (CMC) was administered, *p.o.*, to one group of rats. The other group was injected with indomethacin, 10 mg/kg, *s.c.*, 30 min before the administration of CMC, and the other lot received indomethacin, 10 mg/kg, *s.c.*, 30 min before the administration of DhL. In another lot, gastric mucosal damage was induced by absolute ethanol (1 ml/rat, *p.o.*) according to Robert *et al.*,¹⁷⁾ while the other lot received DhL, 40 mg/kg, 1 h before absolute ethanol. The control rats were given 0.4% CMC orally. The animals were decapitated 1 h

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after the last administration and the stomachs were rapidly removed. The experiment for the PGE study was performed with 3 lots of rats ($n=10$). The control group received the vehicle (0.4% CMC) intragastrically. Two experimental groups received DhL, 40 mg/kg, intragastrically, in acute or subchronic treatment (11 d, twice daily), respectively. The animals were decapitated 1 h after the last administration and the stomachs were rapidly removed. DhL was prepared just before the experiment and suspended in 0.4% CMC. In another experiment, rats were grouped in two lots ($n=8$) for PGE₂ study in subchronic treatment, and were administered with DhL, 40 mg/kg, orally (11 d, twice daily). Animals were sacrificed 1 h after the last administration and their stomachs were removed and opened along the greater curvature.

Induction of Gastric Lesions Gastric lesions were produced according to the method of Robert *et al.*¹⁷⁾ Wistar rats weighing 200–220 g were fasted for 24 h and deprived of water for 19 h prior to the experiments. All rats were housed in wire mesh-bottomed cages throughout the study to prevent coprophagy. Absolute ethanol (1 ml) administered orally was employed as the necrotizing agent, and 1 h later, the animals were decapitated. The stomachs were removed, opened along the greater curvature, and washed gently with ice-cold saline solution. The degree of erosion in the glandular part of the stomach was assessed from a scoring system designed by Marazzi-Uberti and Turba¹⁸⁾ from 0 (no erosions) to 5 (maximal damage). All data are presented as medians and a range for a given number of animals. DhL and indomethacin were prepared just before the experiment and suspended in 0.4% CMC. The control rats were given 1 ml absolute ethanol (*p.o.*). In other lot, DhL (40 mg/kg) was given 60 min before the administration of ethanol. Another group was injected with indomethacin (10 mg/kg, *s.c.*) 30 min before the administration of DhL. In another experiment, rats were grouped in lots for gastric cytoprotective activity study in subchronic treatment, and were administered with DhL, 40 mg/kg, orally (11 d, twice daily). Another group was injected with indomethacin (10 mg/kg, *s.c.*) 30 min before the last administration of DhL.

PGE Radioimmunoassay The samples (stomachs) were weighed and incubated in Krebs-Ringer bicarbonate solution (KRB), with glucose (11.0 mM) as the external substrate, for 1 h in a Dubnoff metabolic shaker under an atmosphere of 5% CO₂/95% O₂ at 37 °C. The ionic composition of the KRB has been reported previously.¹⁹⁾

At the end of the incubation period, tissues were removed and used for protein determination. For PG extraction, the solution in which we had suspended the preparations was acidified to pH 3.0 with 1 N HCl and extracted three times with 1 volume of ethyl acetate. Pooled ethyl acetate extracts were dried under an atmosphere of N₂ and stored at –20 °C until PGE radioimmunoassay.

PGE was quantified by radioimmunoassay using a rabbit antiserum from Sigma Chemical Co. The tissue residues and all the reagents were reconstituted in 0.01 M phosphate buffered saline (pH 7.4) containing 0.15 M NaCl, 0.1% bovine serum albumin (BSA) and 0.1% sodium azide. Fractions (100 µl) of standards or sample extracts were incubated with the antiserum (500 µl) for 30 min at 4 °C. Bound and free forms were separated by a dextran coated charcoal suspension (0.1–1.0%). Sensitivities of these assays were

10 pg/tube. The cross-reactivity of PGE₂ was 90% with PGE₁ and less than 0.1% with other PGs.

Metabolism of [¹⁴C]Arachidonic Acid by Samples After preincubation of the stomachs for 60 min in KRB, they were incubated for 1 h in 2 ml of medium containing 0.25 µCi of [¹⁴C]arachidonic acid (New England Nuclear; 52.9 Ci/mol; 1 Ci=37 Gbq).

At the end of the incubation period, tissues were removed and the remaining incubation medium was acidified to pH 3.0 with 1.0 M HCl. The arachidonic acid metabolites were extracted two times with 2 ml of ethyl acetate. Pooled ethyl acetate extracts were dried under nitrogen. The residue was suspended in chloroform/methanol (2:1, v/v) and applied to silica gel TLC plates. Before the application of extracts, reference compounds, 6-keto-prostaglandin F (PGF_{1α}), PGF_{2α}, thromboxane B₂ and PGE₂ were placed on the plates. The plates were developed in a solvent system of benzene/dioxane/glacial acetic acid (60:30:3.0, v/v/v). The position of authentic PGs was visualized by spraying the dried plates with 10% phosphomolibdic acid in ethanol, followed by heating at 110 °C for 10 min; the plates were scraped off and ¹⁴C radioactivity was measured by liquid scintillation counting. Average *R_f* values were 0.30 for 6-keto-PGF_{1α}, 0.35 for PGF_{2α}, 0.47 for PGE₂, 0.57 for thromboxane B₂ and 0.80 for arachidonic acid. The results are expressed as the percentage conversion of [¹⁴C]arachidonic acid per mg of stomach over 60 min.

Drugs and Chemicals PGs, CMC and indomethacin were purchased from Sigma Chemical Co. and [³H]PGE (specific activity 150–170 Ci/mmol) from NEN Research Products. All other chemicals used were of reagent grade.

Statistics The statistical significance of difference among means was assessed by analysis of variance (ANOVA) with the multiple comparison method by Tukey, and by Student's *t*-test. Differences between means were considered significant at $p<0.05$. In Table 1, all data are presented as medians and a range for a given number of animals. Statistically significant differences between experimental conditions were assessed using the Kruskal-Wallis and Mann Withney test.

RESULTS AND DISCUSSION

The evidence that endogenous PGs play an important role in maintaining normal resistance to luminal injurious factors has been pointed out.²⁰⁾ Different mechanisms underlie the effects of gastroprotective agents. Only certain protective compounds involve the gastric mucosal PG system, either by stimulating biosynthesis or by inhibiting degradation.²¹⁾ Pharmacological studies support the hypothesis that a deficiency of PGE₂ is one of the causes of gastric ulcers. Baumeister *et al.* showed a decrease in PGE₂ accumulation exists in the incubation medium of patients with gastric ulcer or acute gastritis compared with normal subjects. Drugs that decrease endogenous PGE₂ synthesis promote the mucosal damage of the stomach.²²⁾ Orally effective methyl analogues of PGE₁ and PGE₂ are successfully used in the treatment of peptic diseases.²³⁾ We focused our experiment on the mucosal production of PGE and the mucosal release of PGE₂ in acute or subchronic treatment. PGE₂ causes higher mucous²⁴⁾ and bicarbonate production²⁵⁾ and stabilizes the gastric mu-

Table 1. Effect of Administration of Indomethacin (10 mg/kg, s.c.) on the Gastroprotective Action of DhL in Acute and Subchronic Treatments

Treatment prior to administration of ETOH		Damage score	
60 min	30 min	Median*	Range
CMC	CMC	5 (13)	4.5—5
CMC	DhL acute	0.5 ^{a)} (9)	0—0.5
Indomethacin	DhL acute	1 ^{b)} (5)	1—1.5
CMC	DhL subchronic	0.5 ^{a)} (4)	0.4—0.5
Indomethacin	DhL subchronic	1.5 ^{b)} (5)	1—1.5

DhL (40 mg/kg, *p.o.*) was administered in a single dose or for 11 d, twice daily, respectively. All data are presented as medians and range. Number of animals is given in parentheses. * Values denoted by letters differ significantly from those in the CMC—CMC prior ethanol treatment controls. a) $p < 0.02$; b) $p < 0.05$; a) $\neq$ b), $p < 0.05$ by Kruskal-Wallis and Mann Whitney test.

Fig. 1. Effect of DhL in a Single Dose of 40 mg/kg, and Previous Treatment with Indomethacin, 10 mg/kg, s.c. (INDO) or Absolute Ethanol (ETOH) on the Formation of PGE₂ from [¹⁴C]Arachidonic Acid ([¹⁴C]AA)

CMC is the treatment for the control. Each bar represents the mean $\pm$ S.E.M. for 5 rats. Analysis of variance (ANOVA) test was used, as well as posterior comparison by Tukey (HSD). Significant difference from CMC is shown as * $p < 0.001$; a, not significant vs. CMC; b, not significant vs. INDO alone; c, not significant vs. ETOH alone.

cosal barrier²⁶⁾ by different mechanisms, including the secretion of surface-active phospholipids.²⁷⁾ In addition, PGE₂ increases the mucosal blood supply by vasodilation²⁸⁾ and enhances the regeneration rate of gastric mucosa.²⁹⁾

The present study showed that the protective activity of DhL against ethanol-induced gastric mucosal lesions in rats was significantly reduced when indomethacin, a classic inhibitor of PG synthesis, was given before DhL (Table 1). This is in agreement with a previous study.¹⁾ This fact suggested that this agent may indeed protect the gastric mucosa by inducing the formation of protective PGs and that these PGs were responsible, at least in part, for the effect observed. In the experiment performed to study whether DhL affects the mucosal release of PGE₂, in contrast to subchronic treatment, a single dose of DhL did not modify the basal production of PGE₂ in the rat stomach preparation incubated with [¹⁴C]arachidonic acid. On the other hand, DhL did not modify the values decreased by previous treatment with indomethacin or by previous treatment with absolute ethanol (see Fig. 1). These findings are partially similar to the results obtained with carbenoxolone³⁰⁾ and rebamipide.²¹⁾ Although the rat stomach pretreated with indomethacin attenuated the protective activity of carbenoxolone, suggesting that it is par-

Fig. 2. Effect of DhL in Acute (DhL AC) or Subchronic (DhL SUBC) Treatments on the Formation of PGE

CMC is the treatment for the control. Each bar represents the mean $\pm$ S.E.M. for 10 rats. Analysis of variance (ANOVA) test was used, as well as posterior comparison by Tukey (HSD); * $p < 0.01$ vs. control.

Fig. 3. Effect of DhL in Subchronic Treatment on the Formation of PGE₂ from [¹⁴C]Arachidonic Acid ([¹⁴C]AA)

Each bar represents the mean $\pm$ S.E.M. for 8 rats. Student's *t*-test was used. * $p < 0.001$ vs. control (CMC).

tially mediated by endogenous PGs,³⁰⁾ administration of the drug did not increase the eicosanoid content in the gastric mucosa.³¹⁾ In contrast, treatment with carbenoxolone significantly enhanced the secretion of PGE₂ into the gastric juice in peptic ulcer patients.³²⁾ In our study, animals that were administered DhL for 11 d showed significantly increased levels of PGE obtained for radioimmunoassay ($p < 0.01$ vs. control and acute treatment group). The results are shown in Fig. 2. Moreover, the PGE₂ released into the incubation medium from the stomachs of rats subchronically treated with DhL increased significantly ($p < 0.001$). The results can be seen in Fig. 3.

In an attempt to elucidate the factors involved in the mechanism of DhL-induced mucosal gastric protection, we demonstrated that: DhL prevents the formation of gastric mucosal lesions induced by absolute EtOH and by other necrotizing agents and increases the synthesis of gastric mucosal glycoprotein;^{1,3,33)} DhL is a non-antisecretory agent which does not inhibit acid secretion in the pylorus-ligated rat (unpublished observations); DhL increases mucous pro-

duction, confirmed by histological studies, in gastric and duodenal mucosa.^{4,34)} Also, the protective effect of DhL, in acute and subchronic treatments, was counteracted by pretreatment with indomethacin, indicating the participation of endogenous PGs. DhL increases the gastric mucosal generation of PGE and the mucosal release of PGE₂ in subchronic treatment. So, we suggest that the protective activity of DhL against ethanol-induced gastric mucosal damage is mediated, at least in subchronic treatment, by the stimulation of endogenous PGE₂ formation, and that these PGs were responsible for the increased mucous production. Moreover, the protective effect of DhL was not totally abolished by pretreatment with indomethacin, in acute and subchronic treatment, indicating that additional mechanisms are involved in the cytoprotective action of DhL.³⁾

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