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Quercetin prevents oxidative stress and NF-κB activation in gastric mucosa of portal hypertensive rats

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Abstract

The present study was designed to investigate the effects of quercetin on oxidative stress and activation of nuclear factor kappa B (NF-κB) in an experimental model of portal hypertensive gastropathy induced by partial portal vein ligation (PPVL). Portal pressure was significantly elevated in PPVL rats. Transaminase and alkaline phosphatase activities were not significantly modified, indicating absence of liver injury. Histological analysis of gastric sections showed a lost of normal architecture, with edema and vasodilatation. The cytosolic concentration of thiobarbituric acid reactive substances and the lipoperoxidation measurement by chemiluminiscence were significantly increased. Superoxide dismutase activity in gastric mucosa was significantly reduced. Portal hypertensive gastropathy induced a marked activation of NF-κB, accompanied by a decrease in IκB protein levels and a significant induction of nitric oxide synthase (iNOS) protein. Administration of quercetin markedly alleviated histological abnormalities and inhibited oxidative stress and NF-κB activation. IκB decrease and induction of iNOS protein were partially prevented by quercetin. Quercetin treatment, by abolishing the NF-κB signal transduction pathway, may block the production of noxious mediators involved in the pathogenesis of portal hypertensive gastropathy.

Keywords: Gastric mucosa; NF-κB; Nitric oxide; Oxidative stress; Portal hypertension; Quercetin

1. Introduction

Portal hypertension is one of the most disastrous conditions related to chronic hepatic diseases. As the portal pressure elevates, portal-systemic collaterals develop gradually to diverse blood flow from the portal system [1]. Gastroesophageal varices are most prominent collaterals and hemorrhage from ruptured gastroesophageal varices leads to a high morbility and mortality [2]. Portal hypertensive gastropaty (PHG) is now recognized as a distinct clinical entity characterized by mucosal and submucosal vascular dilatation used to describe the endoscopic appearance of gastric mucose, with a characteristic mosaic-like pattern with or without red spots, seen in patients with cirrhotic or noncirrhotic portal hypertension [3]. PHG, usually present in association with either esophageal or gastric varices [4], is one of the leading causes of death in Americans between the ages of 35–54 [5] and approximately 30% of patients afflicted with PHG develop life – threatening gastric hemorrhage, either spontaneous or caused by noxious agents [6].

There is experimental evidence to suggest that gastric mucosal defense mechanisms are impaired in the presence of portal hypertension. Factors such as excessive nitric oxide (NO) production and increased generation of oxygen free radicals and lipid peroxidation have been implicated in its increased susceptibility to injury in PHG [7–9]. Over the last few years, a number of studies have provided evidence of an important role of reactive oxygen species (ROS) in mediating the microvascular disturbance that preceded gastric mucosal injury [10].

ROS may inflict direct damage to vital cell constituents such as lipids, proteins and DNA, but also modulate the pattern of gene expression through functional alterations of transcription factors such as nuclear factor kappa B (NF-

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κB) [11]. NF-κB, in turn, is required for the induction of nitric oxide synthase (iNOS) gene in response to cytokines [12]. NO, a potent endogenous vasodilator synthesized by vascular endothelial cells, contributes to the hyperdynamic circulation, mesenteric hyperemia and vascular hyporesponsiveness to vasoconstrictors in portal-hypertensive states [13,14]. In addition, the vascular hyporeactivity phenomenon observed in hemorrhagic shock may be mediated by NO [15].

Oxidative stress-induced extracellular signal-regulated kinase 2 (mitogen-activated protein kinase) has been shown to be defective in PHG, and this is mediated by an overexpression of mitogen-activated protein kinase phosphatase 1 that normalizes after supplementation with Vitamin E. Use of antioxidants may therefore have therapeutic implications for the management of PHG. Flavonoids are phenolic phytochemicals that represent substantial constituents of the non-energetic part of the human diet and are thought to promote optimal health, partly via their antioxidant effects in protecting cellular components against reactive oxygen species (ROS) [16]. Quercetin (3,5,7,3′4′-pentahydroxy flavon) is one of the most widely distributed flavonoids, present in fruit, vegetables and many other dietary sources [17]. This compound has been reported to scavenge superoxide in ischemia-reperfusion injury [18], to protect against oxidative stress induced by ultraviolet light [19], spontaneous hypertension [20], secondary biliary cirrhosis [21] and bacterial lipopolysaccharide [22] or to inhibit angiogenesis [23] and carcinogenesis [24].

The present study was designed to investigate the effects of quercetin on oxidative stress, activation of NF-κB and up-regulation of NO in an experimental model of portal hypertensive gastropathy.

2. Materials and methods

2.1. Animal and procedures

Male Wistar rats weighing 300–350 g were used for this study. The rats were caged at 24 °C, with a 12 h light–dark cycle and free access to food and water until the time of experiments. Survival surgery and hemodynamic study were performed with the rats under anesthesia with sodium pentobarbital (50 mg/kg body weight i.p.). Portal hypertension was induced by partial portal vein ligation (PPVL) as described by Vorbioff et al. [25]. In brief, the portal vein was isolated and a 3-0 silk ligature was tied around both the portal vein and an adjacent 20 gauge blunt-tipped needle. The needle was then removed and the vein allowed to re-expand. A second loose ligature was left around the portal vein with two endings of the ligature placed on each side in the abdominal cavity. The abdomen was then closed and the animal allowed to recover. All experiments were performed in accordance with the *Guiding Principles for Research Involving Animals* (NAS). Control rats underwent a similar operation but without partial occlusion of portal vein.

After 8 days, quercetin (Sigma, St. Louis, MO) was suspended, immediately before administration, in a 0.2% Tween aqueous solution. Groups of control and portal hypertensive animals received daily a 500 μl i.p. injection of quercetin (50 mg/kg body wt^{-1} in 0.5 ml) or vehicle for the last 7 days of the study. Portal venous pressure was measured on a Wilson biscriptual polygraph (Wilson Medical Electronics Inc. Middletown, Wisconsin, USA).

2.2. Biochemical analysis

The stomach were excised, weighed, and immediately frozen at −70 °C. Frozen tissue from each rat was homogenized in ice-cold phosphate buffer (KCl 140 mM, phosphate 20 mM, pH 7.4) and centrifuged at 3000 rpm for 10 min. Cytosolic superoxide dismutase (SOD) (EC 1.15.1.1) was assayed according to Misra and Fridovich [26] at 30 °C. The rate of autooxidation of epinephrine, which is progressively inhibited by increasing amounts of SOD in the homogenate, is monitored spectrophotometrically at 560 nm. The amount of enzyme that inhibits epinephrine autooxidation at 50% of the maximum inhibition is defined as 1 U of SOD activity.

Oxidative stress was determined by measuring the concentration of thiobarbituric acid reactive substances (TBARS) [27] and the hydroperoxide-initiated chemiluminescence (QL) [28]. The amount of aldehydic products generated by lipid peroxidation was quantified by the thiobarbituric acid reaction using 3 mg of protein per sample. Results were referred as TBARS. The samples were incubated at 90 °C for 30 min after adding 500 μL of 0.37% thiobarbituric acid in 15% trichloroacetic acid, then centrifuged at 4 °C at 2000 × *g* for 15 min. Spectrophotometric absorbance was determined in the supernatant a 535 nm. For the QL determination, 0.5 ml of homogenate were added to 120 mM KCl, 30 mM phosphate buffer (pH 7.4), and 3 mM *tert*-butyl hydroperoxide at 30 °C and assayed for chemiluminiscence in a liquid scintillation counter in the out-of-coincidence mode.Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (AP) were determined by commercial kits (Boehringer Mannheim, Germany).

2.3. Western blot

For Western blot analysis of iNOS and IκB protein formation, stomach tissue was homogenized with 140 mM NaCl, 15 mM EDTA, 10% glycerol, 20 mM and a protease inhibitor cocktail. The mixture was incubated for 30 min at 4 °C and centrifuged 30 min at 17,000 × g and 4 °C. The supernatant was kept as stomach tissue lysate. Samples containing 100 μg of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide) and transferred to nitrocellulose.

Non-specific binding was blocked by preincubation of the nitrocellulose in phosphate-buffered saline containing 5% bovine serum albumin for 1 h. The nitrocellulose was then incubated overnight at 4 °C with polyclonal anti-iNOS or anti-IκB antibodies (Santa Cruz Biotechnology). Bound primary antibody was detected with HRP-conjugated anti-rabbit antibody (DAKO) by chemiluminiscence. The density of the specific iNOS and IκB bands was quantitated with an imaging densitometer.

2.4. Electrophoretic mobility shift assay

Nuclear extracts were prepared from stomach lysates as described previously [29]. Activation of transcription factor NF-κB was examined using consensus oligonucleotides of NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG C-3′). Probes were labeled by T4 polynucleotide kinase. Binding reactions included 10 μg of nuclear extracts in incubation buffer (50 mM Tris–HCl pH 7.5, 200 mM NaCl, 5 mM EDTA, 5 mM mercaptoethanol, 20% glycerol and 1 μg poly (dI-dC)). After 15 min on ice, the labeled oligonucleotide (30,000 cpm) was added and the mixture incubated 20 min at room temperature. For competition studies, 3.5 pmol of unlabeled (cold) NF-κB oligonucleotide (competitor) or 3.5 pmol of labelled NF-κB oligonucleotide mutate (noncompetitor) were mixed 15 min before the incubation with the labelled oligonucleotide. The mixture was electrophoresed through a 6% polyacrylamide gel for 90 min at 150 V. The gel was then dried and autoradiographed at −70 °C overnight. Signals were densitometrically analyzed.

2.5. Histology

For histological examination a piece of the liver and stomach was trimmed and fixed by immersion in 10% buffered formalin for 24 h. The blocks were dehydrated in a graded series of ethanol and embedded in paraffin wax. Serial 3 μm sections were stained with hematoxilin and eosin.

2.6. Statistical analysis

Means and S.E.M.'s were calculated for all data. Significant differences between means were evaluated by analysis of variance and in the case of significance, a Newman–Keul's test was also applied. Significance was accepted at $P < 0.05$.

3. Results

3.1. Portal pressure and transaminase activities

No significant differences were found for any of tested parameters between untreated controls and those treated with quercetin, and hence data were pooled together. There was a statistically significant difference in portal pressure between control animals and those with portal vein ligation (+46%). No significant difference from the control group was observed in PPVL rats treated with quercetin (Table 1). Transaminase and alkaline phosphatase activities did not significantly differ between the different experimental groups.

Table 1
Effects of partial portal vein ligation (PPVL) and quercetin (Q) administration on portal pressure and serum AST, ALT and AP activities

	Control	PPVL	PPVL + Q
Portal pressure (mmHg)	11.9 ± 1.6	19.7 ± 1.9*	13.0 ± 0.9[a]
AST (U/L)	166.0 ± 14.6	159.2 ± 21.3	174.0 ± 15.7
ALT (U/L)	95.1 ± 8.0	91.0 ± 9.2	72.3 ± 6.2
AP (U/L)	191 ± 16	170 ± 20	164 ± 29

Rats were partially ligated or sham operated as described in Section 2. Values are means ± S.E.M. for 6–8 rats. AS: aspartate aminotransferase; ALT: alanine aminotransferase; AP: alkaline phosphatase.
* $P < 0.05$ against control.
[a] $P < 0.05$ against PPVL.

3.2. Histology

Histological analysis of gastric sections showed modifications of normal architecture, with congestion and edema in the submucosa and a proliferation of blood vessel in PPVL animals. Administration of quercetin markedly alleviated histological abnormalities (Fig. 1). No histological alterations were detected in liver of different groups (data not shown).

3.3. Markers of oxidative stress and SOD activity

The cytosolic concentration of TBARS increased in the animals with portal hypertension (PPVL group) (+93%), while values did not significantly differ from the controls in PPVL rats treated with quercetin (Fig. 2). Lipoperoxidation measurement by chemiluminiscence also demonstrated a significant increase in PVL rats (+101%) that was absent in those animals receiving quercetin (Fig. 2). SOD activity tended to decrease in PPVL animals and this effect was prevented by quercetin administration (Fig. 3).

3.4. NF-κB activation and IκB expression

NF-κB binding activity was evaluated by EMSA performed with a NF-κB consensus nucleotide sequence. To confirm that the result shifted band is the specific binding of NF-κB to its sequence specific oligonucleotide, a competition binding assay was performed. As shown in Fig. 4, portal hypertensive gastropathy induced a marked activation of NF-κB (+98%) that was absent in stomach of animals treated with quercetin. Since it has been well documented that activation of NF-κB correlates with rapid proteolytic degradation of IκB, we assessed protein levels

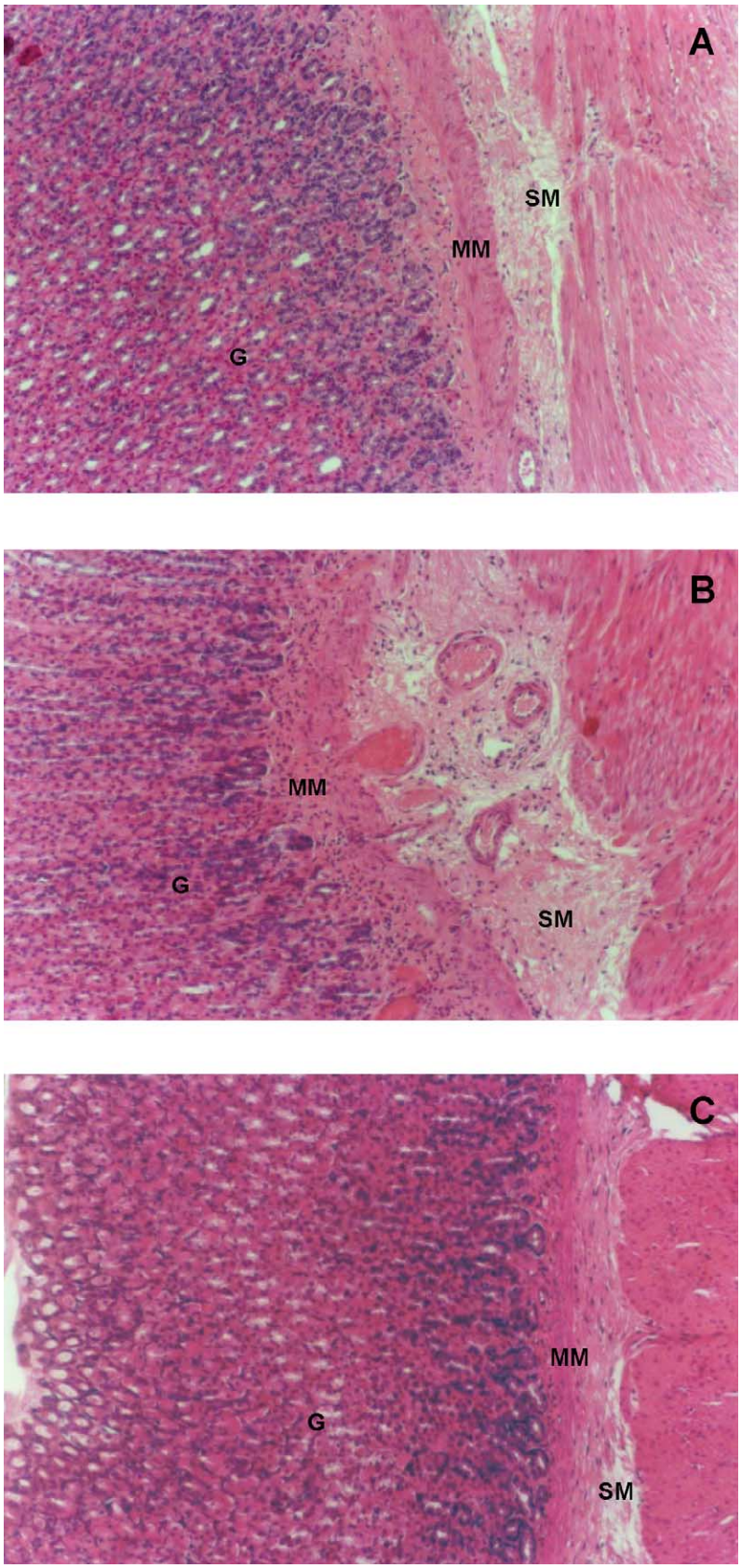

Fig. 1. Micrographs of gastric tissue in a control rats (A), rats with partial portal vein ligation (PPVL) (B) and rats with portal vein ligation and quercetin treatment (PPVL + Q) (C). G: glandular tissue, MM: muscular mucosa, SM: submucosa. Hematoxilin and eosin staining, original magnification, 10×. Congestion, edema and a proliferation of blood vessel were evident in B.

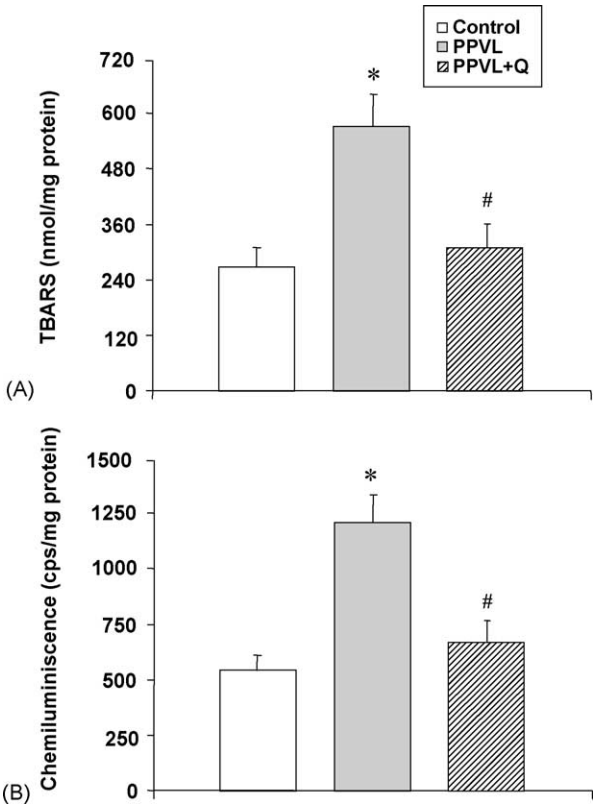

Fig. 2. Effects of partial portal vein ligation (PPVL) and quercetin (Q) administration on gastric TBARS concentration and chemiluminiscence (QL). Values are means ± S.E.M. for 6–8 rats. $^{*}P < 0.05$ against control. $^{\#}P < 0.05$ against PPVL.

of IκB using Western blot analysis. Protein levels were decreased in animals from the PPVL group (−25%) and this effect was partially blocked by quercetin (Fig. 5).

3.5. iNOS expression

To evaluate the effects of experimental gastropathy and quercetin treatment on nitric oxide production, the expression of iNOS was quantified by measurement of protein (Western blot) levels. PPVL coursed with a significant induction of iNOS protein (+346%). This effect was partially abrogated by quercetin (Fig. 6).

4. Discussion

The partial portal vein ligation used in this study is an animal model characterized by prehepatic portal hypertension, with a maintained hepatic structure and a hyperdynamic circulation that develops in a short and predicted period of time [1]. This model has been extensively studied and found to be a useful tool for understanding the pathophysiology of portal hypertension [1,2,30]. Moreover, it can provide measurements in the portal circulation which cannot be performed accurately in humans because of ethical and technical limitations [31].

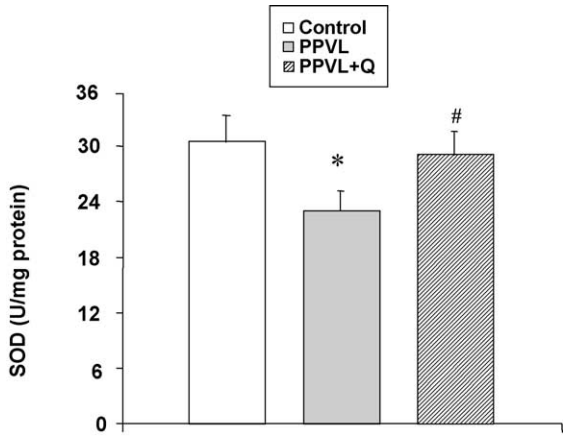

Fig. 3. Effects of partial portal vein ligation (PPVL) and quercetin (Q) administration on gastric superoxide dismutase activities. Values are means ± S.E.M. for 6–8 rats. $^{*}P < 0.05$ against control. $^{\#}P < 0.05$ against PPVL.

In our study, portal hypertension was accompanied by the presence of edema and dilated vessels in the gastric mucosa, but no alteration was detected in liver histology or transaminase activities. This confirms previous findings that the procedure of PPVL, although causing a transient reduction in the metabolic activity of the liver [32], do not produce hepatocellular damage [33] and support the suggestion that portal hypertension seems to be the key factor for the development of PHG, being equally common in portal hypertensive patients with or without liver disease [34].

The fact that partial portal vein ligation results in oxidant injury was first demonstrated by Fernando et al. [7], which concluded that the formation of ROS may be important in the pathogenesis of hemodynamic changes and the development of the hyperdynamic circulation. In models of injury induced by nonsteroidal anti-inflammatory drugs a number of studies have also provided evidence of an important role of ROS in mediating the microvascular disturbance that preceeds gastric mucosal injury [10]. Kawanaka et al. [5] have shown that mitogen-activated protein kinase ERK2 activation is impaired in the gastric mucosa of portal hypertensive rats as a result of the underlying and continual oxidative stress. The potential role of ROS formation in the pathogenesis of PHG is further supported by the prevention of the hyperdymamic circulation induced by *N*-acetylcysteine [7] and the reverse by Vitamin E of the increased susceptibility of the gastric mucosa to alcohol-induced injury in portal hypertensive rats [5].

Oxidative stress has been defined as an imbalance between pro- and antioxidants, and ROS-induced lipid peroxidation can occur either in situations in which scavenging systems are overwhelmed (excessive production of ROS) or by impairing the antioxidant systems [35]. Superoxide dismutase is an intracellular metalloenzyme which owes its antioxidant properties to its elevated capacity of scavenging O_2 radicals. In the present investigation SOD activity was significantly decreased in the gastric mucosa, in line with previous studies which have shown a similar effect in NSAID-induced gastropathy [10,36]. The

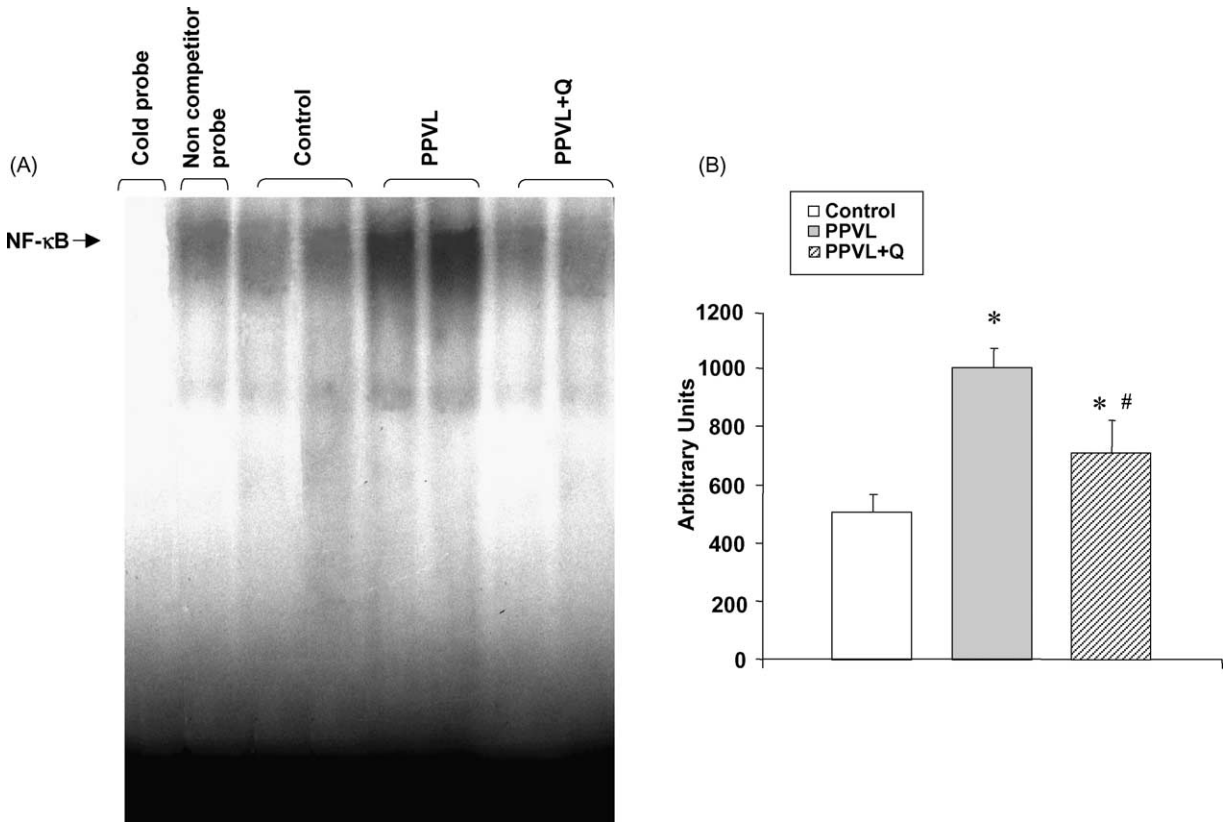

Fig. 4. Nuclear factor κB activation in stomach from the different experimental groups. For the EMSA assay nuclear extracts were incubated with ^{32}P-labelled consensus oligonucleotide, followed by electrophoresis and analysis by autoradiography. Specific binding was verified by addition of unlabeled (cold) oligonucleotide (competitor) or labelled oligonucleotide mutate (noncompetitor). (A) Shows representative EMSA. (B) Shows mean values ± S.E.M. of five different observations. PPVL: partial portal vein ligation; Q: quercetin. $^{*}P < 0.05$ against control. $^{\#}P < 0.05$ against PPVL.

decreases in SOD activity may enhance lipid peroxidation as well as aggravate the injury to gastric mucosa. The decrease in portal pressure, associated to a marked alleviation of histological abnormalities, normalization of SOD activity and inhibition of lipid peroxidation induced by quercetin in the present study confirms that the use of

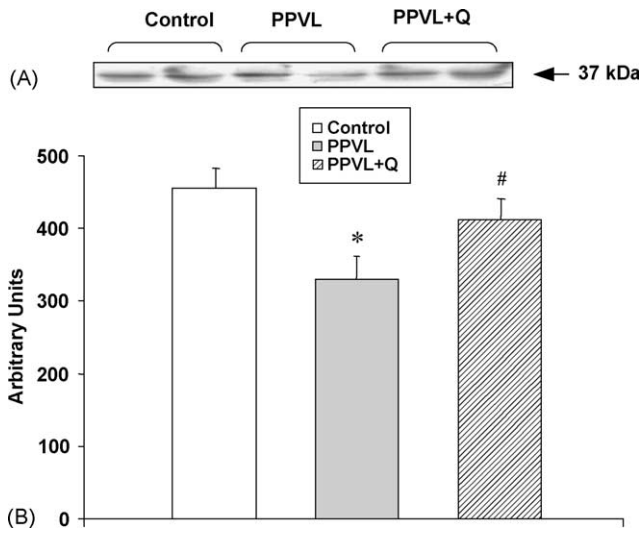

Fig. 5. Western blot analysis of IκB protein in stomach from the different experimental groups. Total cellular protein was separated on 12% SDS-polyacrylamide gels and blotted with anti-IκB antibodies. (A) Shows representative Western blot photographs. (B) Shows mean values ± S.E.M. of five different observations. PPVL: partial portal vein ligation; Q: quercetin. $^{*}P < 0.05$ against control. $^{\#}P < 0.05$ against PPVL.

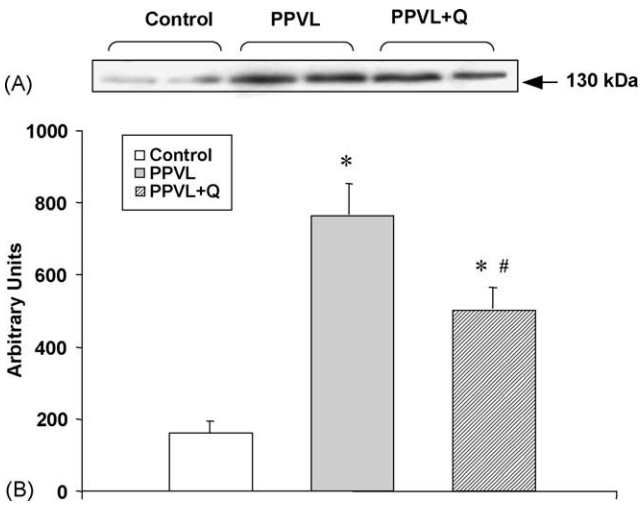

Fig. 6. Western blot analysis of iNOS protein in livers from the different experimental groups. Total cellular protein was separated on 12% SDS-polyacrylamide gels and blotted with anti-iNOS antibodies. (A) Shows representative Western blot photographs. (B) Shows mean values ± S.E.M. of five different observations. PPVL: partial portal vein ligation; Q: quercetin. $^{*}P < 0.05$ against control. $^{\#}P < 0.05$ against PPVL.

antioxidants may provide a new therapeutic modality for protection of the gastric mucosa in portal hypertension.

In addition to the presence of oxidative stress, we observed an increased expression of iNOS in animals with experimental gastropathy, which coincides with previous reports of elevated levels of products of NO metabolism in this model [7,8]. Several studies have demonstrated an increased vascular NO production in different models of portal hypertension at a time when the hyperdynamic circulation is fully developed [37–41] and chronic high blood flow has been shown to increase endothelial NO release in different vascular beds and animal species [42,43]. Although the role of NO as a mediator of the hyperdynamic circulation is well demonstrated [44], the fact that no statistical correlation has been observed between serum NO levels and gastris mucosal damage led to the suggestion that altered gastric mucosa iNOS gene by itself does not account for pathogenesis of PHG [45]. However, NO also react with ROS, such as the superoxide radical, to yield the highly reactive oxidant species peroxynitrite, which further contributes to oxidative stress [46], and peroxynitrite overproduction may therefore increase susceptibility of gastric mucosa to damage [9].

One additional pathway by which ROS and nitric oxide can contribute to PHG progression is through activation of the transcription factor NF-κB. Upon stimulation by a variety of noxious inflammatory or environmental stimuli, activated NF-κB moves to the nucleus and activate target genes mostly related to inflammation, including those encoding cytokines, nitric oxide or adhesion molecules [12]. Thus, it has been demonstrated that stimulation of ICAM expression on endothelial cells, critical in causing NSAID-induced gastropathy, is mediated by activation of NF-κB [47] and this effect could also play a role in the pathogenesis of PHG. Activation of NF-κB is induced by phosphorylation of inhibitor IκB, in response to diverse stimuli including ROS, which leads to its degradation and results in unmasking of nuclear localization signals that allow NF-κB to be translocated into the cell nucleus [48]. It has been proposed that ROS can directly activate NF-κB by degrading or modifying IκB in the cytoplasmic NF-κB–IκB complex [49]. Results in this study demonstrate that in rats with experimentally induced gastropathy, proteolysis of IκB results in activation and nuclear translocation of NF-κB and this is accompanied by iNOS gene up-regulation.

Treatment of rats with quercetin at a dose likely to be achieved in man [50] completely recovered portal pressure to normal. Although it has been suggested that blood pressure reduction of quercetin in animal models of hypertension could be partly explained by direct vasodilator effects [51], this antioxidant molecule also abrogated the activation of NF-κB and partially prevented iNOS protein induction. The fact that quercetin is a potent inhibitor of transcription factors has been previously reported by different authors [52,53] and the suppression of NF-κB activation by quercetin results, in turn, in a down-regulation of iNOS [54]. NSAID gastropathy has been reported to be prevented by blocking the proteolytic degradation of IkB through treatment with proteosoma inhibitors [48] and results from the present study suggest that IkB protein levels are modified by quercetin. Data obtained, however, do not clearly allow to elucidate the importance of this mechanism and, because the key regulatory step in NFκB activation by proinflammatory stimuli is the activation of IKKs (IκB kinases) [55], which in turn phosphorylate IκBs, an analysis of IKKs should be necessary to get a further insight into the mechanisms of action of quercetin. In any case, quercetin pretreatment, by abolishing the NF-κB signal transduction pathway may block the production of noxious mediators involved in the pathogenesis of portal hypertensive gastropathy.

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