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Decreased gastric tone and delayed gastric emptying precede neutrophil infiltration and mucosal lesion formation in indomethacin-induced gastric damage in rats

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Abstract

Gastric antral dysmotility has been implicated in the pathogenesis of indomethacin-induced gastric damage, but the relationship between gastric motor abnormalities and mucosal lesions has not been extensively studied. We investigated whether changes in gastric tone and gastric retention correlate with mucosal lesions and neutrophil migration in indomethacin-induced gastric damage in rats. Indomethacin, either 5 or 20 mg/kg (INDO-5 and INDO-20), was instilled into the stomach, and then gastric damage, neutrophil migration, gastric tone and gastric retention were assessed 1 or 3 h later. Gastric damage was calculated as the sum of the lengths of all mucosal lesions, and neutrophil migration was measured by assaying myeloperoxidase activity. Gastric tone was determined by a plethysmometric method, and gastric retention of either saline or Sustacal® was evaluated by a scintigraphic method. Gastric damage was detectable 3 h after either INDO-5 or INDO-20, but not after 1 h. Neutrophil migration was significantly higher 3 h after INDO-20 as compared with INDO-5 or control group, but not after 1 h. Values of gastric tone 1 and 3 h after either INDO-5 (1 h = 1.73 ± 0.07 ml; 3 h = 1.87 ± 0.03 ml) or INDO-20 (1 h = 1.70 ± 0.02 ml; 3 h = 1.79 ± 0.03 ml) were significantly lower than in controls (1 h = 1.48 ± 0.05 ml; 3 h = 1.60 ± 0.06 ml). Gastric retention of saline was higher 1 h after INDO-5 (58.9 ± 3.3%) or INDO-20 (56.1 ± 3.1%) compared to control (45.5 ± 1.7%), but not after 3 h. There were no differences concerning gastric retention of Sustacal® between the various groups. Indomethacin induced decreased gastric tone and delayed gastric emptying, which precede mucosal lesion and neutrophil infiltration. These results indicate that there is no relationship between these gastric motor abnormalities and mucosal lesion in indomethacin-induced gastropathy.

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- Gastric emptying
- Gastric tone
- Indomethacin
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- Neutrophil

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used drugs in the world (1). NSAID-induced gastric damage is the major side effect of this type of drug. The mechanism of NSAID-induced gastric damage is generally believed to be related to the ability of these agents to inhibit gastric prostaglandin generation (1). However, evidence has been recently produced that leukocyte adherence to the vascu-

lar endothelium (2-4), microcirculatory disturbances, superoxide radicals and protease liberation may be relevant pathogenic mechanisms in NSAID gastropathy (5,6).

Several studies have shown that NSAIDs are associated with altered gastroduodenal motility (6-10). Some data indicate that the administration of NSAIDs is followed by either increased gastric contractility, which correlates with gastric damage (6-9) or decreased intestinal spiking amplitude and disruption of migrating motor complexes (10), with no correlation with gastric damage. More recently, Bassotti et al. (11) showed that gastric antral motility is unchanged after NSAID administration in humans. It follows that the relationship between disturbed gastric motor activity and the extent of NSAID-induced gastric damage remains controversial.

In spite of a number of studies showing different effects of NSAIDs on antral motor activity (6-9,11), the motor function of the proximal stomach and the gastric emptying of liquid meals in indomethacin-induced gastropathy have not been studied extensively. In the present study we determined whether changes in gastric tone and emptying of nutrient or non-nutrient meals correlate with mucosal lesions and neutrophil infiltration in indomethacin-induced gastric damage in rats.

Material and Methods

Animals

Male Wistar rats weighing 220-280 g were fasted for 18-24 h before the experiments. The animals were housed in cages in temperature-controlled rooms and received water and food *ad libitum*. All animal treatments and surgical procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals, National Institutes of Health, Bethesda, MD, USA.

Drugs

The drugs used in this study were indomethacin (Prodome Química e Farmacêutica, São Paulo, SP, Brazil), Tris buffer (Merck, São Paulo, SP, Brazil), 3,3',5,5'-tetramethylbenzidine (Sigma, St. Louis, MO, USA), hexadecyltrimethylammonium bromide (H-TAB) (Sigma), urethane (Sigma), and Sustacal® (Mead-Johnson Nutritionals, Bristol-Myers Squibb, São Paulo, SP, Brazil).

Induction of gastric damage

Gastric damage was induced by intragastric instillation of indomethacin (5 or 20 mg/kg, INDO-5 and INDO-20) dissolved in Tris buffer (1.22% ethylthiomethane in distilled water (w/v), pH 8.0). The control group received only the vehicle (Tris buffer). Animals were killed 1 or 3 h later by decapitation and the stomachs rapidly removed, opened by an incision along the greater curvature and pinned out on a wax platform. The hemorrhagic or ulcerative lesions were counted and their lengths measured with an analogical caliper utilizing a magnifying lens. Gastric damage score (lesion index) was then calculated as the sum of the lengths of all linear lesions (12), which was measured by a single observer (M.H.L.P.S.), who was unaware of the treatment given to the animals. Full-thickness pieces of the gastric corpus were then weighed, frozen and stored at -70ºC until the assay for myeloperoxidase (MPO) activity.

Gastric myeloperoxidase activity

The extent of neutrophil accumulation in the gastric mucosa was measured by assaying MPO activity as previously described (13). Briefly, 50 to 100 mg of gastric tissue was homogenized in 2 volumes of ice-cold buffer (0.1 M NaCl, 20 mM $NaPO_4$, 15 mM NaEDTA), pH 4.7, and centrifuged at 0.8 *g*

for 15 min. The pellet was then subjected to hypotonic lysis (900 µl of 0.2% NaCl solution followed 30 s later by the addition of an equal volume of a solution containing 1.6% NaCl and 5% glucose). After a further centrifugation, the pellet was resuspended in 50 mM $NaPO_4$ buffer, pH 5.4, containing 0.5% H-TAB and re-homogenized. The homogenate was then frozen, thawed three times and centrifuged again at 9.3 *g* for 15 min at 4ºC. MPO activity in the resuspended pellet was assayed by measuring the change in absorbance at 450 nm utilizing tetramethylbenzidine (1.6 mM) and H_2O_2 (0.5 mM). Results are reported as the total number of neutrophils by comparing the absorbance of the tissue supernatant with that of rat peritoneal neutrophils processed in the same way. To this end, neutrophil migration was induced in the peritoneum of rats by injecting carrageenin (300 µg/animal). A standard curve relating neutrophil (>90% purity, 12,500 to 195.3 neutrophils/50 µl) numbers and absorbance was obtained by processing purified neutrophils as described above and assaying for MPO activity.

Gastric volume under a fixed intragastric pressure

Gastric tone was evaluated by assessing variations of gastric volume under a fixed pressure. After 18-24 h of fasting with free access to water, animals were anesthetized with urethane (1.2 g/kg, *ip*) and submitted to a tracheotomy to assure free breathing. A balloon catheter made of fingertips of surgical gloves was introduced *per os* and positioned in the rat proximal stomach, as previously described (14). The opposite end of the catheter was connected to a three-way valve and then to the bottom of a U-shaped glass reservoir equipped with an electronic volume sensor coupled to a plethysmometer (model 7140, Ugo Basile, Varese, Italy). In all experiments, the liquid reservoir level was 4 cm above the animal xiphoid appendix. Since the total volume of fluid in the entire system (stomach plus external reservoir) was known, changes in the volume in the external reservoir reflected those of the stomach. Gastric volume values (in ml) were monitored and recorded every minute. After a 10-min period for stabilization of gastric motility, the accuracy of the gastric volume measured with the plethysmometer was assessed in a group of four animals. A preweighed 1-ml syringe was used to gradually change the balloon volume by either injecting into or withdrawing ionic solution aliquots (0.1 ml) from the system up to ±0.5 ml. A strong correlation ($r^2 = 0.99$) was observed between the graded balloon volume and the values displayed by the plethysmometer for both injection and withdrawal of the ionic solution. Because of its volumetric capacity, the reservoir functions as a barostat, detecting changes in stomach volume under a constant pressure (14).

Three other groups of rats were treated at random with Tris buffer (1.22% ethylthiomethane in distilled water (w/v), pH 8.0), INDO-5, or INDO-20 as described before. One or 3 h after the injections, gastric volume was measured every minute for 10 min, as described before.

Gastric emptying

Gastric emptying was assessed by determining the fractional radioactivity remaining in the stomach after intragastric instillation of either saline or Sustacal® (1.0 ml/100 mg) labeled with 10 MBq of ^{99m}technetium (Instituto de Pesquisas em Energia Nuclear, IPEN, São Paulo, SP, Brazil) coupled to phytate ("Phytosid", Sydma Medical Reagents and Equipment, Ribeirão Preto, SP, Brazil) as an unabsorbable carrier. Three groups of 6-12 rats each were treated at random with Tris buffer, INDO-5 or INDO-20, as described before. One hour or 3 h after these injections, the animals were lightly anesthetized with ether and treated at ran-

dom with saline or Sustacal® dissolved in water (32 mg protein, 93.75 mg carbohydrate and 1.75 mg fat). Test meals were administered by gavage, which was followed by immediate removal of the tube. One or 3 h after these injections, saline or Sustacal® dissolved in water (32 mg protein, 93.75 mg carbohydrate and 1.75 mg fat) was instilled quickly by gavage, with immediate removal of the tube. Thirty minutes after saline administration, or 2 h after Sustacal® administration, animals were killed by decapitation and the stomach and small and large bowels were isolated by consecutive ligatures at the esophagogastric, gastroduodenal, ileocecal and retosigmoidal junctions. The gut segments were excised and inserted into bags made of glove fingers, where they were kept until counting in order to avoid spillage of their contents. Each segment was counted in a gamma camera (Sopha Vision DST, Sopha Medical Vision America, Twinsburg, OH, USA) and the results are reported as number of counts per minute after subtracting the background activity. The radioactivity remaining in the gastric segment was reported as the percentage of the sum of the counts in all gastrointestinal segments, including the stomach.

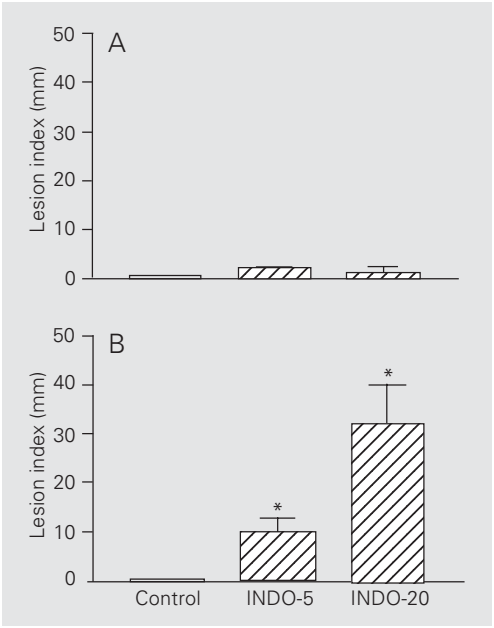

Figure 1. Gastric mucosal damage in rats treated with 5 or 20 mg/kg indomethacin (INDO-5 or INDO-20) or vehicle (control). After 1 h (panel A) or 3 h (panel B), animals were killed and the lesion index (mm) was calculated. Data are reported as the mean ± SEM for N = 5. *P < 0.05 compared to control (ANOVA followed by Newman-Keuls test).

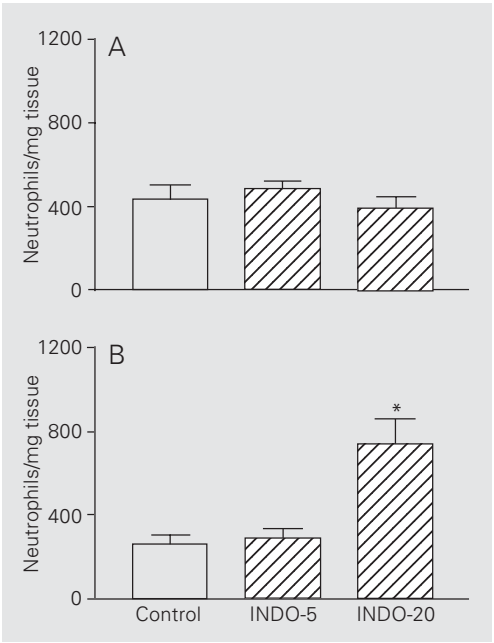

Figure 2. Recruitment of neutrophils in the stomach, measured by a myeloperoxidase (MPO) activity assay, in rats treated with 5 or 20 mg/kg indomethacin (INDO-5 or INDO-20) or vehicle (control). After 1 h (panel A) or 3 h (panel B), animals were killed and samples from the gastric mucosa were weighed, frozen and stored at -70ºC until time of the MPO activity assay. Results are presented as number of neutrophils per mg tissue and reported as mean ± SEM for N = 5. *P < 0.01 compared to control (ANOVA followed by Newman-Keuls test).

Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Newman-Keuls test when appropriate, with the level of significance set at $P < 0.05$. The accuracy of gastric volume measurements by plethysmometry was assessed using Pearson's linear regression.

Results

As shown in Figure 1B, intragastric instillation of both INDO-5 and INDO-20 caused significant ($P < 0.05$) and dose-dependent gastric damage, 3 h after drug administration, with maximal effect at the dose of 20 mg/kg. After 1 h, however, indomethacin administration caused negligible gastric damage with either 20 or 5 mg/kg (Figure 1A).

Figure 2B shows that neutrophil migration in the gastric mucosa was significantly higher ($P < 0.01$) 3 h after INDO-20, as compared with INDO-5 or vehicle (control).

However, there was no difference in neutrophil migration after 1 h in the same groups (Figure 2A).

Values of gastric volume 1 h after either INDO-5 or INDO-20 were significantly higher ($P < 0.01$) than in controls (Figure 3A). In addition, 3 h after indomethacin, gastric volume was significantly higher ($P < 0.01$) in both INDO-5 and INDO-20 groups as compared with control animals (Figure 3B).

Gastric retention of saline 1 h after either INDO-5 or INDO-20 was significantly higher ($P < 0.05$) compared to control (Figure 4A). However, 3 h after indomethacin, there were no significant differences between INDO-5 or INDO-20 and controls (Figure 4C). There were no differences in the gastric emptying of Sustacal® between the various groups either 1 h (Figure 4B) or 3 h after indomethacin administration (Figure 4D).

Discussion

The role of disturbances of gastric motility in the pathogenesis of NSAID-induced gastric damage remains to be elucidated. Takeuchi et al. (8,9) reported increased amplitude of antral contractions in indomethacin-induced gastric lesions in rats and suggested that gastric hypercontractility could play an important role in the pathogenesis of this condition. However, Fioramonti and Bueno (10) showed that indomethacin-induced gastric ulceration was associated with

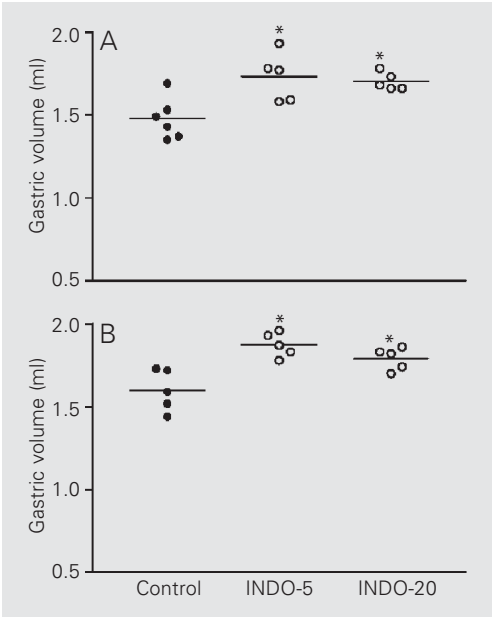

Figure 3. Gastric tone (volumes measured under a fixed intragastric pressure) in rats treated with 5 or 20 mg/kg indomethacin (INDO-5 or INDO-20) or control rats injected with Tris buffer. Measurements were started 1 h (panel A) or 3 h (panel B) after indomethacin administration. Data are individual values of gastric volume (in ml) recorded by plethysmography in anesthetized rats. The individual values were pooled into 10-min intervals. The horizontal bars represent the means. *$P < 0.01$ compared to control (ANOVA followed by Newman-Keuls test).

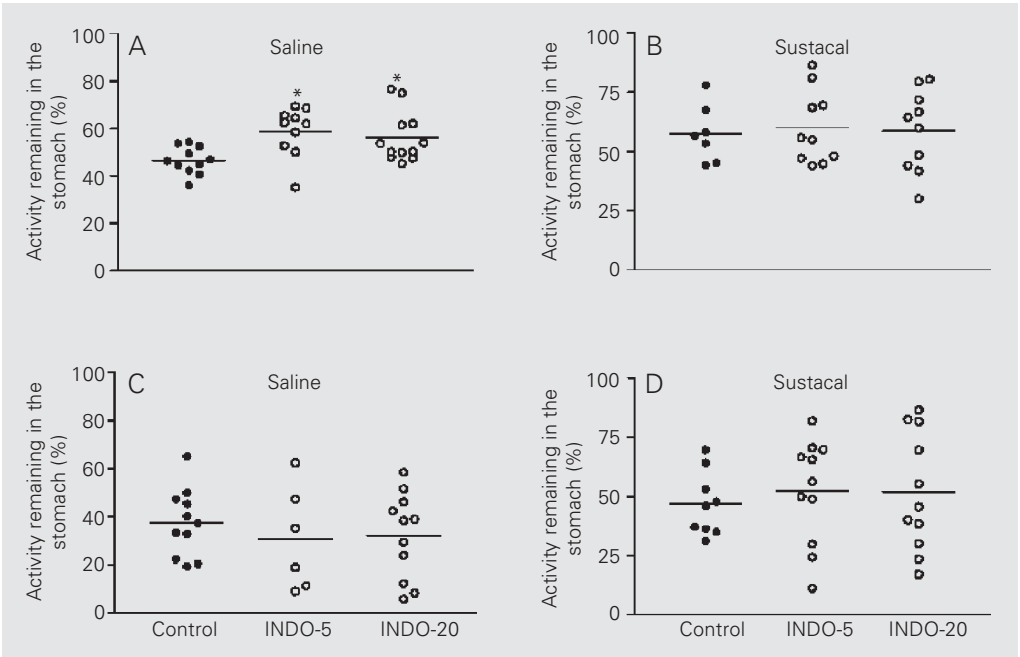

Figure 4. Gastric emptying of saline (panels A and C) or Sustacal® (panels B and D) in rats treated with 5 or 20 mg/kg indomethacin (INDO-5 or INDO-20) or control rats injected with Tris buffer. The studies started 1 h (panels A and B) or 3 h (panels C and D) after indomethacin administration. Data are individual values of the radioactivity remaining in the stomach 30 min after saline administration or 2 h after Sustacal® administration. Meals were labeled with ^{99m}technetium coupled to phytate as an unabsorbable carrier. Results are reported as percent of the total activity in the gastrointestinal tract. Horizontal bars indicate the means. *$P < 0.05$ compared to control (ANOVA followed by Newman-Keuls test).

both a reduction of intestinal spiking amplitude and disruption of the migrating motor complexes, but these motor abnormalities did not correlate with the extent of gastric mucosal damage. More recently, Bassotti et al. (11) were not able to show any difference in gastric antral motility after NSAID administration in men. In the present study we investigated whether changes in gastric motility concerning the proximal stomach and gastric emptying of liquid meals correlated with mucosal lesions and neutrophil infiltration in indomethacin-induced gastric damage and we found that decreased gastric tone and delayed emptying precede but do not seem to correlate with inflammatory and erosive changes in the gastric mucosa induced by indomethacin.

Our results indicate that neutrophil infiltration and gastric erosions were dose-dependent and evident 3 h, but not 1 h, after indomethacin administration, which is consistent with other studies showing that indomethacin caused a dose- and time-dependent increase in the extent of gastric mucosal damage and tissue MPO activity (8,15). Trevethick et al. (15) showed that the ulceration became first apparent macroscopically 2 h after dosing with indomethacin, and Takeuchi et al. (8) observed hemorrhagic lesions only within 4 h after indomethacin administration. Also, in our study, neutrophil migration was detected only with the higher dose (20 mg/kg), but not with the lower dose of indomethacin (5 mg/kg), which conflicts with the result of another study showing that administration of 5 mg/kg indomethacin caused an increase in MPO activity (4). This discrepancy could be ascribed to the differences in the sensitivity of the MPO assay used. However, we cannot rule out the possibility that in our study the gastric damage induced by low doses of indomethacin was a neutrophil-dependent process.

The results of the present study also showed that indomethacin administration caused a reduction in gastric fundus tone, expressed as an increased gastric intraballoon volume with a fixed pressure at all times and with all doses used. These results are in agreement with previous studies that showed a reduction in gastric tone *in vitro* by indomethacin pretreatment (16). Studies performed *in vitro* showed that indomethacin administration can reduce the responsiveness to acetylcholine in both the longitudinal and the circular layers of muscle strips (17). It is noteworthy that a decreased cholinergic reactivity after indomethacin administration could explain our observations. Alternatively, the indomethacin-induced reduction in gastric fundus tone might be explained by an increase in nonadrenergic, noncholinergic relaxation. However, this does not seem to be the case, since Gustafsson and Delbro (18) showed that the vagally induced nonadrenergic, noncholinergic gastric relaxation was significantly reduced *in vivo* by indomethacin. In addition, Corak et al. (17) showed that indomethacin-induced reduction of responsiveness to acetylcholine in both the longitudinal and the circular layers of muscle strips was not affect by L-NAME pretreatment.

We found a significant delay in the gastric emptying of saline 1 h after indomethacin, when neither gastric damage nor neutrophil infiltration had yet been detected. Our results suggest that changes in gastric motor activity are not related to macroscopic gastric mucosal damage. Nevertheless, we cannot completely rule out a contribution of motor abnormality in gastric lesion formation, because we did not measure microscopic damage in the early phase. These results agree with previous gastric emptying studies conducted on both rats (17) and in humans (19) showing that the delay in gastric emptying bears no apparent relationship with gastric mucosal damage. In contrast, gastric tone was still decreased by the time both gastric mucosal damage and neutrophil infiltration had become apparent, 3 h after

indomethacin administration, but was not associated with delayed gastric emptying of non-nutrient or nutrient meals. Decreased gastric tone, as we consistently found after indomethacin, would theoretically be associated with delayed gastric emptying, which we found with saline only 1 h after drug administration (Figure 4). This apparent discrepancy between decreased gastric tone and unaltered gastric emptying of saline might be explained by the effect of indomethacin administration on duodenal motility, which was not evaluated in this study. Fioramonti and Bueno (10) reported that decreased amplitude of spike potentials and abolished fasting migrating motor complexes were shown in indomethacin-induced gastric damage. In addition, Lu et al. (20) demonstrated that after indomethacin administration, intestinal segments with lesions failed to produce peristaltic activity, whereas in those segments with no visible lesions the peristaltic activity was enhanced. Concerning gastric emptying of Sustacal®, we were not able to find any difference between control and indomethacin-treated animals, at any time point or doses utilized. This could be explained by the strong inhibitor effect of the fat and protein components of Sustacal® on duodenal receptors, which could slow gastric emptying through a variety of motor mechanisms, including decreased gastric tone, and therefore mask any possible effect of indomethacin.

As far as the clinical meaning of our results is concerned, it is noteworthy that at least 10 to 20% of patients taking NSAIDs have gastric mucosal lesions and upper gastrointestinal symptoms, while the overall prevalence of dyspepsia may reach up to 50% of patients on NSAIDs (21,22). The mechanisms underlying NSAID-related dyspepsia associated or not with gastric mucosal lesions are poorly understood and current interpretations lack a scientific basis (23). Since our results show the occurrence of motor abnormalities concerning both gastric proximal function and emptying in an animal model of NSAID-induced gastric damage at a time when the animals have not yet developed gastric mucosal damage, it is tempting to speculate whether gastric motor abnormalities play a role in the origin of some symptoms in NSAID-associated dyspepsia. In order to confirm this hypothesis, further studies on humans are needed. Recently, Holtmann et al. (24) showed that a failure to increase sensory thresholds during treatment with aspirin is associated with the development of dyspepsia.

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