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ORIGINAL ARTICLE

Carbenoxolone gastroprotective mechanism: participation of nitric oxide/$_c$GMP/K_{ATP} pathway in ethanol-induced gastric injury in the rat

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ABSTRACT

Carbenoxolone, a semi-synthetic triterpenoid, exhibits gastroprotective activity related to the participation of nitric oxide (NO); however, the complete NO/$_c$GMP/K_{ATP} channels pathway for carbenoxolone is unknown. Therefore the aim of this study was to examine the NO/$_c$GMP/K_{ATP} channels pathway as the gastroprotective mechanism of carbenoxolone in the ethanol-induced gastric injury model in the rat. Oral administration of carbenoxolone (30 mg/kg, p.o.) exhibited gastroprotective effect against ethanol-induced gastric injury in rats. Pretreatment with N^G-nitro-L-arginine methyl ester (L-NAME, 70 mg/kg, i.p.); 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ, guanylate cyclase inhibitor, 10 mg/kg, i.p.); or glibenclamide (K_{ATP} channels inhibitor, 1 mg/kg, i.p.) reversed the gastroprotective effect of carbenoxolone for ethanol-induced gastric injury. Furthermore, gastric prostaglandins and NO levels increased after carbenoxolone administration in ethanol-induced gastric injury in rats. In conclusion, our results suggest that the increase of NO levels in gastric tissue after pretreatment with carbenoxolone activates the NO/$_c$GMP/K_{ATP} channels pathway, the principal gastroprotective mechanism of carbenoxolone.

INTRODUCTION

Carbenoxolone, a semisynthetic triterpenoid obtained as the succinate sodium salt of 18-β-glycyrrhetic acid from *Glycyrrhiza glaba* [1], has been shown to induce anti-inflammatory [2], antiulcer actions [2–5]; accelerate ulcer healing in humans [2]; and reduce glucose production during hyperglucagonemia in diabetic patients [6]. Several studies related to mediators implicated in the gastroprotective effects of carbenoxolone have been done. Carbenoxolone exhibits gastric cytoprotective action partly through the participation of endogenous prostaglandins (PGs) [4]. The gastroprotection of carbenoxolone is diminished but not abolished by the cyclooxygenase inhibitor indomethacin [7] and carbenoxolone increased $PGF_{1\alpha}$ and PGE_2 [8,9]. Furthermore, carbenoxolone has been shown to induce an increase in pH duodenal and HCO_3^- secretion in a dose-dependent manner [10].

Nitric oxide (NO), a gas mediator with vasodilatador properties interacts in gastric mucosa with neuropeptides and PGs to maintain mucosal integrity in basal conditions; this gas mediator participates in defending gastric mucosa by regulating gastric mucosa blood flow, acid, and alkaline mucus secretion [11]. NO activates guanylyl cyclase to increase cyclic guanosine monophosphate ($_c$GMP) levels in several tissues. Recently, the activation

of ATP sensitive potassium channels (K_{ATP}) by this pathway has been described in gastroprotection; Medeiros et al. [12] report that sildenafil protects against ethanol-induced gastric injury through the NO/$_c$GMP/K_{ATP} channels pathway. In addition, the contribution of NO to the gastroprotective effect of carbenoxolone on ethanol-induced gastric injury in the rat has been described [4,5]. However, the complete NO/$_c$GMP/K_{ATP} channels pathway has not been studied in the gastroprotective mechanism of carbenoxolone. Therefore, the aim of this study was to examine the activation of NO/$_c$GMP/K_{ATP} channels pathway in terms of the gastroprotective effect of carbenoxolone in the ethanol-induced gastric injury model in the rat.

MATERIAL AND METHODS

Drugs

Carbenoxolone, N^G-nitro-L-arginine methyl ester (L-NAME), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ) and glibenclamide were purchased from Sigma Aldrich (St Louis, MO, USA).

Animals

All experiments were performed with male Wistar rats (200–225 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 Handling (NOM-062-ZOO-1999) and in compliance with the international rules on care and use of laboratory animals. The number of rats per group was at least six. Rats were fed standard laboratory chow and tap water ad libitum. The rats were placed singly in cages with wire-net floors and fasted 24 h before experimentation, but allowed free access to tap water.

Acute gastric ulcers induced by absolute ethanol

Ulceration was induced according to the method described by Robert [13]. Briefly, intragastric instillation of 1 mL of absolute ethanol was administered. Two hours after ethanol administration, the rats were killed in a CO_2 chamber. The stomach and duodenum were removed, inflated with 10 mL of 4% paraformaldehyde, and placed in 4% paraformaldehyde for at least 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 (×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 [14].

Effect of N^G-nitro-L-arginine methyl ester pretreatment in carbenoxolone gastroprotection

To investigate the involvement of endogenous NO in the gastroprotective effect of carbenoxolone, L-NAME (70 mg/kg dissolved in saline solution) was intraperitoneally administered 30 min before carbenoxolone (30 mg/kg, p.o.); 30 min after carbenoxolone administration, the gastric mucosal lesions were induced and measured as described [14].

Effect of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one pretreatment in carbenoxolone gastroprotection

To investigate the role of $_c$GMP in carbenoxolone gastroprotective effect, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ, 10 mg/kg dissolved in dimethylsulfoxide 0.01%) was intraperitoneally administered 30 min before carbenoxolone (30 mg/kg, p.o.). Thirty minutes later, the gastric mucosal lesions were induced and measured as described. The dose of ODQ was selected from previous studies by Medeiros et al. [12].

Effect of glibenclamide pretreatment on carbenoxolone gastroprotection

To investigate the involvement of K_{ATP} channels on gastroprotection induced by carbenoxolone, we administered glibenclamide (1 mg/kg, dissolved in NaOH 0.05 M, i.p.) 30 min before carbenoxolone administration (30 mg/kg, p.o.). Thirty minutes later, the gastric lesions were induced and measured as described. The dose of glibenclamide was selected in accordance with previous studies by Medeiros et al. [12].

Measurement of nitrites/nitrates levels

Eighteen rats were divided into three groups of six animals each: groups 1 and 2 received saline (5 mL/kg, p.o.); group 3 received carbenoxolone (30 mg/kg, p.o.); 30 min later group 1 received saline and groups 2 and 3 received absolute ethanol (1 mL) by gastric gavage. After 2 h the rats were sacrificed in a CO_2 chamber. The stomach was removed, and a sample of the corpus region of the stomach was excised, weighed, and added to a tube containing 1 mL of sodium phosphate buffer (10 mmol/L; pH 7.4). The tissue sample was minced with scissors for 30 s and placed in a shaking water bath (37 °C) for 20 min. The samples were centrifuged (9000 *g*) for 1 min, and the concentration

of nitrites in the supernatant was determined by nitrate/nitrite colorimetric assay using the Griess reaction at 540 nm [15].

Measurement of endogenous levels of PG E_2 (PGE_2)

Three groups of at least six rats received saline, vehicle, or carbenoxolone (30 mg/kg, p.o.); 30 min later the two latest groups were gavaged with absolute ethanol (1 mL). After 2 h the rats were sacrificed in a CO_2 chamber. The stomach was removed, and a sample of the corpus region of the stomach was excised, weighed, and added to a tube containing 1 mL sodium phosphate buffer (10 mmol/L; pH 7.4). The tissue sample was minced with scissors for 30 s and placed in a shaking water bath (37 °C) for 20 min. The samples were centrifuged (9000 ***g***) for 1 min, and the concentration of PGE_2 in the supernatant was determined by ELISA [16].

Statistical analysis

All data are expressed as mean ± SEM. Groups were compared using a one-way analysis of variance followed by the student-Newman–Keuls test. Values of $P \leq 0.05$ were considered to show significant differences between means.

RESULTS

Oral administration of carbenoxolone exhibited a gastroprotective effect on ethanol-induced gastric injury in rat, while L-NAME reversed the carbenoxolone-induced gastroprotective effect (*Figure 1*). In addition, gastric injury induced by ethanol showed decreased gastric NO levels, measured as nitrites/nitrates, compared with basal conditions, while treatment with carbenoxolone on ethanol-induced gastric injury rats reestablished gastric NO levels as basal group (*Table I*). Gastric PGs diminished after ethanol-induced gastric injury compared with basal conditions, while pretreatment with carbenoxolone in rats treated with ethanol to induce gastric damage increased PGs levels in gastric tissue (*Table I*).

Table I Levels of gastric nitrites/nitrates and PGE_2.

Treatment	Nitrites/nitrates (ng/g of tissue)	PGE_2 (ng/g of tissue)
Saline + saline	119.60 ± 18.67	139.20 ± 34.4
Saline + ethanol	61.44 ± 7.40*,**	68.26 ± 4.7*,**
Carbenoxolone + ethanol	117.50 ± 9.76	145.10 ± 10.09

Levels of gastric nitrites/nitrates and PGE_2 for basal conditions (saline + saline), control group (saline + ethanol) and carbenoxolone treated group (30 mg/kg, p.o.; Carbenoxolone + ethanol). Data are presented as mean ± SEM ($n = 6$). *$P \leq 0.05$ vs. saline + saline, **$P \leq 0.05$ vs. carbenoxolone + ethanol.

The inhibition of guanylate cyclase by ODQ significantly reversed the carbenoxolone-induced gastroprotective effect (*Figure 2*), evidencing the role of $_c$GMP in the gastroprotective effect of this compound. In addition, glibenclamide, a K_{ATP} channels inhibitor, prevented the gastroprotective effect of carbenoxolone (*Figure 3*).

DISCUSSION

The results showed that carbenoxolone exerted its gastroprotective effect due to the increase of gastric NO

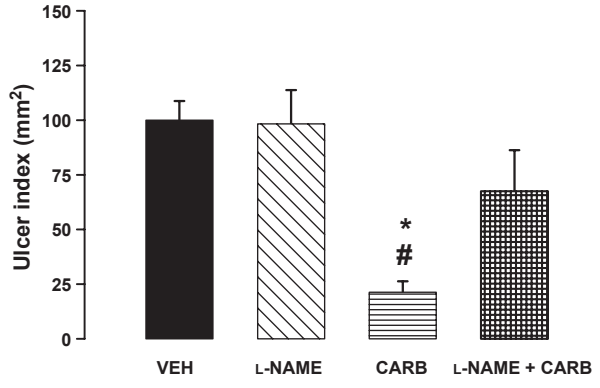

Figure 1 Effects of N^G-nitro-L-arginine methyl ester (L-NAME, 70 mg/kg, i.p.) on carbenoxolone (CARB; 30 mg/kg, p.o.) gastroprotection on gastric lesions induced by ethanol in rats. Data are presented as mean ± SEM ($n = 6$). *$P \leq 0.05$ vs. vehicle (VEH), #$P \leq 0.05$ vs. L-NAME + Carbenoxolone (L-NAME + CARB).

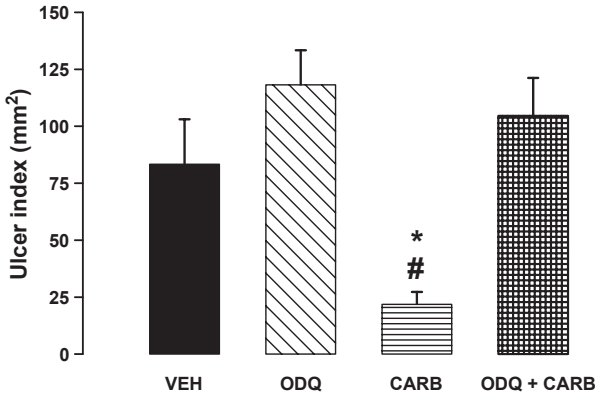

Figure 2 Effects of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ, 10 mg/kg i.p.) on carbenoxolone (CARB; 30 mg/kg, p.o.) gastroprotection on gastric lesions induced by ethanol in rats. Data are presented as mean ± SEM ($n = 6$). *$P \leq 0.05$ vs. vehicle (VEH), #$P \leq 0.05$ vs. ODQ + Carbenoxolone (ODQ + CARB).

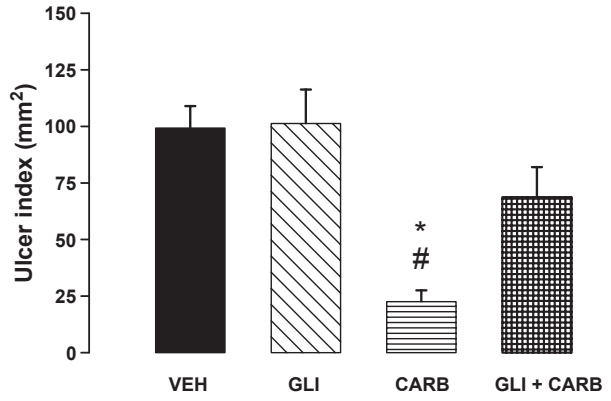

Figure 3 Effects of glibenclamide (1 mg/kg, i.p.) on carbenoxolone (CARB; 30 mg/kg, p.o.) gastroprotection on gastric lesions induced by ethanol in rats. Data are presented as mean ± SEM ($n = 6$). $^{*}P \leq 0.05$ vs. vehicle (VEH), $^{\#}P \leq 0.05$ vs. glibenclamide + Carbenoxolone (GLI + CARB).

levels, which stimulates guanylate cyclase soluble and activates K_{ATP} channels in the gastric microcirculation. Consequently, NO/$_c$GMP/K_{ATP} channels pathway contributes to the gastroprotective mechanism of carbenoxolone in ethanol-induced gastric injury model in rats.

The NO/$_c$GMP/K_{ATP} channels pathway has been found to participate in the cardiovascular system (regulating vascular tone and platelet function), the nervous system (neurotransmission and, possibly, long-term potentiation and depression) [17] and antinociception [18–20]. Furthermore, sildenafil, a phosphodieterase-type 5 inhibitor, exhibits a gastroprotective effect on ethanol-induced gastric injury through the activation of the NO/$_c$GMP/K_{ATP} channels pathway [12].

Previous reports have shown the participation of NO in carbenoxolone-induced gastroprotection in the rat [4,5]; however, gastric NO levels were not measured. Our results demonstrated an increase in gastric NO levels by carbenoxolone treatment in ethanol-induced gastric injury reported as nitrates/nitrites. It is known that ethanol induces severe histopathological changes in oxyntic mucosa of mouse and rat stomach: acute erosive hemorrhagic lesions, vascular congestion, edema, and necrosis [17,21], but it is also known that NO modulates several elements of gastric mucosal defense [22], therefore NO levels increased by carbenoxolone can be related with an increase the mucosal defense factors. It is also known that ethanol administration induces injury by constricting venules, an effect that is reversed by PG exogenously administered [23]. Absence of blood flow develops extensive gastric mucosa damage within a short period after being in contact with absolute ethanol [24,25].

NO and PGs play a key role in modulating gastric microcirculation [26]; mucosal microcirculation is essential for the capture of oxygen and nutrients and the elimination of toxic substances in gastric tissue. Mucosal blood perfusion is physiologically dependent on NO synthesis [27]. NO and PGs stimulate mucus synthesis due a direct action on gastrin cells [28] and the stimulation of parietal cells [29], respectively. Carbenoxolone increases mucus secretion to protect the gastric mucosa from injury [30]. The participation of PGs and NO in the carbenoxolone-induced gastroprotective effect has been shown in previous reports [4,7]. Furthermore, we have demonstrated carbenoxolone-augmented gastric NO and PGs' levels on ethanol-induced gastric injury, which could be related to the increase of mucus secretion induced by carbenoxolone pretreatment. However, data are contradictory in terms of PG level changes after ethanol-induced gastric damage: some studies suggest an increase in PG levels [31], while others report a decrease [32]. Our data agree with Zhao et al.'s results [32] in which PGs decreased during ethanol-induced gastric damage; the discrepancy between increases and decreases could be related to the ethanol concentration used to induce gastric damage. Thus, additional experiments are required to elucidate the effect of ethanol concentration on PG levels.

Leukocyte accumulation is considered necessary for the appearance of mucosal inflammatory diseases in the intestinal tract [33]; adhesion of neutrophils on areas of inflammation begins with the adhesion of circulating cells to the endothelial lining of small blood vessels [16]. Two main mechanisms through neutrophil adherence induce gastric mucosal injury: First, the factors that trigger the adherence of neutrophils to the vascular endothelium also trigger the activation of neutrophils leading to the liberation of oxygen-derived free radicals and proteases; leukocyte adherence to the vascular endothelium may also obstruct capillaries, resulting in reduced gastric mucosal blood flow and predisposing the mucosa to injury [34–36].

Leukocyte adhesion to the endothelium and subsequent migration depend on the expression of specific adhesion molecules, such as L-, E- and P-selectin or intercellular molecular adhesion 1, to the surface of each cell type, an event that follows the activation of cytokines such as TNF-α (tumoral necrosis factor alpha) [37,38].

Recently, leukocyte adhesion and extravasation induced by TNF-α in vivo microcirculation was reduced by gap junction blockers such as carbenoxolone [38].

The ability of carbenoxolone to decrease leukocyte adhesion, which induces gastric injury, may be related to its ability to increase gastric NO. The role of NO in leukocyte adherence is evidenced via enhanced leukocyte–endothelium interactions in inducible nitric oxide synthase knockout mice subjected to endotoxemia [39]. Moreover, erosion of the gastric mucosa is related to an important reduction in mucosal NOS activity [40]. In control conditions, NOS inhibition increased the permeability of the ulcerated tissue to luminal acid and abolished hyperemia in response to acid back diffusion [41]. Nitroglycerin treatment favored the healing of acetic acid- [42] and stress- [22] induced ulcers in rats. The importance of NO in regulating mucosal blood flow led to the development of NO-releasing NSAIDs, such as NO aspirin and NO naproxen, which are associated with reduced gastric injury compared with the parent drug [43,44].

Activation of K_{ATP} channels by NO/$_c$GMP has been poorly studied in the gastroprotective mechanism for several drugs that induce an increase of gastric NO. Gastroprotective mechanisms have been poorly studied for natural products or semisynthetic drugs. This is the first study where the NO/$_c$GMP/K_{ATP} channels pathway has been identified in the gastroprotective mechanism for a semi-synthetic compound.

In conclusion, carbenoxolone induces an increase of gastric PGs and NO and its pharmacological mechanism occurs by activating the NO/$_c$GMP/K_{ATP} channels pathway.

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