The Natural Flavonoid Quercetin Ameliorates Cerulein-Induced Acute Pancreatitis in Mice

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Many plant-derived flavonoids including quercetin exhibit antioxidant and anti-inflammatory properties. Proinflammatory cytokines and oxidative stress play an important role in acute pancreatitis. This study aimed to evaluate the effect of quercetin on cerulein-induced acute pancreatitis in mice. Animal groups were pretreated with quercetin (25, 50, 100 mg/kg, per os (p.o.)), thalidomide (200 mg/kg, p.o.) or vehicle (2% dimethyl sulfoxide (DMSO)) 1 h before hourly (×5) intraperitoneal injections of cerulein. A saline (0.9%, NaCl)-treated control group was included for comparison. Cerulein significantly enhanced the serum levels of amylase and lipase, and pancreatic myeloperoxidase activities, malondialdehyde and the proinflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6, as well as the pancreatic wet weight/body weight ratio. Cerulein significantly reduced the serum levels of IL-10. Histological assessment of the pancreas showed tissue edema, neutrophil infiltration, acinar vacuolization, and cell necrosis and a marked increase in the immunoreactivity staining for TNF-α. Pretreatment with quercetin or thalidomide significantly attenuated the severity of cerulein-induced acute pancreatitis as evidenced by effective reductions in the pancreatic wet weight/body weight ratio, biochemical indices, proinflammatory cytokines, myeloperoxidase activity, malondialdehyde formation, and an increase in anti-inflammatory cytokine IL-10. Quercetin treatment also markedly suppressed the histological changes such as pancreatic edema, inflammatory cell infiltration, acinar cell necrosis, and the expression of TNF-α. Taken together, these results indicate that quercetin ameliorates the severity of cerulein-induced acute pancreatitis by acting as an anti-inflammatory and antioxidant agent.

Key words acute pancreatitis; flavonoid; quercetin; cerulein; cytokine; mouse

Acute pancreatitis is characterized by activation of digestive proteases, widespread inflammatory cell infiltration, leukocyte activation, the release of various inflammatory mediators, and acinar cell necrosis and is often associated with significant morbidity and mortality.1,2 The premature intra-acinar activation of digestive enzymes is a key event in the pathogenesis of acute pancreatitis. The inflammatory response is partly caused by the release of chemokines from acinar cells, which is followed by recruitment of helper T lymphocytes and macrophages, leading to pancreatic edema and accumulation of neutrophils. A role for oxidative stress in the pathogenesis of pancreatic disease has been reported.3,4 The local and systemic release of inflammatory mediators, including cytokines, complement, and nitric oxide in excess may lead to the development of the systemic inflammatory response syndrome (SIRS) and/or systemic acute respiratory distress syndrome (ARDS).5—7 Repeated attacks of acute pancreatitis have the potential to evolve into chronic disease characterized by fibrosis and loss of pancreatic function.8 There are no specific therapies for acute pancreatitis. Medical management is aimed at the control of symptoms, prevention of severe complications, and possibly endoscopic stone removal if common bile duct stones are present or suspected.9 Agents that affect oxidative stress, inflammation, and acinar cell injury during the early phase of acute pancreatitis may arrest the pathologic progression to severe pancreatitis.

Studies in the past addressed the beneficial effects of plant-derived flavonoids like green tea catechins, emodin and baicalein in experimental pancreatitis.10—12 Quercetin (3,5,7,3′,4′-pentahydroxylflavone) is a naturally occurring plant flavonoid abundantly present in onions, fruits, and Chinese herbs. Several studies pointed out the beneficial biological activities of quercetin which include antioxidant, anti-inflammatory, antiatherosclerotic, and antitumor properties.13—15 Quercetin has been shown to inhibit carbachol-stimulated amylase release and to a partial extent the amylase release induced by agonists such as the cholecystokinin C-terminal octapeptide, calcium ionophore A23187, and phorbol ester tetradecanoylphorbol-13-acetate. The ability of quercetin to decrease agonist-stimulated amylase release has been attributed, at least in part, to quercetin-induced inhibition of PKC activity.16 In addition, quercetin was shown to block HSP70 expression and inhibit the activities of serine proteases (trypsin, thrombin, and urokinase) in vitro.17,18 Despite these potential beneficial effects, quercetin has not been evaluated so far in experimental pancreatitis. The present study therefore evaluated its potential to prevent pancreatic injury in cerulein-induced acute pancreatitis in mice, an experimental model that simulates human acute pancreatitis.19

MATERIALS AND METHODS

Chemicals and Drugs Quercetin, cerulein, hexadecyltrimethylammonium bromide, o-dianisidine dihydrochloride, hydrogen peroxide, 1,1,3,3-tetramethoxyxpropane, formalde-
hyde, dimethyl sulfoxide (DMSO), eosin and hematoxylin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Thalidomide was from Funed (Brazil). Quercetin was dissolved in 2% DMSO in saline.

**Animals** Male Swiss mice weighing 25—30 g were kept at a constant room temperature (ca. 23 °C) with light–dark cycles of 12 h and were allowed free access to water and standard laboratory chow. The animals were fasted for 12 h before beginning the experiments. Experimental protocols were approved by the Institutional Committee on Care and Use of Animals for Experimentation (No. 23/08) in accordance with the guidelines of the National Institutes of Health, Bethesda, MD, U.S.A.

**Cerulein-Induced Pancreatitis** The mice were divided into six groups (n = 8 in each group): group 1, normal control; group 2, vehicle (2% DMSO)-treated cerulein control; groups 3, 4, and 5, cerulein+quercetin-treated (25, 50, 100 mg/kg, per os (p.o.), respectively); and group 6, cerulein+thalidomide (200 mg/kg, p.o.). Acute pancreatitis was induced by 5 injections of cerulein (50 μg/kg, intraperitoneally (i.p.)) at intervals of 1 h.20 Quercetin and thalidomide were administered 1 h before the cerulein administration. The normal control mice were given saline (0.9%, NaCl) solution intraperitoneally instead of cerulein. Six hours after the last injection of cerulein or saline, mice were anaesthetized with pentobarbital (40 mg/kg, i.p.), blood samples were drawn, the animals were exsanguinated, and the pancreas was quickly removed and frozen at −70 °C until use.

**Determination of Pancreatic Edema** The pancreatic weight/body weight ratio was evaluated as an estimate of the degree of pancreatic edema.21

**Determination of Serum Parameters** Blood samples were collected 6 h after the last cerulein administration and then centrifuged at 3000 g for 10 min at 4 °C. The serum amylase and lipase levels were determined with a colorimetric method using a commercial kit for amylase (Labtest, Minas Gerais, Brazil) and lipase (Bioclin, Minas Gerais). Serum tumor necrosis factor (TNF-α), interleukin (IL)-1β, IL-6, and IL-10 levels were measured in enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Quantikine, R&D Systems, Minneapolis, MN, U.S.A.).

**Myeloperoxidase Assay** The myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocytes in the pancreas was determined according to a previously described method.23 Samples of pancreatic tissue were homogenized in 50 mM of phosphate buffer (pH 6), containing 0.5% hexadecyltrimethylammonium bromide (HTAB). The samples were freeze-thawed thrice with sonication between cycles and then the samples were centrifuged at 10000 g for 15 min. Aliquots of supernatant were added to the reaction mixture containing 0.167 mg/ml of o-dianisidine and 0.0005% H₂O₂ solution, which were prepared in 50 mM of phosphate buffer. The resulting change in absorbance at 460 nm was measured spectrophotometrically for 5 min. One unit of MPO activity was defined as that degrading 1 mmol of peroxide per min at 25 °C. The activity was expressed in units per milligram of tissue.

**Malondialdehyde Assay** The concentration of pancreatic lipid peroxidation was determined by estimating malondialdehyde (MDA) using the thiobarbituric acid test.23 The pancreatic tissue was homogenized in KCl 0.15 M (pH 7.4). The homogenate was maintained in a water bath for 60 min at 37 °C. Perchloric acid (35%) was added to the homogenate and centrifuged for 10 min at 14000 rpm. The supernatant was mixed with 1.2% thiobarbituric acid, and the mixture was heated at 98 °C for 30 min. After cooling to room temperature, the absorbance was measured at 532 nm. The standard curve was obtained using 1,1,3,3-tetramethoxypropane. The results were expressed as nanomoles of MDA per gram of wet tissue.

**Immunohistochemistry** Immunohistochemical analysis of the expression of TNF-α was performed. Sections of the pancreas (4 μm) were transferred to a gelatin-coated slide. The tissue sections were deparaffinized, and endogenous peroxidase activity was blocked by incubation with 3% H₂O₂ (10 min). Non-specific protein binding was blocked by incubating the tissue sections with goat serum (1:200 in phosphate buffered saline (PBS) for 45 min). The slides were then incubated overnight with primary rabbit anti-TNF-α (Sigma, U.S.A.) diluted 1:400 in PBS plus bovine serum albumin (BSA). The slides were gently washed with Tris-buffered saline (pH 7.4) and incubated with alkaline phosphatase-conjugated secondary antibody (EnVision/AP K1396, DakoCytomation kit). The reaction was developed by applying a solution containing levamisole on the slides to block human alkaline phosphatase activity and Fast Red Substrate (EnVision/AP, DakoCytomation).

**Histological Examinations** Samples of pancreatic tissue were fixed in 10% buffered formalin solution, embedded in paraffin using standard methods, cut into 5-μm sections, stained with hematoxylin-eosin, and then assessed under light microscopy and examined blind by a morphologist for grading the histological alterations. Pancreatic edema, leukocyte infiltration, acinar vacuolization, and necrosis were described with scores ranging from 0 to 3 as described by Dembiński et al.24

**Statistical Analysis** Statistical analysis was performed using analysis of variance (ANOVA) followed by Kruskal–Wallis or Student–Newman–Keul as post-hoc tests. The non-parametric data are expressed as median (with low and high ranges), and parametric data as mean±S.E.M. A p value of less than 0.05 was considered to represent a statistically significant difference.

**RESULTS**

The secretagogue cerulein (5×50 μg/kg), administered intraperitoneally induced acute pancreatitis in mice as evidenced by changes in biochemical and histological parameters. Cerulein caused a significant enhancement in serum levels of amylase and lipase enzyme and in the pancreatic weight/body weight ratio as compared with saline-treated controls (Fig. 1). These elevations were significantly less in mice pretreated with quercitin (25, 50, 100 mg/kg) (Figs. 1A—C). Thalidomide (200 mg/kg), the reference antiinflammatory drug included in the study also resulted in significant reductions in serum amylase and lipase activities as well as in the pancreatic weight/body weight ratio (Fig. 1).

In vehicle-treated cerulein controls, the pancreatic MPO activity, MDA formation, and serum levels of TNF-α, IL-1β, and IL-6 were significantly elevated, whereas the levels of serum IL-10 were significantly decreased (Figs. 2, 3). Similar
to thalidomide, treatment with quercetin (25, 50, 100 mg/kg) significantly reduced the cerulein-evoked increase in pancreatic MPO activity and MDA, and the serum levels of TNF-\(\alpha\), IL-1\(\beta\), IL-6, and IL-10 (Figs. 2, 3). In addition, quercetin (25, 50, 100 mg/kg) significantly enhanced the cerulein-evoked reduction in serum IL-10 (Fig. 3D).
Representative TNF-α immunostaining of the pancreas for different treatments are shown in Fig. 4. In control mice, the pattern of TNF-α staining was very faint (Fig. 4A). On the other hand, while there was high intensity staining for TNF-α in the acinar cells, inflammatory cells, and blood vessels of the pancreas in the cerulein group (Fig. 4B), in mice pretreated with quercetin (100 mg/kg) or thalidomide (200 mg/kg), TNF-α immunostaining intensity was much less (Figs. 4C, D).

Histological examination of normal controls showed normal architecture and the absence of edema, leukocyte infiltration, acinar vacuolization, and necrosis (Fig. 5A, Table 1). In contrast, pancreatic sections from cerulein-administered mice revealed extensive tissue damage characterized by significant disruption of the architecture with acinar cell vacuolization, extensive acinar cell necrosis, and inflammatory cell infiltration and thus received significantly higher scores (Fig. 5B, Table 1). Pretreatment with quercetin (100 mg/kg) and thalidomide (200 mg/kg) significantly protected the pancreas from histological damage induced by cerulein, as indicated by lower histological scores (Figs. 5C, D, Table 1).

**Table 1. Effects of Quercetin Treatment on Morphological Signs of Pancreatic Damage**

<table>
<thead>
<tr>
<th>Group</th>
<th>Edema (0—3)</th>
<th>Inflammatory infiltration (0—3)</th>
<th>Acinar vacuolization (0—3)</th>
<th>Necrosis (0—3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>0 (0—0)</td>
<td>0 (0—0)</td>
<td>0 (0—0)</td>
<td>0 (0—0)</td>
</tr>
<tr>
<td>Vehicle + cerulein</td>
<td>2 (1—3)*</td>
<td>2 (1—3)*</td>
<td>2 (0—3)*</td>
<td>1.5 (0—3)*</td>
</tr>
<tr>
<td>Quercetin 100 mg/kg + cerulein</td>
<td>0 (0—0)*</td>
<td>0 (0—1)*</td>
<td>0 (0—0)*</td>
<td>0 (0—0)</td>
</tr>
<tr>
<td>Thalidomide</td>
<td>0 (0—1)*</td>
<td>0 (0—1)*</td>
<td>0 (0—1)*</td>
<td>0 (0—1)*</td>
</tr>
</tbody>
</table>

Median scores with ranges (min–max) of the results on 6 animals in each group are shown. *p<0.05 compared with the normal control group, **p<0.05 compared with the vehicle + cerulein group (analysis of variance followed by the Kruskal–Wallis test).

**DISCUSSION**

The present study demonstrated that the flavonoid quercetin attenuates the severity of cerulein-induced pancreatitis in mice. In particular, we demonstrated that quercetin treatment reduces pancreatic inflammation and associated tissue injury through suppression of neutrophil infiltration, TNF-α, IL-1β, and IL-6 cytokine production, and TNF-α expression, besides increased IL-10 cytokine production.

Cerulein-induced pancreatitis is an experimental mouse model of human acute pancreatitis characterized by increased serum amylase and lipase activities, release of proinflammatory mediators and cytokines, and histological alterations.19) Cerulein, an analogue of cholecystokinin (CCK) acting through the CCK receptors, yields exaggerated stimulation of acinar cells, which leads to prematuration of trypsinogen, followed by lysosomal degradation of intracellular organelles within autophagic vacuoles in acinar cells and marked interstitial edema.20,21 The transcription factor nuclear factor (NF)-κappa B (NF-κB); cytokines TNF-α, IL-1β, and IL-6; platelet-activating factor (PAF), a neuropeptide acting through the tachykinin neurokinin 1 (NK1) receptor; free radicals; and hydrogen sulfide are known to take part in the pathogenesis of cerulein-induced pancreatitis.1,6,26,27)

TNF-α, IL-1β, and IL-6 were found to be enhanced in both experimental pancreatitis as well as in pancreatitis patients.28—30) TNF-α plays a pivotal role in severe acute pancreatitis, acting early in the disease course. IL-1β and IL-6 constitute the principal mediators in the synthesis of acute-phase proteins, in addition to transitioning the acute inflammatory response to a chronic response.20,31) In this study, quercetin pretreatment significantly attenuated cerulein-induced TNF-α, IL-1β, and IL-6 responses. Quercetin is believed to be the most effective orally active antioxidant and the present observations of quercetin activity are consistent with the proposal that hydroxyl groups of flavones in positions 3’, 4’, 5 and 7 are associated with TNF-α inhibition.32—34)

IL-10 is known to inhibit the activation of macrophages and T cells and to decrease proinflammatory cytokine production in acute pancreatitis.35,36) Administration of exogenous IL-10 protects the pancreas against acute damage.37,38) Increased serum levels of IL-10 have been shown to correlate with attenuation of disease severity.39)

Furthermore, immunohistochemical staining for TNF-α showed that quercetin could inhibit its expression. Thus the present study revealed that quercetin ameliorates acute pancreatitis by suppressing proinflammatory cytokines TNF-α, IL-1β, and IL-6, and TNF-α expression and the release of
the antiinflammatory cytokine IL-10.

Excessive reactive oxygen species (ROS) and nitrogen species (RNS) produced by nitric oxide synthase (NOS), and isoforms of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, or as by-products of the mitochondrial electron-transport chain, have been implicated in the pathogenesis of acute pancreatitis. Oxidative stress is a deleterious process that can be an important mediator of damage to cell structures, including lipids, membranes, proteins, and DNA. Antioxidants such as N-acetylcysteine, raxofelast, and pyrrolidine dithiocarbamate are efficient inhibitors of NF-κB activation in animal models of pancreatitis. In the present study, quercetin potently suppressed the neutrophil-mediated MPO enzyme and lipoperoxidation as evidenced by reduced MDA formation, events that reflect its antioxidant activity. Studies showed that quercetin has the capacity to inhibit NF-κB activation and thereby the production of inflammatory mediators.

Thalidomide, the positive control used in this study, is a synthetic derivative of glutamic acid that has been shown to inhibit TNF-α production, modulate adhesiveness in microvascular beds through the modification of surface cell adhesion molecules, and suppress the NF-κB activity. Thalidomide ameliorated pancreatic injury almost to a similar degree as quercetin. This finding with thalidomide is consistent with the observations of Malleo et al. that showed its efficacy in the cerulein model of experimental pancreatitis.

Repeated attacks of acute pancreatitis have the potential to evolve into chronic disease characterized by fibrosis and loss of pancreatic function. Acute pancreatitis is potentially fatal, and the currently practiced therapeutic regimens specific to pancreatitis reduce general lethality only to a small extent. Patients undergoing therapeutic endoscopic retrograde cholangiopancreatography (ERCP) are at a higher risk of developing post-ERCP pancreatitis than patients undergoing diagnostic ERCP. Clinical studies have suggested that prophylactic administration of nonsteroidal antiinflammatory drugs (NSAIDs like indomethacin and diclofenac), steroids, somatostatin, and the antibiotics (beta-lactams and quinolones) is useful in preventing pancreatitis in patients undergoing therapeutic ERCP. While steroids administered as prophylaxis against ERCP offered no such beneficial effect, NSAIDs, somatostatin, and antibiotics have in randomized studies shown favorable results by reducing the mortality as a result of sepsis. Therapeutic agents specific to pancreatitis have failed to show any advantages so far. In view of the role played by inflammatory mediators and oxidative stress in the development of acute pancreatitis, we consider that quercetin may be an ideal candidate for drug therapy against acute pancreatitis by virtue of its wide spectrum of pharmacologic activity (antioxidant, antiinflammatory, antiatherosclerotic and antitumor properties) and the ability to suppress cerulein-induced acute pancreatitis in a prophylactic regimen.

Quercetin at the tested oral doses of 25, 50, and 100 mg/kg in mice did not manifest any clinical signs of toxicity and therefore the doses employed in this study could be considered safe and nontoxic to treat acute pancreatitis. In this context, the literature also indicates no signs of clinical toxicity or decrease in body weights in rats treated with quercetin at the subcutaneous dose of 30, 90, or 270 mg/kg/d over 14 d.

In conclusion, this study provided the first evidence showing that quercetin attenuates the development of cerulein-induced acute pancreatitis through antiinflammatory and antioxidant mechanisms, reducing the infiltration of neutrophils, generation of inflammatory cytokines, and increasing antiinflammatory cytokines. The protease inhibitory effect of quercetin on the inflammatory response in experimental acute pancreatitis deserves future study.

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