Anti-Oxidant Mechanisms Involved in Gastroprotective Effects of Quercetin
M. J. Martín, C. La -Casa, C. Alarcón-de-la-Lastra, J. Cabeza, I. Villegas and
V. Motilva
Laboratory of Pharmacology, Faculty of Pharmacy, Seville University,
c/Prof. García Gonzalez s/n, 41012-Sevilla, Spain
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The anti-ulcerogenic and anti-oxidant effects of various flavonoids have been frequently reported. We investigated the cytoprotective properties of quercetin, a natural flavone, in gastric mucosal injury induced by 50% ethanol, since in this experimental model the pathogenesis of the lesions has been related with production of reactive oxygen species. The involvement of neutrophil infiltration and the capacity of this flavonoid to restrain the oxidative process produced in the gastric tissue after ethanol administration were also investigated. Oral pretreatment with the highest dose of quercetin (200 mg/kg), 120 min before absolute ethanol was the most effective anti-ulcer treatment. Thiobarbituric acid reactive substances in the gastric mucosa, an index of lipid peroxidation, were increased by ethanol injury, but the increase was inhibited by the administration of 200 mg/kg of quercetin. This dose also induced a significant enhancement in the levels of mucosal non-protein SH compounds (important anti-oxidant agents) and in glutathione peroxidase activity. Exposure of the gastric mucosa to 50% ethanol induced a significant increase in myeloperoxidase activity, an index of neutrophil infiltration. However, quercetin was not able to modify the increase in enzymatic activity generated by the necrotizing agent. The activity of superoxide dismutase enzyme involved in several antioxidant processes was also not significantly modified after quercetin treatment.

These results suggest that the anti-ulcer activity of quercetin in this experimental model could be partly explained by the inhibition of lipid peroxidation, through decrease of reactive oxygen metabolites. However, the inhibition of neutrophil infiltration or the increase of superoxide dismutase activity does not appear to be involved in gastroprotective effect of this flavonoid.

Introduction
The flavonoids comprise a large group of unique compounds that are widely distributed in the plant kingdom. In recent years, they have been reviewed for their wide biological activities, focusing in particular on the potential therapeutic use of this class of molecule as anti-inflammatory, anti-allergic, antiviral, anticancer or immunostimulant drugs (Havsteen, 1993). This pharmacological potential is probably due at the capability of flavonoids to interact with important cellular processes in which key enzymes such as cyclo-oxygenase, lipo-oxygenase, phospholipase A₂, NADH-oxidase or glutathione reductase are involved (Bombardelli and Morazzoni, 1993). Other interesting studies also reported the capacity of some flavonoids to interact with oxygen activated species since they are strong scavengers of lipid radicals (Wolf et al., 1994).

Quercetin is a naturally occurring flavonoid with several pharmacological effects, including a pronounced anti-ulcerogenic activity against different experimental models such as absolute ethanol (Alarcón de la Lastra et al., 1994), acetic acid (Motilva et al., 1992), restraint-stress and pyloric occlusion (Martín et al., 1993).

Lipid peroxidation mediated by oxygen free radicals is believed to be an important cause of destruction and damage to cell membranes and it is involved in the pathogenesis of acute mucosal injury induced by ethanol, ischaemia-reperfusion and indomethacin (Kviety et al., 1990; Salim, 1990). Quercetin has been reported as a very strong antilipoperoxidant compound (Larson, 1988; Bombardelli and Morazzoni, 1993). In addition, glutathione (GSH) is an important constituent of intracellular protective mechanisms against

Reprint requests to Dr. M. J. Martín Calero.
Telefax: 34-5-233765.

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a number of noxious stimuli, and it is known to be a major low molecular weight scavenger of free radicals in cytoplasm. Sulphydryl- (SH) containing compounds, and also agents that modify SH groups, prevent the acute haemorrhagic erosions caused by ethanol, non-steroidal anti-inflammatory drugs (NSAIDs) or stress in animal models (Szabo et al., 1987). In the same way, various antioxidant enzymes such as superoxide dismutase (SOD), an important radical superoxide scavenger, and glutathione peroxidase (GSH-Px), an enzyme involved in the elimination of hydrogen peroxide and lipid hydroperoxides, play an important role in cell protection. Recently, the role of neutrophils has been proposed in the gastric lesions induced by NSAIDs (Wallace et al., 1990), acetic acid (Motilva et al., 1996) or ethanol (Kvietys et al., 1990). These leukocytes adhere to endothelial cells, thereby blocking capillaries and damaging the endothelial integrity through the release of proteases, leukotrienes and active oxidants (Jacobson, 1992). These findings prompted us to study the cytoprotective properties of quercetin in gastric mucosal injury produced by 50% (v/v) ethanol. Changes in lipid peroxidation were determined by measuring thiobarbituric acid production, GSH levels and the activity of GSH-Px and SOD. We also measured myeloperoxidase activity in the gastric mucosa as a marker of neutrophil infiltration.

**Materials and Methods**

**Animal groups and drug preparation**

Male and female Wistar rats, 180–200 g, supplied by the Animals Service of the University of Seville, were used throughout this study. The animals, 8–10 per group, were deprived of food for 24 h before the experiments but had free access to water. They were placed in single cages which had wire-net floors to prevent coprophagy. The temperature was maintained at 22–24 °C and humidity at 70–75% in a controlled room.

Quercetin (Sigma Chem. Co., Mo. USA) was suspended in distilled water with Tween 20 (1%). It was prepared freshly each time and administered at different doses (50, 100 and 200 mg/kg) by intragastric route. Control rats received the vehicle orally in a comparable volume (1 ml/100 body weight).

**Protection against 50% (v/v) ethanol**

Ulceration was induced as described by Wong et al., instilling 1 ml/100 g animal of 50% (v/v) ethanol in distilled water with Tween 20 (1%). Quercetin was administered 2 h before the p.o. administration of ethanol. One hour after the experimental period, the animals were sacrificed using an overdose of anaesthetic, and their stomachs were removed and opened along the greater curvature. Their lesions were examined macroscopically. The number and severity of haemorrhagic lesions per stomach were assessed in accord with the following scoring system: 0) no lesions; 1) one haemorrhagic ulcer length <5 mm and thin; 2) one haemorrhagic ulcer length >5 mm and thin; 3) more than one ulcer grade 2; 4) one ulcer length >5 mm and width >2 mm; 5) two or three ulcers of grade 4; 6) from four to five ulcers of grade 4; 7) more than six ulcers of grade 4; 8) complete lesion of the mucosa. Mean scores for each group were calculated and expressed as ulcer index (UI).

**Sulphydryl determination: total and non-protein SH contents**

The amount of mucosal sulphydryls was measured in the gastric mucosa of rats according to the method described by Garg et al. (1991).

After the experimental period, the animals were sacrificed and their stomachs removed, opened, rinsed in ice-cold sodium phosphate buffer (0.2 M, pH=8.0), and quickly placed on an ice-cold surface for scraping off the total glandular mucosa. The scrapings were then suspended in 1 ml sodium phosphate buffer, homogenized, made up to 2 ml with buffer, and centrifuged at 1800 × g for 10 min at 4 °C. The supernatant sulphydryl content was determined. The protein sulphydryl levels were obtained by subtracting the non-protein sulphydryl values from that of the total sulphydryls. Light absorbance at 412 nm, against a reagent blank, was measured with a spectrophotometer (Perkin-Elmer Lambda 3). Sulphydryl concentration was calculated from freshly prepared standard curves of glutathione (GSH, Sigma Chem. Co.) and the results were expressed as μmol glutathione/g tissue.
Determination of lipid peroxidation

The levels of thiobarbituric acid (TBA) reactants in the gastric mucosa were measured according to the modified method of Ohkawa et al. (1979). Briefly, groups of quercetin-treated rats were killed under deep ether anaesthesia and the stomachs removed. The mucosa was scraped with glass slides, weighed, and homogenized in 10 ml KCl (10%). The homogenate was supplemented with 8.1% sodium lauryl sulphate, 20% acetic acid and 0.8% TBA, and boiled for 1 h. After cooling, the reactants were supplemented with n-butanol and pyridine (15:1 v/v), shaken vigorously for 1 min and centrifuged for 10 min at 2600xg. Absorbance was measured spectrophotometrically at 532 nm and the results were expressed as nmol TBA mg protein.

Enzymatic assay

Myeloperoxidase activity

Neutrophil infiltration in vivo has previously been assessed by measuring granulocyte-specific enzymes such as myeloperoxidase (MPO) in tissue (Grisham et al., 1990). Briefly, one sample from the gastric corpus was excised from each animal and rapidly rinsed with ice-cold saline, blotted dry, and frozen at -70°C. The tissue was thawed, weighed and homogenized in 10 volumes of 50 mM phosphate-buffered saline (PBS), pH=6.0. The homogenate was centrifuged at 20000xg, 20 min at 4°C. The pellet was again homogenized in 10 volumes of 50 mM PBS, pH=6.0, containing 0.5% hexadecyl-trimethylammonium bromide (HETAB) and 10 mM EDTA. This homogenate was subjected to one cycle of freezing/thawing and a brief period of sonication.

Homogenate (0.5 μl) was added to a 0.5 ml reaction volume containing 80 mM PBS, pH=5.4, 0.5% HETAB and 1.6 mM 3,3',5,5'-tetramethylbenzidine (TMB) . The mixture was incubated at 37°C for 5 min and the reaction started by the addition of 0.3 mM H2O2. Each tube containing the complete reaction mixture was incubated for exactly 3 min at 37°C. The reaction was terminated by the sequential addition of catalase (20 μg/ml) and 2 ml of 0.2 mM Na-acetate, pH=3.0. The changes in absorbance at 655 nm were measured with a spectrophotometer. 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.

Glutathione peroxidase activity

GSH-Px activity was quantified by the method of Yoshikawa et al. (1993). From scrapings of glandular stomach mucosa of control and treated animals, the anti-oxidative enzyme was evaluated by following the decrease in absorbance at 365 nm induced by 0.25 mM H2O2 in the presence of reduced glutathione (10 mM), NADPH, (4 mM), and 1U enzymatic activity of glutathione reductase in PBS buffer, pH 7.8. The absorbance changes were read between minutes 3 and 10. Results were expressed as nmol/min/mg protein. Protein concentration was calculated following the Bradford (1976) assay.

Superoxide dismutase

The enzymatic activity of SOD is based on the inhibition of the reduction of cytochrome c according to the method of McCord and Fridovich (1969). Samples of gastric mucosa were homogenized (1:150) in a mixture of 50 mM PBS and 100μM EDTA (pH 7.8). The homogenate was supplemented with 0.1% Triton. The assay method used 10 μM ferricytochrome c, 50 μM xanthine, as source of O2−, and sufficient milk xanthine oxidase (5 nM) to give a rate of increase in absorbance of 0.025/min at pH 7.8 and 25°C. The reaction kinetic was measured in a spectrophotometer Shimadzu UV-160 A at 550 nm at a rate of 0–80 seconds. Results were expressed as U/mg protein. One unit of SOD is defined as the amount of enzyme that causes 50% inhibition of cytochrome c reduction.

Statistical analysis

Data from 6–10 experiments were pooled and expressed as arithmetic means ± SEM. The data were evaluated using Student’s t-test for paired data and the non-parametric Mann-Whitney U-test (UI determination). P values were considered significant at p<0.05.
Results

Fig. 1 shows the protective effects of quercetin on ethanol-induced gastric lesions. Oral administration of 50% v/v ethanol induced multiple, elongated, reddish bands of lesions in the corpus mucosa along the long axis of the stomach. The lesion index was 4.66 ± 0.61. In this experimental model, oral pretreatment with the highest dose of quercetin (200 mg/kg), 120 min before 50% ethanol, prevented ulceration. The U. I. was significantly lower than in the animal group receiving only ethanol (2.5 ± 0.72, p<0.05). Using TBA-reactive substances in the gastric mucosa as an index, lipid peroxidation increased significantly (p<0.001) from a basal concentration of 3.53 ± 0.92 nmol/mg protein to 12.61 ± 1.82 nmol/mg protein after administration of 50% ethanol. Quercetin significantly decreased (p<0.001) the levels of TBA-reactive substances in the gastric mucosa, 5.49 ± 1.75 nmol/mg protein, approaching the base value.

In normal rats, the levels of non-protein and total SH groups in the gastric mucosa were 1.91 ± 0.17 and 14.93 ± 1.34 μmol GSH/g tissue respectively (Table I). In agreement with the previous observations of Takeuchi et al. (1988), the necrotizing agent caused a marked reduction in the mucosal non-protein SH content in this experimental model (0.90 ± 0.15 μmol GSH/g tissue, p<0.001). In contrast, administration of the highest dose of quercetin (200 mg/kg) was able to restore the levels of this mucosal fraction completely (1.68 ± 0.66 μmol GSH/g tissue, p<0.05).

Table II shows gastric mucosal values of enzymatic activities. Ethanol induced a considerable increase of MPO and SOD activities with respect to the control group (p<0.05), and quercetin was not able to modify the enzymatic activity generated by this agent. No important changes could be observed after quercetin administration. In contrast, glutathione peroxidase activity was significantly decreased in the mucosa following gastric injury, and treatment with quercetin (200 mg/kg) resulted in a significant increase of this enzymatic activity compared with the group that received ethanol alone.

Discussion

Available data suggest that oxygen free radicals are a fundamental factor causing tissue injury during the pathogenesis of various disorders of the digestive tract (Smith and Kvietys, 1988). Concentrated ethanol is noxious for the stomach, affecting the gastric mucosa topically by disrupting its barrier causing pronounced microvascular changes in few minutes after its application. Rapid and strong venoconstriction is accompanied by rapid and vigorous arteriolar dilation and this combination of microvascular events induce marked engorgement of mucosal capillaries (Oates and Hakkinem, 1988;
Glavin and Szavbo, 1992). Some authors have demonstrated that oxygen-derived free radicals are directly implicated in the pathogenesis of ethanol-induced mucosal damage and impair healing of the lesions (Pihan et al., 1987; Terano et al., 1986, Salim, 1990). Increased purine degradation, possibly associated with a shift from the dehydrogenase to the xanthine oxidase pathway, is proposed as a possible mechanism for ethanol-stimulated free radical production which initiates the process of lipid peroxidation. Under our experimental conditions, exposure of the gastric mucosa to the damaging agent induced a significant enhancement of SOD activity. This suggests that xanthine oxidase-derived oxyradicals are also involved in 50% ethanol-induced injury. However, quercetin treatment did not produce modifications of enzymatic activity.

Numerous papers show that among flavonoids there are strong scavengers of lipid radicals (Bombardelli and Morazzoni, 1993; Wolf et al., 1994). Quercetin has been found to be an important anti-oxidant agent (Afanasiev et al., 1989; Robak et al., 1988) and also has well-established properties against lipid peroxidation (Negré-Salvayre et al., 1991; Bombardelli and Morazzoni, 1993). Our findings are in agreement with these observations, since the levels of lipid peroxides in treated groups were significantly lower than animals that received ethanol alone.

In addition, it is known that the cytoprotective effect of various drugs is partly mediated by an enhancement of non-protein sulphydryl compounds in the gastric mucosa (Szabo and Vattay, 1990). Sulphydryl compounds such as reduced glutathione (GSH) bind free radicals that form following tissue injury by noxious agents such as ethanol (Takeuchi et al., 1988) or aspirin (Szabo et al., 1987). Similar results were obtained in animals subjected to stress (Konturek et al., 1990). Depletion of GSH results in enhanced lipid peroxidation and excessive lipid peroxidation can cause increased GSH consumption. By contrast, an augmentation in gastric non-protein SH content limits the production of oxygen-derived free radicals, and could be related with cellular protection (Itoh and Guth, 1985). In this experimentation the highest dose of quercetin produced an enhancement of mucosal non-protein SH content. Thus, it seems that there is an association between the gastroprotective effect of quercetin and the increase of sulphydryl compounds.
Our results also revealed that GSH-Px activity decreases in gastric mucosa after ethanol-treatment. GSH-Px is an important enzyme which plays a key role in the elimination of H$_2$O$_2$ and lipid hydroperoxides in the gastric mucosal cells. The anti-oxidant activity of this enzyme is coupled with the oxidation of reduced glutathione, which can subsequently be reduced by glutathione reductase with NADPH as the reducing agent. Thus, inhibition of this enzyme may result in the accumulation of H$_2$O$_2$ with subsequent oxidation of lipids. By contrast, quercetin treatment (200 mg/kg) induced a significant increase in GSH-Px activity after ethanol administration. The enhancement of GSH levels and GSH-Px activity suggests that the anti-ulcerogenic effect of quercetin may appear through glutathione metabolism.

Although the anti-inflammatory properties of quercetin have been observed on numerous occasions (Bombardelli and Morazzoni, 1993) in our experimental model, this flavonoid did not induce any changes in MPO enzymatic activity, as index of neutrophil infiltration, generated by ethanol. The circulating neutrophils play a critical role in the pathogenesis of NSAID (Wallace et al., 1990) and acetic acid mucosal lesions (Motilva et al., 1996). Neutrophils might contribute to gastric ulceration by several possible mechanisms such as production of reactive oxygen metabolites, release of proteases and lipid mediators, e.g. leukotrienes and PAF, that affect vascular tone and permeability, exacerbating tissue ischaemia (Beck et al., 1988, Guth, 1992). Under our experimental conditions, ethanol induced a considerable neutrophil infiltration in gastric mucosa. These findings are in agreement with previous observations (Kvietys et al., 1990), and suggest that the neutrophil-derived reactive metabolites, are also involved in the pathogenesis of ethanol-induced gastric lesions.

In summary, the present results suggest that the gastroprotective effect of quercetin in the experimental lesions induced by 50% ethanol could be mediated by its anti-oxidant properties reducing the induction of lipid peroxidation and restoring the base levels of anti-oxidative molecules such as non-protein SH compounds. However, no significant modifications in the ethanol-induced leucocyte infiltrate nor oxygen free radical reduction via the SOD pathway have been observed.


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