INTRODUCTION

Stress accompanies the life of humans and other organisms affecting various physiological processes predominately including the activation of hypothalamic-pituitary-adrenal axis (HPA), which results in an excessive stimulation of the adrenal glands and the release of catecholamines and glucocorticoids (1, 2). Stress of diverse etiology is a well-recognized factor that impairs the integrity of gastrointestinal (GI) mucosa and leads to formation of microbleedings and...
material and methods

All procedures conducted in this study conformed to the guidelines of the Committee for Research and Animal Ethics of the Jagiellonian University and were run according principles of Helsinki Declaration.

The study was conducted on 183 white Wistar rats of both genders and weighing on average between 200 – 250 g. Rats were fasted for 18 hours before the start of each experiment but were allowed free access to water. The animals were fed with standard granulate with free access to water at standardized temperature and humidity.

Determination of gastric secretion in vivo

Gastric secretory tests were performed in 30 rats surgically equipped with metal cannula in about 14 days before the conduction of the secretory experiments as described in our previous study (18). Briefly, rats were anesthetized with pentobarbital (50 mg/kg i.p.), their stomach was exposed and an incision at the border of the forestomach and corpus was made. A metal fistula with a diameter of 4.0 mm and a length of 3.0 cm was placed in the incision site and gently sutured. Likewise, the abdominal cavity and skin were subsequently closed by suturing. All secretory experiments were carried out 14 days after the day of surgery when animals had fully recovered. Prior to each test, the animals with gastric fistula were deprived of food for 18 hours. For gastric content collection, rats were placed in individual Bollman-type cages to prevent coprophagy which is observed in these species during a period of a longer starvation. To study the secretory activity of gastric mucosa the fistulas were opened and the stomachs washed with about 5 – 10 ml of pre-warmed saline kept at 37°C. During the test, the animals received a continuous subcutaneous infusion of saline administered at a speed of 4.0 ml/h, to compensate for fluid lost with this secretion. Curcumin was administered in graded doses of 2.5, 10, 25, 50 and 100 mg/kg i.g. in a volume of 1.0 ml using a metal orogastric tube. The control animals received vehicle (1.0 ml of saline i.g.). For determination of the effect of curcumin on stimulated gastric secretion, the effect of curcumin on pentagastrin- or histamine-stimulated gastric secretion was examined. After the collection of two basal secretory samples of gastric juice the separate groups of rats were administered with pentagastrin in a dose of 250 µg/kg s.c. or histamine in a dose of 2 mg/kg s.c. and the gastric acid secretion was determined similarly as described above. In tests with pentagastrin- and histamine-stimulated gastric secretion, curcumin was applied i.g. in a single dose of 50 mg/kg and the gastric juice collection was continued for total of 120 min in four 30 min intervals. The control group of rats received 1.0 ml of vehicle (saline) given i.g. instead of curcumin. The volume of each 30 min sample was measured and the hydrogen ion concentration was determined by titration a sample of each fluid with 0.1N of NaOH to pH 7.0, using an auto-burette (Radiometer, Copenhagen, Denmark). The acid output was measured and calculated for each fraction and the results were expressed in µmol/30 minutes.

Stress-induced gastric mucosal damage and experimental groups

Stress-induced gastric mucosal damage was caused in rats by our modification of a method described earlier by Takagi and Okabe (19, 20). The rats were restrained in special cages and they were then immersed to the xiphoid level in water at 23°C. Previous studies have documented that both, water immersion and restraint act synergistically in the mechanism of formation of water immersion and restraint stress (WRS)-induced gastric

Curcumin, a yellow-color substance obtained from Curcuma longa is the main component of the root of the plant which for centuries has been universally used as a spice, a colorant, a cosmetic and even as the medication due to its anti-inflammatory and anti-cancer properties (11). Curcumin was reported to exhibit a therapeutic effect in various tumors including gastrointestinal (GI) ones, most inflammatory bowel diseases (IBD) and found effective in anti-inflammatory drugs, such as indomethacin, aspirin and phenylbutazone, which in contrast to curcumin can exert serious GI-side effects. Outside of GI-tract, curcumin reduced the organ inflammation and has been shown to inhibit the activity of enzymes such as cyclooxygenase-2 (COX-2), lipoxygenase (LOX) and the inducible isoform of nitric oxide synthase (iNOS) (13, 14). This compound has also been shown to reduce the production of proinflammatory cytokines such as interferon-γ and tumor necrosis factor-α (TNF-α) and exerts the profound inhibitory effect on the activity of transcription factors such as nuclear factor κB (NF-κB) and activator protein-1 (AP-1); all considered as signaling molecules essential in cell inflammatory response (15). Curcumin was reported to exhibit a therapeutic effect in inflammatory bowel diseases (IBD) and found effective in various tumors including gastrointestinal (GI) ones, most likely due to its high bioavailability for the cells of GI-tract organs (16, 17). Despite the proven multi-target, anti-inflammatory properties of curcumin, only a few experimental studies have until now been conducted to determine the mechanism of curcumin against experimental gastric mucosal lesions induced by various ulcerogenes and to study the potential role of gastric acid secretion and vasoactive mediators and pro- and anti-inflammatory factors in this protection.

Therefore, the aim of our present study was to examine whether curcumin affects gastric acid secretion and exhibits gastroprotective activity against gastric lesions by stress. Our study were essentially 3-folds: 1) to determine the effect of curcumin on basal and pentagastrin or histamine-stimulated acid secretion in rats with chronic gastric fistula; 2) to examine the contribution of major protective factors endogenous prostaglandin (PG), nitric oxide (NO) and neuropeptides released from sensory afferents in the mechanism of curcumin-induced protection against WRS-induced gastric lesions, and 3) to assess the gastric mucosal expression of gastric mucosal proinflammatory mediators such as TNF-α, iNOS and COX-2 in the intact gastric mucosa and that pretreated with curcumin and exposed to WRS.

hemorrhagic lesions so called ‘stress ulcerations’, whose pathogenesis was so far not thoroughly described (3, 4). The development of stress-induced gastric lesions is a frequent complication of diseases with severe clinical outcomes, such as extensive burns, shock, sepsis, brain trauma, surgeries and human terminal states (5, 6). The acute superficial gastric erosions in response to stress may progress to deeper lesions reaching muscularis mucosae. Previous studies revealed that the increased secretion of catecholamines from the adrenal medulla and a reduction in the generation of endogenous PG in the gastric mucosa possibly resulting from the activation of HPA and increased secretion of glucocorticoids can suppress the GFB resulting in vasoconstriction and lead to an increase in gastric acid secretion in the stomach. The enhanced gastric secretory activity in response to various stressors has been shown to exacerbate the course of stress-induced gastric damage, thus contributing to the pathogenesis of stress lesions (7-10).
mucosa lesions (21, 22). After being exposed to 3.5 h of WRS, the animals were anesthetized with pentobarbital (50 mg/kg i.p.), their abdominal cavity was opened and the gastric blood flow (GBF) was assessed. The stomachs were removed from all subjects and dissected along the greater curvature. Then mucosal damage was assessed macroscopically by planimetry (Morphomat, Carl Zeiss, Berlin, Germany) and the number of gastric lesions was calculated in each stomach of rat belonging to different experimental groups.

Experimental groups and pharmacological treatments for determination of the role of endogenous prostanoids (PG), nitric oxide (NO) and sensory nerves in protection by curcumin

Three major series (A, B and C) rats were selected to determine the contribution of endogenous PG, NO and sensory neuropeptides to the mechanism of protection induced by curcumin applied in a dose of 50 mg/kg i.g. which was efficient in our preliminary dose-dependent studies with curcumin in inhibiting WRS-induced gastric lesions by about 50%.

The involvement of endogenous PG in gastroprotective effects of curcumin was determined in series A of animals each consisting of 5 – 7 rats per each subgroup and pretreated either with:

1) vehicle (1 ml of 0.9% NaCl i.g.);
2) curcumin (50 mg/kg i.g.);
3) the non-selective COX-1 and COX-2 inhibitor, indomethacin (5 mg/kg i.p.); the selective COX-1 inhibitor, SC-560 (5 mg/kg i.g.); and the selective COX-2 inhibitor, rofecoxib (10 mg/kg i.g.) administered alone or in combination with curcumin (50 mg/kg i.g.).

The importance of NO in curcumin-induced gastroprotection was determined in rats of series B pretreated with L-NNA, an inhibitor of NO-synthase (23, 24) applied with or without vehicle or curcumin (50 mg/kg i.g.) and exposed to 3.5 h of WRS. Rats in this series B received:

1) vehicle (saline);
2) curcumin (50 mg/kg i.g.);
3) L-NNA (20 mg/kg i.p.) administered with or without curcumin (50 mg/kg i.g.), and
4) L-arginine (200 mg/kg i.g.) administered to rats pretreated with L-NNA (20 mg/kg i.p.) and combined 30 min later with curcumin (50 mg/kg i.g.) and exposed to 3.5 h of WRS.

The involvement of sensory neuropeptides in protective effect of curcumin was determined in rats of series C with intact or capsaicin-induced functional ablation of sensory afferent fibers as described before (23, 24). Briefly the irreversible ablation of visceral-afferent fibers was evoked by the subcutaneous application of capsaicin in neurotoxic doses according to the method described elsewhere (23). For this purpose, capsaicin was administered in gradually increasing dosages, 25, 50 and 50 mg/kg s.c. (a total dose of 125 mg/kg) each dose being injected within 3 consecutive days. All injections were performed under pentobarbital anesthesia to reduce respiratory impairment including bronchospasm that can be commonly observed when toxic doses of capsaicin were applied (23). The effectiveness of denervation was verified by a blink test, which involves the loss of corneal reflex after the direct application of a drop of diluted capsaicin to the conjunctival sac. All rats without corneal reflex were used for testing 2 weeks after the last dose of capsaicin was administered. Capsaicin-denervated rats (series C) received vehicle (saline) or curcumin (50 mg/kg i.g.) applied alone or combined with or exogenous CGRP (10 µg/kg s.c.), a major neuropeptide released from sensory nerve endings, and were 30 min later exposed to 3.5 h of WRS, similarly as those with intact sensory nerves. The implication of vanilloid VR-1 receptor was determined in rats pretreated with capsaepine (5 mg/kg i.g.), the vanilloid transient receptor potential vanilloid receptor (TRPV1) receptor antagonist (25) alone or combined with curcumin (50 mg/kg i.g.) and given 30 min prior the exposure to 3.5 h of WRS.

Examination of gastric blood flow (GBF) in WRS rats pretreated with vehicle (control) and curcumin

At the termination of each experiment the animals were anesthetized with pentobarbital (50 mg/kg i.p.) and GBF was measured using H2 gas clearance technique as described in our previous studies (26). Briefly, the GBF was measured in three places of intact gastric oxyntic mucosa and that exposed to WRS but not involving gastric lesions. The mean of 3 determinations was calculated and the GBF was expressed as percentage of the flow in the intact gastric mucosa as described before (27).

Determining plasma gastrin levels with radioimmunoassay

After the GBF determination was completed blood samples was withdrawn from vena cava, centrifuged for 15 minutes at 4000 rpm and the blood plasma was stored at –20°C until the RIA analysis as described in our previous studies (28). Briefly, the plasma concentration of gastrin was determined using the principle of method originally proposed by Yalow and Berson (29) with anti-gastrin antibodies (rabbit serum 4562, Copenhagen, Denmark) in a final dilution of 1:280000. The antibodies used in the tests recognized gastrin-17 and gastrin-34 in an equal measure (26). The effectiveness of detecting gastrin, evaluated as the sensitivity of gastrin assay, amounted to 2.5 pmol/L, while the precision of the method was determined at 88 – 92%.

Determination of mRNA expression for TNF-α, iNOS and COX-2 in intact gastric mucosa and that pretreated with vehicle- or curcumin in rats exposed to WRS

The expression of mRNA TNF-α, iNOS and COX-2 was determined by RT-PCR in intact gastric mucosa and those collected from rats pretreated with vehicle (saline) or curcumin administered in increasing dosages ranging from 2.5 mg/kg up to 50 mg/kg. Samples of the gastric mucosa (about 200 mg) were collected in Eppendorf tubes at 0°C using laboratory glass slides and then immediately immersed in liquid nitrogen. The collected samples were stored at –80°C until molecular tests. The RNA was isolated from the gastric mucosa using the method originally described by Chomczynski and Sacchi using Trizol (Invitrogen, Carlsbad, USA) according to the manufacturer’s protocol (30). Pre-standard cDNA was synthesized from total cellular DNA (µg) using the Reverse Transcription System (Promega, Madison, USA). The PCR reaction was conducted in an automatic DNA thermo cycler using 1 µg cDNA and Promega PCR reagents. To amplify the TNF-α, iNOS and COX-2 DNA specific DNA primers were used (Sigma-Aldrich, St. Louis, USA). The sequence of the TNF-α, iNOS and COX-2 primers is presented in Table 1. In order to verify the integrity of the RNA, a control amplification of β-actin was performed using the same samples (Table 1). PCR products were separated by electrophoresis in a 2% agarose gel containing 0.5 µg/ml of ethidium bromide and then visualized when exposed to UV light. The location of the expected PCR products was confirmed by using the control set of PCR products (O’Gene Ruler 50 bp DNA). The expression of β-actin, TNF-α/β-actin, iNOS/β-actin and COX-2/β-actin obtained from the immunoreactive areas of gastric mucosa from intact rats and those subjected to curcumin (50 mg/kg i.g.) applied 30 min prior 3.5 hour WRS was compared using the densitometry method (Gel-Pro Analyzer, Fotodyne Incorporated, USA).
Statistical analysis

The statistical analysis was performed on the basis of a nonparametric Mann-Whitney test. The results were reported as mean ± S.E.M. and, the results for parameters with a significance level of P < 0.05 were considered as significant.

RESULTS

Effect of vehicle (saline) or curcumin on basal and pentagastrin- and histamine-stimulated gastric acid in rats with chronic gastric fistula

The results of secretory tests with vehicle (saline, control) and curcumin administered i.g. in increasing doses ranging from 2.5 mg/kg up to 100 mg/kg i.g., on basal gastric secretion in rats equipped with chronic gastric fistula are presented in Table 2. The gastric acid output in the vehicle-control rats reached the value of 135 ± 21 µmol/30 min. Curcumin administered in a dose of 2.5 mg/kg i.g. failed to affect the basal acid output, however, when curcumin was administered i.g. in higher doses of 10, 25, 50 and 100 mg/kg, the significant dose-dependent reduction (P < 0.05) in basal acid output was observed by approximately 30%, 45%, 56% and 68%, respectively, compared to the value recorded in vehicle-control animals.

As presented in Fig. 1, basal gastric H⁺ concentration in vehicle (control) group of rats was 84.5 ± 9.0 µmol/30 min and this H⁺ concentration was significantly increased (P < 0.01) by the single administration of pentagastrin (250 µg/kg s.c.). The intragastric administration of curcumin in a dose of 50 mg/kg i.g. resulted in a statistically significant reduction of H⁺ concentration within the time period up to 240 min compared to the corresponding value obtained in the vehicle-control rats administered with pentagastrin (P < 0.05). The results of secretory tests presented in Fig. 2, demonstrate that the basal gastric acid secretion in the vehicle-control group of rats reached the value of 79 ± 6 µmol/30 min and this H⁺ concentration was significantly increased by histamine (2 mg/kg s.c.) (P < 0.01). The i.g. administration of curcumin in a dose of 50 mg/kg, significantly decreased the gastric H⁺ ion concentration over the time period of 240 min compared to the corresponding values obtained for the vehicle-control group stimulated with histamine (P < 0.05).

As shown in Fig. 3, the exposure of rats to 3.5 h of WRS caused numerous gastric lesions as reflected by the mean lesion number and significantly decreased the gastric blood flow (GBF) (P < 0.05). The value of GBF in stressed animals reached the value about 26% lower comparing to the value recorded in intact gastric mucosa (not shown). Plasma gastrin level which in intact rats not exposed to stress reached the value of 16 ± 2.5 pM/L, was significantly increased in vehicle-control rats exposed to WRS whose plasma gastrin averaged 25 ± 3.1 pM/L (P < 0.05). Intragastric application of curcumin in a dose of 2.5 mg/kg failed to significantly influence the number of WRS-induced gastric lesions, the GBF and plasma gastrin levels compared to the corresponding value obtained in vehicle-control group (Fig. 3). In contrast, the i.g. application of curcumin in higher doses of 10, 25, 50 and 100 mg/kg resulted in a significant reduction in the number of WRS-induced gastric lesions, the GBF and plasma gastrin levels compared to the corresponding value obtained in vehicle-control group (Fig. 3).

Table 2. The effect of graded doses of curcumin administered intragastrically (i.g.) on basal acid secretion in rats with chronic gastric fistula. The results are mean values ± S.E.M. obtained from seven rats per each group. An asterisk indicates a statistically significant (P < 0.05) change in the gastric acid secretion compared with the values obtained in vehicle (control).

<table>
<thead>
<tr>
<th>Study group</th>
<th>Gastric concentration of H⁺ (µmol/30 min)</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>135 ± 21</td>
</tr>
<tr>
<td>Curcumin 2.5 mg/kg i.g.</td>
<td>125 ± 14</td>
</tr>
<tr>
<td>Curcumin 10 mg/kg i.g.</td>
<td>82 ± 11*</td>
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<tr>
<td>Curcumin 25 mg/kg i.g.</td>
<td>73 ± 9*</td>
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<tr>
<td>Curcumin 50 mg/kg</td>
<td>61 ± 5*</td>
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<tr>
<td>Curcumin 100 mg/kg</td>
<td>48 ± 5*</td>
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Table 1. The sequence of primers, the annealing temperature and the size of PCR products of factors determined by RT-PCR in gastric mucosa of rats pretreated with vehicle (control) and curcumin (50 mg/kg i.g.) and exposed to 3.5 h of WRS.
Fig. 1. The effect of curcumin administered i.g. in a dose of 50 mg/kg on the pentagastrin (250 µg/kg)-stimulated gastric acid output in rats with chronic gastric fistula. The results are mean values ± S.E.M. obtained from seven rats. An asterisk indicates a statistically significant increase in gastric acid secretion compared to the control values (P < 0.05). The double asterisks indicate a statistically significant reduction in gastric acid secretion compared to the concentration of gastric H⁺ secretion following the application of vehicle (control) or curcumin at 120 min of observation in rats with pentagastrin-stimulated gastric acid secretion (P < 0.05).

Fig. 2. The effect of curcumin administered in a dose of 50 mg/kg i.g. on histamine stimulated (2 mg/kg s.c.) gastric acid secretion in rats with chronic gastric fistulas. The results are mean values ± S.E.M. obtained from seven rats per each group. An asterisk indicates a statistically significant increase (P < 0.05) in gastric acid secretion compared to the control values. The double asterisks indicate a statistically significant (P < 0.05) reduction in gastric H⁺ secretion compared to that stimulated with histamine in rats administered with vehicle (control) or curcumin at 120 min of observation.
Fig. 3. The mean number of WRS-induced gastric lesions and the alternation in the gastric blood flow (GBF) and plasma level of gastrin in rats pretreated with vehicle (saline, control) or curcumin administered intragastrically (i.g.) in graded doses ranging from of 2.5 mg/kg up to 100 mg/kg and exposed 30 min later to 3.5 h of WRS. The results are mean ± S.E.M. obtained from 5 – 7 rats per experimental group. An asterisk indicates a statistically significant change compared with vehicle (control) (P < 0.05).

Fig. 4. The mean number of lesions, the alternations in gastric blood flow (GBF) in rats pretreated with curcumin administered i.g. in a dose of 50 mg/kg with or without indomethacin (5 mg/kg i.p.), SC-560 (5 mg/kg/i.g.) and celecoxib (10 mg/kg i.g.) and exposed to 3.5 h of WRS. Results are mean ± S.E.M. from 5 – 6 rats per each experimental group. An asterisk indicates a statistically significant change compared with the vehicle-control values (P < 0.05). Cross indicates a statistically significant change compared with the corresponding values obtained in animals without curcumin administration (P < 0.05). Asterisk and cross indicate a statistically significant change compared with the corresponding values obtained in animals administered with COX-1 and COX-2 inhibitors (P < 0.05).
As shown in Fig. 4, the i.g. application of curcumin (50 mg/kg) significantly reduced the mean lesion number and caused a significant increase in the GBF in rats exposed to WRS (P < 0.05) with the extent similar to those presented in Fig. 3. The application of rofecoxib, the selective COX-2 inhibitor, SC-560, the selective inhibitor of COX-1 activity or indomethacin, the non-selective COX-1 and COX-2 inhibitor, all applied alone significantly increased the number of gastric lesions induced by WRS and also significantly reduced the accompanying alterations in GBF as compared with those recorded in vehicle-control group (Fig. 4). When curcumin was combined with rofecoxib, SC-560 or indomethacin, respectively, the number of WRS-induced gastric lesions was significantly increased and a significant reduction in GBF was observed as compared with the corresponding values obtained in animals administered with curcumin alone (P < 0.05) (Fig. 4).

As shown in Fig. 5, the pretreatment with curcumin (50 mg/kg i.g.) similarly as in Figs. 3 and 4, significantly reduced the number of gastric lesions induced by WRS and significantly increased the GBF as compared to respective values obtained in rats pretreated with vehicle (P < 0.05). The intraperitoneal (i.p.) application of L-NNA in a dose of 20 mg/kg failed to significantly alter in the number of WRS-induced gastric lesions and GBF compared to the corresponding values obtained in the vehicle-control mucosa in animals exposed to WRS. When curcumin was combined with L-NNA, the protective effect of curcumin was significantly reduced as manifested by a significant increase in the mean number of WRS-induced gastric lesions and by the significant reduction in GBF compared to the respective values obtained in rats pretreated with curcumin alone (P < 0.05) (Fig. 5). The combined administration of L-arginine and curcumin in the presence of L-NNA restored the protective effect of curcumin as documented by significant reduction in the number of WRS-induced gastric damage and an increase in GBF compared to the corresponding values observed in rats treated with combination L-NNA and curcumin without L-arginine administration (P < 0.05) (Fig. 5).

Fig. 6 shows that the i.g. administration of curcumin in a dose of 50 mg/kg resulted in a significant reduction in the number of WRS-induced lesions accompanied by a significant increase in GBF (P < 0.05), similarly as presented in Figs. 3 – 5. The combination of CGRP (10 µg/kg s.c.) added to curcumin applied i.g. in a dose of 50 mg/kg resulted in a further significant reduction (P < 0.05) in the number of WRS-induced lesions and a significant increase in GBF (P < 0.05) when compared with curcumin applied alone (Fig. 6). The number of gastric lesions was significantly increased and the GBF was significantly decreased in WRS exposed rats with capsaicin denervation comparing with those recorded in rats with intact sensory nerves (P < 0.05) (Fig. 6). Both the decrease in lesion number and an increase in GBF observed in rats pretreated with curcumin were significantly decreased in those with capsaicin denervation and both the decrease in lesion number and the accompanying increase in GBF were, in part, restored when CGRP was administered together with curcumin in rats with capsaicin denervation (P < 0.05) (Fig. 6).

Fig. 5. The mean number of gastric lesions and alterations in gastric blood flow (GBF) in rats pretreated i.g. with curcumin in a dose of 50 mg/kg alone or combined with L-NNA injected i.p. in a dose of 20 mg/kg with or without the combination with L-arginine (L-Arg) applied i.g. in a dose of 200 mg/kg and exposed 30 min later to 3.5 hours of WRS. The results are mean ± S.E.M. from seven rats. An asterisk indicates a statistically significant change as compared to the control values (P < 0.05). A cross indicates a statistically significant change as compared to animals, in which only curcumin was administered (P < 0.05). A double cross indicate a statistically significant change as compared to animals concomitantly treated with L-NNA and curcumin (P < 0.05).
The macroscopic appearance of the rat gastric mucosa pretreated with vehicle (saline) or curcumin in gastric mucosa rats with intact sensory nerves and those with capsaicin-denervated sensory fibers administered intragastrically (i.g.) with curcumin (50 mg/kg) alone or in combination with CGRP (10 µg/kg s.c.) and exposed 30 min later to WRS. The results are mean values ± S.E.M. observed in 6–7 rats per each experimental group. An asterisk indicates a statistically significant change as compared to the vehicle-control values (P < 0.05). The asterisk and cross indicates a statistically significant change in as compared with rats with intact visceral sensory fibers administered with curcumin alone (P < 0.05). A single cross indicates a statistically significant change as compared with respective values in rats with intact sensory nerves administered with curcumin (P < 0.05). The double crosses indicate a significant change as compared with the values in group of animals with capsaicin denervation who received curcumin only (P < 0.05).

As shown in Fig. 9, the expression of mRNA for COX-2, TNF-α and iNOS in the intact gastric mucosa was weak and in majority of cases negligible but the exposure of vehicle-pretreated rats to 3.5 hours of WRS resulted in a significant increase in the expression of mRNA for these proinflammatory factors in the gastric mucosa (P < 0.05). The administration of curcumin in a dose of 2.5 mg/kg i.g. failed to significantly alter the signal expression of mRNAs for COX-2 and TNF-α but slightly decreased signal expression for iNOS as compared to those observed in vehicle-pretreated gastric mucosa in rats exposed to WRS (P < 0.05) (Fig. 9, left panel). A densitometry assessment of the ratio of mRNA for COX-2, TNF-α and iNOS over mRNA for β-actin confirmed that curcumin at the dose of 2.5 mg/kg failed to significantly affect the expression of COX-2 or TNF-α (Fig. 9A, 9B and 9C, right panel) and tended to decrease the mRNA expression of iNOS as compared to that in vehicle-pretreated gastric mucosa in rats exposed to WRS (P < 0.05) (Fig. 9D, right panel). The i.g. administration

Fig. 6. The mean number of WRS-induced lesions and the accompanying changes in gastric blood flow (GBF) in rats with intact and capsaicin-denervated sensory fibers administered intragastrically with curcumin (50 mg/kg) alone or in combination with CGRP (10 µg/kg s.c.) and exposed 30 min later to WRS. The results are mean values ± S.E.M. observed in 6–7 rats per each experimental group. An asterisk indicates a statistically significant change as compared to the vehicle-control values (P < 0.05). The asterisk and cross indicates a statistically significant change in as compared with rats with intact visceral sensory fibers administered with curcumin alone (P < 0.05). A single cross indicates a statistically significant change as compared with respective values in rats with intact sensory nerves administered with curcumin (P < 0.05). The double crosses indicate a significant change as compared with the values in group of animals with capsaicin denervation who received curcumin only (P < 0.05).
of curcumin in doses of 10 mg/kg or 50 mg/kg decreased the signal expression of mRNA for iNOS, COX-2 or TNF-α in the gastric mucosa of rats exposed to 3.5 h of WRS (Fig. 9A-9D, left panel). A densitometry analysis confirmed a significant reduction in the ratio of mRNAs for iNOS, COX-2 and TNF-α over mRNA β-actin as compared with the respective values of the ratio of mRNA for these proinflammatory mediators in vehicle-control animals (P < 0.05) (Fig. 9A-9D, right panel).

**DISCUSSION**

The integrity of gastric mucosa exposed to damaging agents depends on both, maintenance of the hydrophobic lining of the epithelial cell membrane as well as on the functional complexity of mucosal defence mechanisms including the maintenance of mucosal blood flow, secretion of protective mucus and bicarbonates, fast restitution and proliferation of gastric cells, the biosynthesis of endogenous prostaglandins (PG), sulphydryl compounds, the endothelial and epithelial nitric oxide (NO) and hydrogen sulfide (H₂S) biosynthesis (31).

Numerous physiological factors and mechanisms are involved in the maintenance of gastric mucosal defence against damage caused by noxious stimuli. Among these factors and mechanisms is an enhanced cyclooxygenase activity (COX), which synthesizes endogenous PG, originally considered as prototypes of protective agents responsible for the concept of gastric cytoprotection later called up as gastroprotection (32). The substrate for COX enzyme is the arachidonic acid from which PG, in particular vasoactive endogenous prostaglandin E₂ (PGE₂) and prostacyclin (PGI₂) are formed (33, 34). It has been shown that PGE₂ protects the gastric mucosa against formation of gastric lesions by an enhancing of GBF, mucus secretion and the secretion of bicarbonate ions (HCO₃⁻) resulting in gastric protection, in part, also mediated by neutralization of acid secretion by these arachidonate metabolites (35). There are two isoforms of COX, the constitutive isoform COX-1, which derives PG involved in gastroprotection and the inducible
isoform COX-2, which raises PG under the inflammatory conditions (36). It is believed that in contrast to PG derived from COX-1, the abundance of PG resulting from COX-2 activity associated with inflammation can intensify inflammatory reactions in the gastric mucosa. This deleterious effect of COX-2 derived PGs can be mediated in particular by the recruitment of inflammatory cells and an enhancement in vascular permeability that can lead to the mucosal edema and the increase of transmission of pain and fever (37).

Another vasoactive factor derived from vascular endothelium and/or produced by epithelial cells of the gastric mucosa is NO which has been implicated in the mechanism of gastric integrity, gastroprotection and ulcer healing. Previous studies have shown that NO in mucosa is formed by the activity of NO synthase (NOS) (34). There are three isoforms of this enzyme in the gastric mucosa including endothelial NO synthase (eNOS), inducible NO synthase (iNOS) and neuronal NO synthase (nNOS). The release of NO resulting from cNOS activity can afford gastroprotection mainly due to a potent vasodilatation and an increase in gastric microcirculation caused by this gaseous molecule. The vasodilatory effect of NO contributes to gastroprotection and ulcer healing by an increase of both, blood flow and mucus secretion as well as the inhibitory effect on platelet aggregation and leukocyte recruitment resulting in the protection of gastric mucosa against damaging agents (38). The apparent reduction in the activity of cNOS following the application of a non-specific NOS inhibitor increases the susceptibility of gastric mucosa to damage in the presence of corrosive and irritating agents (39). In contrast, the iNOS isoform is known to synthesize excessive amounts of NO under inflammatory conditions mainly in macrophages (38). The enhanced NO derived from iNOS expression and activity plays an important role in non-specific immune mechanisms (39). Interestingly iNOS expression has been observed not only in macrophages, but also in epithelial cells lining the GI-tract (40-42). The iNOS expression in the mucosal epithelial cells of the stomach and the duodenum has been associated with the inflammatory response initiated by infectious agents, toxic substances or stress (43, 44). An excessive amount of NO released in inflammatory conditions increases the interaction of NO with superoxide anion (O$_2^-$) that leads to the formation of a highly reactive peroxynitrite (ONOO$^-$), which in turn, can lead to cellular damage through oxidation of phospholipids, DNA structural damage and the activation of enzymatic and regulatory proteins and proteases (46, 47).

It is widely accepted that besides PG and NO, also neuropeptides released from capsaicin-sensitive C-afferent fibers are also involved in the mechanism of gastric mucosal integrity. These fibers were shown to regulate the gastric blood flow as well as the metabolism and activity of the secretory cells of the mucous membrane (47). Vasoactive neuropeptides, released from these fibers including calcitonin gene related peptide (CGRP) or substance P (SP), have been shown to affect directly the vascular smooth muscle cells causing vasodilatation, or they can exert indirect vasodilatory effect via release of NO (48, 49).

Capsaicin affects the activity of visceral-afferent fibers depending on the dose, because when applied in small doses, it increases the activity and secretion of CGRP and SP (50). In contrast, high neurotoxic doses of capsaicin were shown to cause functional ablation of sensory fibers and an inability to release of vasoactive neurotransmitters (50). We have confirmed the

![Fig. 8.](image-url) Fig. 8. The mean number of WRS-induced gastric lesions and alternations in the gastric blood flow (GBF) in rats pretreated with curcumin administered in a dose of 50 mg/g i.g. with or without the combination with capsazepine applied i.g. in a dose of 5 mg/kg. The results are mean values ± S.E.M. in 5 – 7 animals per each group. An asterisk indicates a statistically significant change compared with the vehicle-control values (P < 0.05). A cross indicates a statistically significant change as compared with curcumin alone (P < 0.05).
involvement of vasoactive C-fibers in the mechanism of protection of gastric mucosa against ethanol- and stress-induced gastric damage by demonstration that these lesions induced by ethanol and stress were augmented in rats with capsaicin denervation. In addition, this capsaicin-induced functional ablation of sensory nerves also impaired the protective activity of various physiological and pharmacological gastroprotectants (8, 9, 51-53).

Previous studies revealed that the stimulation of the parietal cells can predispose the gastric mucosa to damage by topical and non-topical ulcerogenes such as aspirin, acidified taurocholate and stress (54). This is why we assessed the effect of intragastrically administered curcumin on basal and stimulated gastric acid secretion. In our preliminary studies, we found that the intragastric administration of curcumin dose-dependently inhibited basal histamine- and pentagastrin-stimulated gastric acid secretion. To date, only a few studies have been conducted to determine the effect of curcumin on gastric acid secretion and to check whether this potential antisecretory activity of curcumin can contribute to the protective efficacy of this compound. For instance, Kim et al. (55) have demonstrated that the *Curcuma longa* extract protected the gastric mucosa against ulceration with extent similar to that achieved with the treatment with histamine H$_2$-receptor antagonist ranitidine and inhibited gastric acid secretion in rats with pylorus ligation ultimately leading to gastric ulcerations. Mahattanadul et al. (56) have also indicated that curcumin and bisdemetoxycurcumin can inhibit the basal gastric acid secretion and this effect can, at least in part, explain an improvement in the healing of chronic gastric ulcerations observed in animals treated with these compounds. Interestingly, the healing effect of curcumin has been found comparable to the inhibitory activity of another histamine H$_2$-receptor antagonist, cimetidine (56). Interestingly, curcumin afforded protection against indomethacin-induced gastric damage resulting also, in part, from the inhibition of gastric acid secretion (57). Xueting et al. (58) have demonstrated that a complex of zinc and curcumin (zinc(II)-curcumin) dose-dependently inhibited stress-induced gastric lesions while suppressing gastric acid secretory activity with the extent similar to that of proton pump inhibitor, lansoprazole.

**Fig. 9.** The expression of mRNA for COX-2, TNF-α and iNOS compared to the expression of mRNA β-actin in the gastric mucosa of intact rats (line 1) and those pretreated with vehicle (saline, control) (line 2) or curcumin administered intragastrically (i.g.) in doses of 2.5, 10 and 50 mg/kg and 30 min later exposed to 3.5 hours of WRS (lines 3 – 5) (left panel) and the semi-quantitative densitometry analysis of signal expression for these factors in intact gastric mucosa and those pretreated with vehicle (control) or curcumin and compromised by WRS (right panel). The results are mean values ± S.E.M. in 3 – 4 animals per each group generated in 3 tests. An asterisk indicates a statistically significant difference compared to the values obtained in gastric mucosa of intact rats (P < 0.05). Cross indicates a statistically significant difference compared with the values obtained in rats treated with vehicle (saline) (P < 0.05).
Our present study has demonstrated that curcumin attenuates the WRS-induced gastric mucosal lesions and that this effect is accompanied by an increase in GBF and plasma gastrin concentration. Xueting et al. (58) studied the protective activity of the Zn(I)-curcumin complex in a stress model involving gastric mucosa lesions. This protective activity of Zn(I)-curcumin was associated with a reduction of mRNA expression of proinflammatory cytokines TNF-α and IL-6 in the gastric mucosa, an increase in the proliferative activity of gastric mucosal cells and a strengthening of antioxidant mechanisms (58). Moreover, the Zn(I)-curcumin complex increased the activity of ROS scavenging enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx), resulting in a reduction in malondialdehyde (MDA) formation, which is considered as an index of lipid peroxidation (58). Previous studies revealed that ROS-induced lipid peroxidation and oxidation of proteins are implicated in the pathophysiological mechanisms predisposing gastric mucosa to stress-induced gastric lesions (58-61). It has been shown that curcumin reduces oxidative stress and the formation of acute gastric damage by preventing the fall in the enzyme activity of SOD and the reduction in the expression of proinflammatory cytokines TNF-α and IL-1β. Moreover, the curcumin-induced protection was accompanied by the reduction in iNOS activity, the generation of free nitrogen radicals, the inhibition of apoptosis and an increase in cell proliferation in gastric mucosa (56, 58, 62, 63).

Herein we have demonstrated that gastroprotective effects of curcumin against the formation of stress lesions were accompanied by a pronounced rise in plasma concentration of gastrin. This hormone secreted by G cells of the APUD cells of gastric mucosa has been shown to exhibit a trophic effect on mucosa resulting in increasing cell proliferation and exerted the gastroprotective effect against damaging effect of ethanol and aspirin (64-66). Curcumin exhibits strong antioxidant, anti-inflammatory and anti-cancer activity through the regulation of gene expression activated by NF-k B (60). It has also been shown that the treatment with Zn(II)-curcumin afforded protection of the stomach against damaging action of mucosal irritants and its application attenuated formation of stress ulcers in the stomach (56).

Previous studies have confirmed that PGs synthesized by COX-1 play a major role not only in the mechanisms underlying the gastric mucosal integrity but also in the mechanisms of gastroprotection, the adaptation of the gastric mucosa to damaging agents and in the mechanism of acceleration of healing process of chronic ulcers (67-69). To determine the involvement of endogenous PG in gastroprotection by curcumin in our present study, we employed indomethacin (COX-1 and COX-2 inhibitor), celecoxib (COX-2 inhibitor) and SC-560 (COX-1 inhibitor). All these COX-1 and COX-2 inhibitors significantly increased the number of WRS damage and these effects were accompanied by a significant reduction in GBF. In physiological conditions, the synthesis of PG depends mainly on the constitutive activity of COX-1 and the PGs derived from COX-1 activity are responsible for maintaining the integrity of the gastric mucosa and adequate GBF (67). The blockade of COX-1 activity in our previous study resulted in the inhibition of generation of endogenous PG and tended to increase the damage caused by ischemia-reperfusion (68). In our study treatment with the selective and non-selective COX-1 and COX-2 inhibitors also intensified WRS gastric mucosal damage and attenuated the GBF. There has been increasing experimental evidence supporting the notion that PGs synthesized by COX-2 are also involved in the gastroprotective and gastric ulcer healing mechanisms of growth factors (70). An increased expression of mRNA COX-2 in the gastric mucosa has been observed in the healing of these acute lesions (68). In another study, Gretzer et al. (71) have indicated that PGs synthesized not only by the COX-1, but also by COX-2 are responsible for the adaptive gastroprotection induced by mild irritants. Our present study shows for the first time that PGs synthesized both by COX-1 and COX-2 could participate in the gastroprotective mechanism of curcumin because the selective and the non-selective COX-1 and COX-2 inhibitors eliminated the hyperemic and protective activities evoked by this compound.

The endogenous NO could be another candidate to contribute to the mechanism underlying the gastroprotective activity of curcumin. In our present study, blocking of the activity of NO synthase in gastric mucosa through the application of L-NNA resulted in elimination of the gastroprotective activity of curcumin against WRS-induced lesions, and this effect of L-NNA was accompanied by a significant reduction in GBF. The application of L-arginine, a substrate for NO synthase, in combination with L-NNA restored the curcumin-induced protection and the accompanying rise in GBF. This suggests the involvement of endogenous NO synthesized by cNOS in the gastroprotective effect of curcumin against WRS-induced gastric lesions. Since vasoactive NO can be released from vascular endothelium and epithelial cell as well as from afferent capsaicin-sensitive fibers (72-74), therefore, the hypothesis that the activity of the visceral-afferent fibers of vagus and spinal nerves play a role in these protective mechanisms of curcumin cannot be ruled out. These afferent and efferent fibers are a component of the reflex arcs of vasomotor reflexes that trigger mucosal and reflexive anti-inflammatory defence mechanisms via release of vasoactive CGRP (75). This neuropeptide has been shown to regulate GBF, post-damage secretion, and enhanced gastroprotection and ulcer healing processes (76). Capsaicin, a substance isolated from red pepper, when applied in small doses it triggers gastroprotective activity, but when administered in high doses it induces functional ablation of afferent fibers that leads to depletion of vasoactive mediators. It is known that capsaicin-induced functional ablation of sensory nerve endings increases susceptibility of mucosal gastric mucosa to the damaging activity of mucosal irritants, stress and ischemia/reperfusion, and has also been shown to delay the healing of experimental pre-existing ulcers (72, 77, 78). Furthermore, recent studies have demonstrated the colocalization of CGRP and the vanilloid receptor (TRPV-1) in the gastric mucosa of mice (79). Previous studies revealed that TRPV-1 is a ligand-gated Ca++ channel which controls the release of the neurotransmitters of visceral-afferent fibers including CGRP and that these receptors are activated mainly by capsaicin. Both capsaicin and TRPV-1 receptors have been proposed to play a role in the mechanisms underlying sensitization to painful stimuli and seem to account for the development of neuropathic and tissue pain (78-82). Studies on mice deprived of the TRPV-1 gene revealed a significant exacerbation of inflammatory reaction in these animals with experimental colitis (72, 84).

Our study documented that the concurrent treatment with CGRP in the presence with curcumin increased the hyperemic and gastroprotective activities of curcumin against WRS-induced damage over those observed in curcumin alone. We confirmed that capsaicin denervation increased the susceptibility of gastric mucosa to the damaging effects of stress, eliminated the protective activity of curcumin and profoundly reduced GBF. However, the combination of CGRP with curcumin in rats with capsaicin denervation restored the gastroprotective and hyperemic activities of curcumin. This study has demonstrated for the first time that neurotransmitters of visceral-sensory fibers exhibiting vasoactive action such as CGRP and NO synthesized possibly by cNOS could account for curcumin-induced gastroprotection and an increase in the GBF observed in gastric
mucosa of rats compromised by WRS. Thus, curcumin exhibiting vasoactive and anti-oxidizing activities can be added to the growing list of antioxidants such as melatonin, L-tryptophan and medicinal plant extracts containing a high amount of antioxidants recently implicated in the mechanism of cytoprotection of upper GI-tract (78).

The administration of capsapine, the TRPV-1 antagonist which by itself failed to influence WRS-induced gastric damage in our study, attenuated the gastroprotective and hyperemic effects of curcumin. This suggests that TRPV-1 receptor can be involved in the gastroprotective mechanisms of curcumin. It is of interest that the TRPV1 receptor has also been implicated in the anti-inflammatory mechanisms of curcumin in rats with experimental colitis because the concurrent treatment with capsapine suppressed the anti-inflammatory effects of curcumin in healing of this colitis (84). The gastroprotective and anti-inflammatory activity of curcumin through the activation of the TRPV1 receptor may be due to the direct activation of this receptor by the curcumin. This assumption is based on the fact that the same vanilloid ring pharmacophore as in case of capsaicin has been identified in the chemical structure of curcumin. Thus, the structure similarities in vanilloid receptors and the structure of curcumin is most probably responsible for activating the TRPV1 receptor by this compound as has been proposed recently (83). Recently, curcumin attenuated the elevated proapoptotic proteins caspase 3 and Bax expression in cytoplasm and nucleus of hepatocytes of gentamicin-injected rats while the lowered expression of antiapoptotic protein Bcl-2 was increased (85). Thus, curcumin successfully prevented the deleterious effects on liver function by enhancing anti-oxidant defense system, suppression of oxidative stress and attenuation of inflammation and apoptosis (86).

In summary, our present study shows that curcumin exhibits dose-dependent gastroprotective and hyperemic properties accompanied by an increase in GFB and plasma gastrin levels. Interestingly, the intragastric administration of curcumin inhibits both basal and secretagogue-stimulated gastric acid secretion and this effect may contribute to gastroprotective activity of curcumin. Exposure to stress increased gastric mucosal expression of proinflammatory factors mRNA for COX-2, TNF-α and iNOS in the gastric mucosa but pretreatment with curcumin reduced expression of these proinflammatory factors TNF-α, COX-2 and iNOS in the gastric mucosa exposed to WRS. The mechanism by which curcumin protects the gastric mucosa may involve cooperation between the endogenous PG and NO and the activity of capsaicin-sensitive afferent fibers releasing CGRP and the stimulation of TRPV-1 by this anti-oxidizing and anti-inflammatory compound.

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