

Role of eicosanoids, nitric oxide, and afferent neurons in antacid induced protection in the rat stomach

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Abstract

The mechanism underlying the mucosal protective effect of antacids is still unclear. This study shows that in rats the aluminum containing antacid, hydrotalcit, induces dose dependent protection against gastric mucosal damage caused by ethanol or indomethacin which is considerably enhanced by acidification. Hydrotalcit did not increase gastric mucosal formation or the intraluminal release of prostaglandins, and did not prevent the increase in mucosal leukotriene C₄ formation in response to ethanol. Pretreatment with indomethacin did not attenuate the protective effect of unmodified or acidified hydrotalcit. Furthermore, hydrotalcit significantly reduced the gastric damage caused by indomethacin even when it was administered up to 2 hours after the ulcerogen. In indomethacin treated rats, simultaneous administration of hydrotalcit did not affect the concentrations of indomethacin in serum or inflammatory exudates nor did it attenuate the inhibition of prostaglandin release into the exudates. In hydrotalcit treated rats there was no attenuation of the increase in sulphidopeptide leukotriene release or decrease in leukocyte influx into inflammatory exudates elicited by indomethacin administration. Functional ablation of afferent neurons and inhibition of endogenous nitric oxide partially antagonised the protective effect of unmodified, but not of acidified, hydrotalcit. It is concluded that (i) the protective effect of unmodified and acidified hydrotalcit is independent of the eicosanoid system; (ii) protection against indomethacin induced gastric lesions does not require treatment before dosing of the ulcerogen and does not interfere with absorption and anti-inflammatory actions of indomethacin; (iii) endogenous nitric oxide and afferent neurons contribute partly to the effect of unmodified, but not of acidified, hydrotalcit suggesting that different mechanisms mediate their mucosal protective activity.

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Aluminum containing antacids have been shown to protect the gastric mucosa against a variety of noxious agents. This protective effect is not mediated by their acid buffering capacity, however, since it occurs in both acid related¹⁻⁵ and acid independent mucosal injury.^{1-3,5-9} Furthermore, recent investigations have shown that acidification of aluminum containing antacids, which results in complete loss of acid neutralising capacity, does not diminish, and even noticeably enhances, their protective activity.^{3-5,9} The

mechanism underlying antacid induced gastroprotection has not been fully explained. It has been suggested that stimulation of endogenous prostaglandin formation in the stomach mediates, at least partly, the protective effect.^{1,7,10} We have investigated whether treatment with the aluminum containing antacid, hydrotalcit (Mg₆ Al₂ (OH)₁₆ CO₃ × 4 H₂O), affects gastric mucosal generation of eicosanoids *ex vivo* and *in vivo* and whether a functioning prostaglandin system is a prerequisite for the mucosal protective effect in rats. In addition, we have investigated whether the hydrotalcit induced protection against lesions caused by indomethacin is related to reduced absorption of the ulcerogen.

Recently, nitric oxide (NO) has been described as a potent endothelium derived vasodilating mediator (for review see¹¹). Endogenous NO contributes to the maintenance of gastric mucosal integrity¹² and is involved in the modulation of the gastric mucosal microcirculation.¹³ Furthermore, NO is a major mediator of the prevention of gastric mucosal damage elicited by the stimulation of afferent neurons in response to capsaicin¹⁴ and of the gastroprotective effect of the antiulcer drug, carbenoxolone.¹⁵ We have, therefore, investigated whether the inhibitors of NO biosynthesis, N^G-nitro-L-arginine (L-NNA) and its methyl-ester (L-NAME)¹¹ modify the mucosal protective effect of unmodified and acidified hydrotalcit. In addition, studies were performed to investigate whether protection elicited by hydrotalcit involves stimulation of capsaicin sensitive afferent neurons.

Methods

MATERIALS

Hydrotalcit was obtained from Bayer AG (Leverkusen, Germany). Indomethacin, L-NNA, L-NAME, prostaglandins, leukotriene C₄ (LTC₄), and all other chemicals were purchased from Sigma chemicals Co (St Louis, MO, USA). [³H]-PGE₂, [³H]-6-keto-PGF_{1α}, [³H]-LTC₄, and [¹⁴C]-indomethacin were from New England Nuclear Co (Dreieich, Germany).

Two hydrotalcit preparations were used in the study. Unmodified hydrotalcit (neutralising capacity 30.7 mval HCl/g¹⁶) was suspended in 0.25% methylcellulose (MC). Acidified hydrotalcit was prepared by titration of hydrotalcit suspensions in 0.25% MC to pH 2.5 using 6 N HCl. Previous studies have shown that acidification to pH 2.5 results in solubilisation, loss of neutralising capacity, and maximum increase in the protective potency of aluminum containing antacids.⁹ Equilibration was achieved

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over 24 hours. Both hydrotalcit suspensions were prepared in such a way that the desired dose was administered in 5 ml/kg.

EXPERIMENTAL PROTOCOL

Male Wistar rats (weighing 180–220 g) were used in all experiments. They were housed in a controlled environment ($22 \pm 1^\circ\text{C}$) with a 12 hour light/dark cycle. Rats were deprived of food for 24 hours but had free access to water before the start of the experiments.

GASTRIC MUCOSAL PROTECTION STUDIES

Ethanol induced gastric lesions

Groups of six rats were treated orally with graded doses of unmodified (80–750 mg/kg) or acidified (40–200 mg/kg) hydrotalcit 60 minutes before intragastric instillation of 1.5 ml of absolute ethanol. Because of the volume of the compound, 750 mg/kg unmodified hydrotalcit is the maximum dose that can be introduced into the stomach. Five minutes after the ethanol instillation, the stomach was removed under ether anaesthesia and mucosal damage was assessed in a blinded manner by calculation of a lesion index based on a 0–3 scoring system as described elsewhere.¹⁷ Previous studies have shown that the mucosal damage is maximal within 5 minutes of ethanol exposure and no worsening occurs after that.¹⁷ Furthermore, pilot experiments have ascertained that identical protective effects of unmodified and acidified hydrotalcit can be shown when a 5 minute or 60 minute observation period after ethanol challenge is used. Control rats were treated with the same volume of the corresponding vehicle (5 ml/kg of 0.25% MC or 0.25% MC acidified to pH 2.5).

To investigate the time course of protection, groups of four to 12 rats were treated orally with unmodified (750 mg/kg) or acidified (200 mg/kg or 90 mg/kg) hydrotalcit. Initial protection by 90 mg/kg of acidified hydrotalcit is equal to the protection elicited by 750 mg/kg of unmodified hydrotalcit. Rats were challenged with intragastric absolute ethanol (1.5 ml) 1, 2, 3, 5, 8, 11, or 16 hours after hydrotalcit administration and gastric mucosal damage was assessed 5 minutes later.

Pretreatment with indomethacin

Groups of rats were treated with subcutaneous indomethacin (20 mg/kg) 60 minutes before oral administration of unmodified (750 mg/kg) or acidified (200 mg/kg) hydrotalcit. One hour later, ethanol was instilled intragastrically and rats were killed 5 minutes later. Mucosal damage was assessed and gastric mucosal fragments were incubated for determination of prostaglandin formation.

Pretreatment with inhibitors of NO biosynthesis

Groups of rats were injected intravenously with L-NNA (40 mg/kg, dissolved in 2.5 ml/kg of saline) 10 minutes before oral administration of graded doses of unmodified (250–750 mg/kg) or

acidified (50–200 mg/kg) hydrotalcit. Thirty minutes after administration of the protective agent, ethanol (1.5 ml) was introduced intragastrically and mucosal damage was assessed 5 minutes later. Controls were injected with saline (2.5 ml/kg) instead of L-NNA and were treated orally with 0.25% MC or 0.25% MC acidified to pH 2.5. Since 40 mg/kg of L-NNA aggravates ethanol induced mucosal damage, the attenuation by L-NNA of the protective effect of hydrotalcit was investigated by comparison with controls injected intravenously with L-NNA (40 mg/kg) and treated orally with MC or acidified MC.

Further experiments similar to those described above were performed with L-NAME (40 mg/kg, orally) 30 minutes before oral dosing of unmodified (750 mg/kg) or acidified (200 mg/kg) hydrotalcit. In additional sets of experiments, the effects of intravenous injection (50 mg/kg) of L-NNA on the protective effect of acidified hydrotalcit and the effect of graded doses (10–50 mg/kg, intravenously) of L-NNA on the protection conferred by unmodified hydrotalcit were studied. Finally, the specificity of the L-NNA (40 mg/kg, intravenously) effect was investigated using intravenous injections of L-arginine or D-arginine (400 mg/kg each, dissolved in 2.5 ml/kg of saline) before administration of unmodified hydrotalcit (750 mg/kg).

Functional ablation of afferent neurons

Rats were treated with a subcutaneous injection of capsaicin (25 and 50 mg/kg on day 1, 50 mg/kg on day 2). Capsaicin was dissolved in 10% ethanol, 10% Tween 80, and 80% saline (vol/vol/vol). Control animals received equal volumes (0.5 ml) of vehicle. All injections were given under ether anaesthesia. Rats were used for experiments 14 days after this treatment. On the day before the experiments, the effectiveness of the treatment was ascertained by evaluating the reduction in the protective wiping movements in response to intraocular instillation of a 0.1 mg/ml solution of capsaicin as described previously.¹⁸ Furthermore, in some rats in each experimental group, the loss of protection against ethanol induced gastric damage in response to oral capsaicin (0.5 mg/kg) was assessed.

Indomethacin induced gastric lesions

Rats were treated orally with graded doses of unmodified (80–750 mg/kg) or acidified (80–320 mg/kg) hydrotalcit 60 minutes before administration of indomethacin (20 mg/kg, orally). Five hours after dosing of indomethacin, the stomach was removed and mucosal damage was assessed. Then mucosal fragments were excised and incubated for determination of prostaglandin formation. In an additional set of experiments, gastric mucosal damage in rats pretreated (60 minutes) with unmodified (750 mg/kg) or acidified (200 mg/kg) hydrotalcit was assessed 16 hours after administration of indomethacin (20 mg/kg, orally).

To investigate whether a functioning prostaglandin system is a prerequisite for the protective effect of hydrotalcit, studies were performed in

which unmodified hydrotalcit (750 mg/kg) was administered orally 30, 60 or 120 minutes after dosing of the ulcerogen indomethacin (20 mg/kg orally). Rats were sacrificed 5 hours after indomethacin administration and mucosal damage and prostaglandin formation were assessed.

GASTRIC EICOSANOID MEASUREMENTS

Formation of eicosanoids by mucosal tissue ex vivo
In groups of rats treated with unmodified (80–750 mg/kg) or acidified (200 mg/kg) hydrotalcit and challenged with intragastric instillation of ethanol (1.5 ml) mucosal fragments were excised from areas between the necrotic bands, blotted, and weighed and tissue aliquots of 40 mg were incubated in oxygenated Tyrode solution at 37°C for 10 minutes. Release of PGE₂, 6-keto-PGF_{1α}, and LTC₄ into the incubation medium was measured using specific radioimmunoassays as described in detail previously.¹⁷ In further experiments, gastric mucosal eicosanoid formation was determined 60 minutes after oral administration of unmodified (750 mg/kg) or acidified (200 mg/kg) hydrotalcit without ethanol challenge.

Release of eicosanoids into the gastric lumen in vivo
Rats were treated orally with unmodified hydrotalcit (750 mg/kg). One hour later the animals were anaesthetised with urethane and the abdomen was opened by a midline incision. The oesophagus and pylorus were ligated. Three ml of ice cold water containing indomethacin (10 µg/ml) to prevent eicosanoid formation during handling of the stomach were introduced into the gastric lumen via a needle inserted through the forestomach. Gastric contents were carefully removed. After centrifugation, the supernatant was extracted using a 3:3:1 mixture of ethyl acetate/isopropanol/0.1 N HCl. The evaporated extracts were then analysed for their content of 6-keto-PGF_{1α} and PGE₂ using specific radioimmunoassays.¹⁷ Amounts of prostaglandins were calculated using dilution curves of

authentic standard prostaglandins that were extracted identical to gastric juice samples. To exclude the possibility that hydrotalcit present in the gastric lumen interferes with measurements of the prostaglandins, various amounts of exogenous 6-keto-PGF_{1α} and PGE₂, respectively, were added to aliquots of gastric contents obtained from rats 60 minutes after oral treatment with unmodified hydrotalcit (750 mg/kg) or MC (0.25%). These samples were extracted and analysed by radioimmunoassays as described above.

INDOMETHACIN BIOAVAILABILITY STUDIES

To investigate whether administration of hydrotalcit interferes with the absorption of indomethacin, concentrations of indomethacin in serum and inflammatory exudates as well as effects of indomethacin on accumulation of eicosanoids and leukocytes in inflammatory exudates were assessed.

SERUM INDOMETHACIN CONCENTRATIONS

Groups of rats were treated orally with unmodified hydrotalcit (750 mg/kg) or vehicle 60 minutes before administration of indomethacin (20 mg/kg, orally). Blood samples (0.2 ml) were obtained by cardiac puncture 0.5, 1, 2, 3, 4, and 5 hours after dosing of indomethacin. In additional experiments, blood samples were obtained 5 hours after administration of indomethacin (20 mg/kg, orally) in rats pretreated with acidified hydrotalcit (160 mg/kg, 60 minutes). Blood samples were centrifuged and indomethacin concentrations were measured in aliquots (10 µl) of serum using a radioimmunoassay developed in our laboratory. Anti-indomethacin antibodies have been raised in a rabbit immunised with an indomethacin-bovine serum albumin-conjugate synthesised using the carbodi-imide method. The antiserum could be used for a sensitive radioimmunoassay (detection limit 58 pg, 50% replacement of [¹⁴C]-indomethacin by 505 pg indomethacin). The indomethacin metabolite O-desmethyloindomethacin showed a relative cross reaction of 33%, N-deschlorobenzoyloindomethacin did not interfere in amounts up to 50 ng. Previous studies in rats have shown the virtual absence in plasma of significant quantities of these metabolites.¹⁹ The material measured by the radioimmunoassay represents, therefore, unmetabolised indomethacin. Serum concentrations of indomethacin were calculated using dilution curves of authentic indomethacin generated in the same volume (10 µl) of serum obtained from rats not treated with indomethacin.

Inflammatory exudates

Rats were treated orally with unmodified hydrotalcit (750 mg/kg) or vehicle (MC, 2.5 ml/kg) 60 minutes before subcutaneous implantation of sterile polyester sponges soaked in 2% carrageenin. Immediately after sponge implantation, indomethacin (20 mg/kg) was administered orally. The sponges were removed

Figure 1: Effect of hydrotalcit on ethanol or indomethacin induced gastric lesions. Rats were treated orally with unmodified (um) or acidified (acid) hydrotalcit 60 minutes before oral administration of ethanol (1.5 ml) or indomethacin (20 mg/kg). Acidified MC had no protective effect. Results are expressed as percentage inhibition of damage produced by ethanol (lesion index 47 (4), n=12) or indomethacin (lesion index 21 (3), n=12) in vehicle treated control rats. Each point represents the mean (SEM) of 6 experiments.

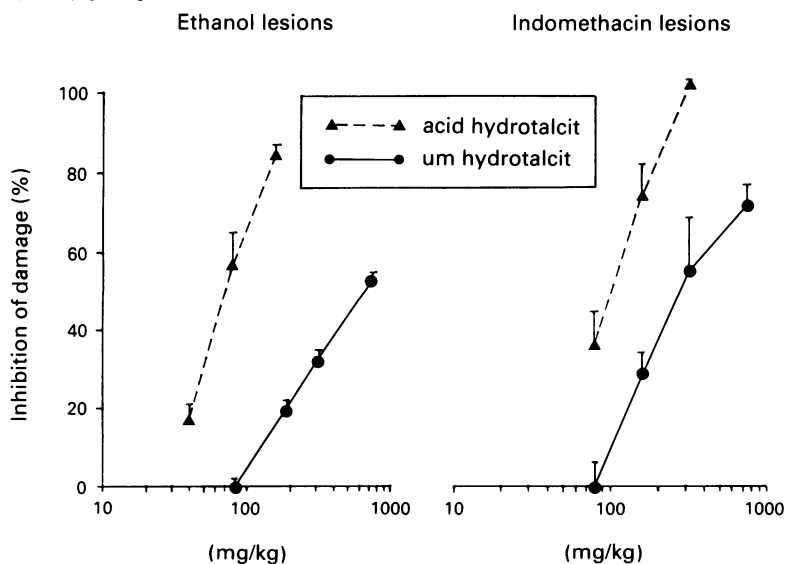
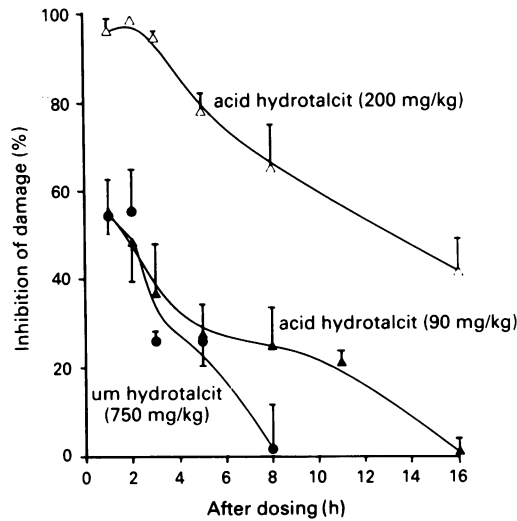


Figure 2: Time course of the protective effect of hydrotalcit. Rats were treated orally with unmodified (um) or acidified (acid) hydrotalcit and were challenged with intragastric instillation of ethanol (1.5 ml) 1–16 hours later. Five minutes after ethanol instillation gastric mucosal damage was assessed. Lesion index in vehicle treated control rats was 45 (3). Results are shown as the percentage inhibition of damage and represent the mean (SEM) of 4–12 rats in each group.

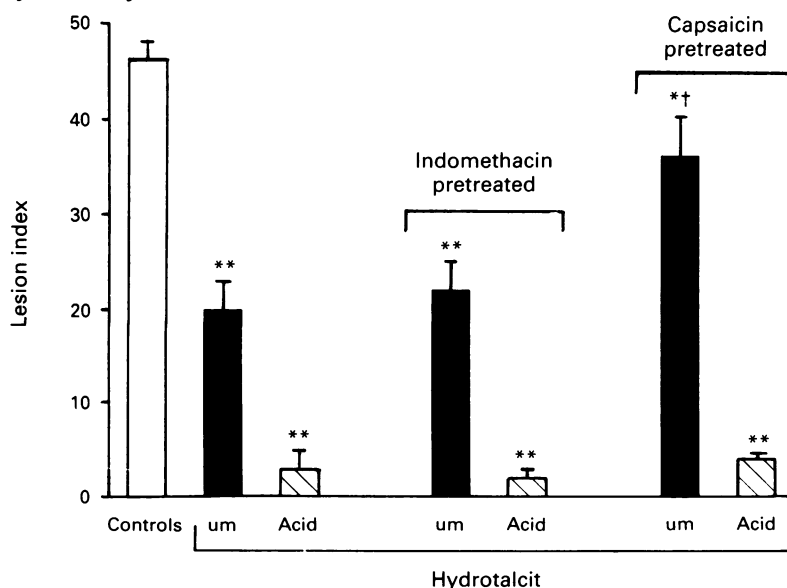


5 hours after implantation and immersed in 2 ml of phosphate buffered saline containing heparin (5 U/ml). They were then squeezed and the exudates were centrifuged at 1500 g at 4°C for 10 minutes. PGE₂ and sulphidopeptide leukotrienes in the supernatants were determined radio-immunologically. The occurrence of PGE₂ (data not shown) and sulphidopeptide leukotrienes (mixture of LTC₄, LTE₄, and LTD₄) in inflammatory exudates has previously been confirmed using high performance liquid chromatography.²⁰ The sediments of the exudates were resuspended in 1 ml of heparinised phosphate buffered saline and leukocyte counts were determined using a Neubauer counting chamber and Türk's solution for cell staining. The methods used have been described in detail previously.²⁰

STATISTICAL ANALYSIS

All data are expressed as mean (SEM) of n values. Comparisons between groups were made by Student's *t* test or the Wilcoxon rank test for parametric or non-parametric data. Levels of significance of differences in decline of protection in the time course study were calculated by

Figure 3: Effects of pretreatment with indomethacin and capsaicin on hydrotalcit induced protection. Indomethacin (20 mg/kg, subcutaneously) was administered 60 minutes before oral administration of unmodified (um, 750 mg/kg) or acidified (acid, 200 mg/kg) hydrotalcit. Sixty minutes after dosing of hydrotalcit, rats were challenged with intragastric ethanol (1.5 ml) and mucosal damage was assessed 5 minutes later. In further sets of experiments, rats pretreated with capsaicin to cause functional ablation of afferent neurons received oral unmodified (750 mg/kg) or acidified (200 mg/kg) hydrotalcit 60 minutes before ethanol challenge. Each bar represents the mean (SEM) of six experiments. **p*<0.05, ***p*<0.001 v controls; †*p*<0.01 v rats treated with unmodified hydrotalcit only.



analysis of covariance. A value of *p*<0.05 was considered significant.

Results

PROTECTIVE EFFECT OF HYDROTALCIT AGAINST ETHANOL INDUCED LESIONS

Both unmodified and acidified hydrotalcit induced dose dependent protection against gastric mucosal damage caused by ethanol. As shown in Figure 1, acidification of hydrotalcit considerably enhanced the protective effect. While the maximum dose of unmodified hydrotalcit (750 mg/kg) which can be introduced into the stomach only partially prevented ethanol induced gastric mucosal damage, complete protection could be achieved with acidified hydrotalcit (Figs 1 and 2). Furthermore, protection by acidified hydrotalcit lasted considerably longer than that of unmodified hydrotalcit. Thus, acidified hydrotalcit (200 mg/kg) reduced ethanol induced damage 16 hours after drug administration by 47% (lesion index 24 (4), *n*=8 v 45 (3), *n*=6, in vehicle treated control rats, *p*<0.002). In contrast, the protective effect of unmodified hydrotalcit was lost 8 hours after dosing. The decline of protection over time as determined by the slopes of the curves shown in Figure 2 was significantly (*p*<0.05) different between acidified (90 mg/kg) and unmodified (750 mg/kg) hydrotalcit despite identical initial protection.

Effects of indomethacin and functional ablation of afferent neurons

Pretreatment with indomethacin (20 mg/kg, subcutaneously) significantly (*p*<0.001, *n*=6 in each group) reduced gastric mucosal formation of 6-keto-PGF_{1α} in rats treated with unmodified hydrotalcit (750 mg/kg) and challenged with ethanol from 691 (54) to 28 (17) and of PGE₂ from 689 (72) to 33 (14). In rats treated with acidified hydrotalcit (200 mg/kg) before ethanol, indomethacin pretreatment reduced (*p*<0.001, *n*=6 in each group) mucosal formation of 6-keto-PGF_{1α} from 804 (112) to 40 (25) and of PGE₂ from 732 (66) to 31 (19). Despite the appreciable inhibition of prostaglandin formation, subcutaneous administration of indomethacin (20 mg/kg) did not produce gastric mucosal lesions within the observation period of 2 hours. Furthermore, this treatment did not attenuate the protective effect of unmodified or acidified hydrotalcit (Fig 3). Functional ablation of afferent neurons by pretreatment with capsaicin partially counteracted the protective effect of unmodified (750 mg/kg) but did not affect that of acidified hydrotalcit (200 mg/kg) (Fig 3).

Effect of inhibitors of NO biosynthesis

Pretreatment with the NO biosynthesis inhibitor L-NNA (40 mg/kg) partially antagonised the protective effect of unmodified hydrotalcit as indicated by a shift to the right of the dose response curve for protection (Figure 4). The effect was significant (*p*<0.05) with the 500 mg/kg and 750 mg/kg dose of hydrotalcit. In contrast,

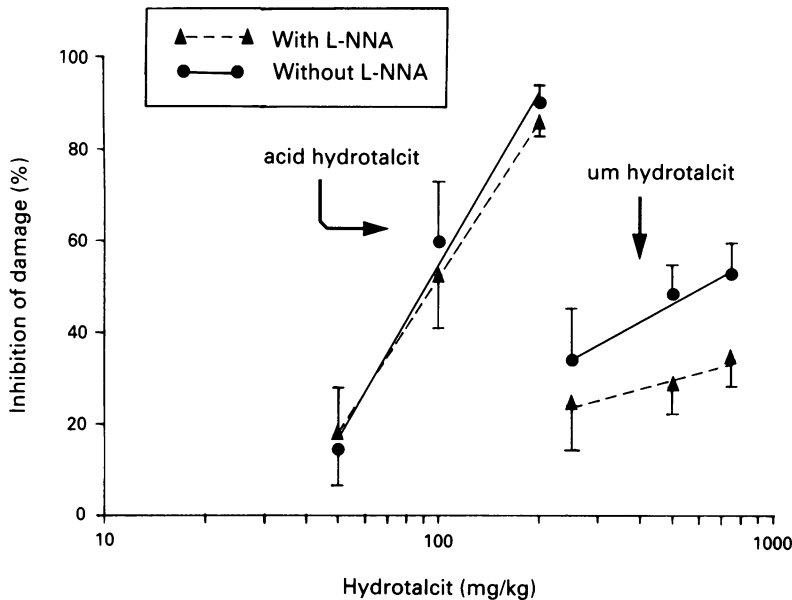
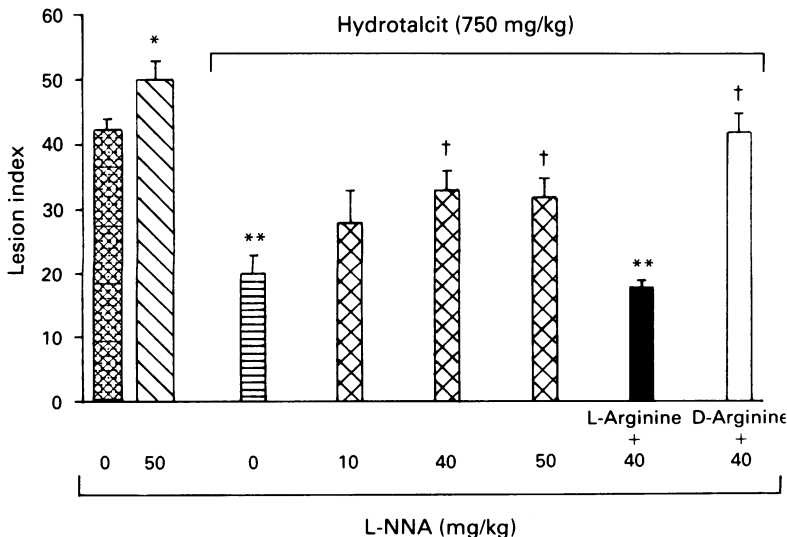


Figure 4: Effect of L-NNA on hydrodrotalcit induced protection. Rats were injected intravenously with L-NNA (40 mg/kg) or saline (2.5 ml/kg) 10 minutes before oral treatment with graded doses of unmodified (um) or acidified (acid) hydrodrotalcit and were challenged with ethanol (1.5 ml) 30 minutes later. All experiments included corresponding control rats treated with L-NNA or saline and methyl cellulose (MC) or acidified MC (5 ml/kg each) instead of hydrodrotalcit. Each point represents the mean (SEM) of six experiments.

L-NNA (40 mg/kg) had no effect on the protection conferred by acidified hydrodrotalcit at any of the doses studied (Fig 4). Thus, acidified hydrodrotalcit (200 mg/kg) completely prevented ethanol induced mucosal damage without L-NNA (lesion index 4 (1), n=6) or after pretreatment with 40 mg/kg of L-NNA (lesion index 6 (0.7), n=6), or 50 mg/kg of L-NNA (lesion index 5 (1), n=9), despite the fact, that L-NNA significantly aggravated mucosal injury in rats not treated with hydrodrotalcit (Fig 5).

The attenuation of the protective effect of unmodified hydrodrotalcit by L-NNA was dose dependent. The maximal effect was reached with 40 mg/kg of L-NNA, resulting in a 39% reduction of protection. Increasing the dose to 50 mg/kg did not further augment the inhibitory effect of L-NNA, indicating that only part of the protective effect of unmodified hydrodrotalcit is sensitive to inhibition of NO biosynthesis (Fig 5). Concurrent administration of the NO-synthase substrate L-arginine abolished the

Figure 5: Attenuation of the protective effect of unmodified hydrodrotalcit by pretreatment with intravenous L-NNA (10 minutes) and the effect of L-arginine and D-arginine (400 mg/kg each, intravenously). Each bar represents the mean (SEM) of six experiments. * $p < 0.05$, ** $p < 0.001$ v control rats treated with methyl cellulose (MC) (orally) and injected with saline (2.5 ml/kg, intravenously); † $p < 0.02$ v rats treated with hydrodrotalcit only. Each bar represents the mean (SEM) of six experiments.



inhibitory effect of L-NNA, while the inactive enantiomere D-arginine was without effect (Fig 5). Identical results were obtained using L-NAME to inhibit endogenous NO production. Thus, L-NAME (40 mg/kg) significantly attenuated protection by unmodified hydrodrotalcit (750 mg/kg) by increasing the lesion index from 17 (2) (n=6) in rats treated with hydrodrotalcit only to 31 (3) (n=6, $p < 0.01$) in rats pretreated with L-NAME. In contrast, the protective effect of acidified hydrodrotalcit was not affected by L-NAME (lesion index 2 (0.6) v 4 (1) in rats treated with acidified hydrodrotalcit (200 mg/kg) only, n=6 per group).

EFFECT OF HYDROTALCIT ON GASTRIC EICOSANOID FORMATION

In rats treated with unmodified (750 mg/kg) or acidified (200 mg/kg) hydrodrotalcit, but not challenged with ethanol, mucosal generation of 6-keto-PGF_{1α} and PGE₂ was not different from that in vehicle treated controls. Furthermore, in ethanol challenged rats, none of the doses of unmodified (80–750 mg/kg) or acidified (200 mg/kg) hydrodrotalcit studied increased ex vivo gastric mucosal formation of 6-keto-PGF_{1α} or PGE₂, and even reduced formation of PGE₂ after ethanol exposure, although the effect reached statistical significance for unmodified hydrodrotalcit (750 mg/kg) only. Mucosal generation of LTC₄ was significantly increased after ethanol exposure confirming previous results,¹⁷ and this effect was not attenuated by pretreatment with protective doses of unmodified or acidified hydrodrotalcit. Results for the maximally effective doses of hydrodrotalcit are shown in the Table.

Previous studies had shown that measurements of prostaglandins in gastric juice using direct radioimmunoassay do not provide reproducible results. However, using extraction into organic solvents before radioimmunoassay, graded amounts of 6-keto-PGF_{1α} and PGE₂ added to samples of rat gastric contents could be recovered quantitatively and the levels of endogenous prostaglandins fell on the regression lines calculated. Furthermore, thin layer chromatography analysis had shown that immunoreactive material extracted from rat gastric contents cochromatographs exclusively with the corresponding authentic standard prostaglandins. In additional sets of experiments, it was shown that oral administration of unmodified hydrodrotalcit does not affect measurements of prostaglandins in gastric contents in that it neither binds prostaglandins released into the gastric lumen nor unspecifically interferes with the analytical procedure. Using this method, it was found that treatment with unmodified hydrodrotalcit in a dose inducing significant protection does not increase the release of 6-keto-PGF_{1α} and PGE₂ into the gastric lumen in vivo (Table). Since LTC₄ is not stable in the presence of acid, the effects of hydrodrotalcit on leukotriene release into the gastric lumen were not assessed.

INDOMETHACIN INDUCED GASTRIC LESIONS

Hydrodrotalcit dose dependently protected against indomethacin induced gastric lesions, with

Effect of hydrotalcit on gastric mucosal formation and intraluminal release of eicosanoids in rats challenged or not challenged with ethanol

	Mucosal fragments						Gastric contents	
	Not challenged			Challenged			Not challenged	
	Controls (n=10)	um Ht (n=6)	acid Ht (n=6)	Controls (n=10)	um Ht (n=6)	acid Ht (n=6)	Controls (n=6)	um Ht (n=6)
6-keto-PGF _{1α}	1048 (39)	998 (96)	1204 (87)	932 (161)	594 (90)	689 (82)	1.1 (0.4)	1.2 (0.3)
PGE ₂	758 (92)	731 (58)	815 (75)	912 (51)	577 (30) [†]	712 (91)	0.6 (0.2)	0.5 (0.1)
LTC ₄	32 (8)	29 (7)	33 (4)	310 (30)**	242 (46)*	228 (51)*	ND	ND

Rats were treated orally with unmodified (um Ht, 750 mg/kg) or acidified (acid Ht, 200 mg/kg) hydrotalcit. Controls received MC (5 ml/kg). Sixty minutes later the stomach was removed, mucosal fragments were excised, and incubated in Tyrode solution at 37°C. Additional groups of rats were challenged with ethanol (1.5 ml, orally) 5 minutes before sacrifice. In further experiments, gastric contents were aspirated 60 minutes after oral dosing of unmodified hydrotalcit or MC, extracted and analysed for their content of prostaglandins. Results, shown as ng/g/10 minutes (mucosal fragments) or ng per stomach (gastric contents) represent the mean (SEM) of (n) experiments in each group. *p<0.01, **p<0.001 v corresponding rats not challenged with ethanol; [†]p<0.001 v MC-treated controls; ND=not determined.

acidification markedly enhancing the effect (Fig 1). Coadministration of hydrotalcit did not attenuate the inhibitory action of indomethacin on the gastric mucosal formation of 6-keto-PGF_{1 α} and PGE₂ (Fig 6). Protection by hydrotalcit was also observed when the drug was administered up to 2 hours after dosing of indomethacin (Fig 6). Oral administration of indomethacin (20 mg/kg) alone produced minor gastric mucosal damage (lesion index 5 (3), n=4) within 2 hours. This may explain the slightly higher lesion index in rats receiving hydrotalcit 2 hours after indomethacin. Protection by hydrotalcit occurred despite full suppression of the endogenous prostaglandin system. Thus, after treatment with a single dose of indomethacin (20 mg/kg, orally) near maximal (p<0.001 v controls) inhibition of mucosal prostaglandin formation (ng/g/10 minutes, n=6 in each group) is reached within 15 minutes (6-keto-PGF_{1 α} : 45 (18); PGE₂: 43 (22) and is maintained for 9 hours (6-keto-PGF_{1 α} : 37 (20); PGE₂: 32 (19)) compared with controls (6-keto-PGF_{1 α} : 852 (69); PGE₂: 799 (29)).

To exclude the possibility that hydrotalcit delays rather than prevents indomethacin induced gastric mucosal damage, injury was assessed 16 hours after administration of the

ulcerogen. After this period, the lesion index (n=4 in each group) was 21 (2) in indomethacin (20 mg/kg, orally) treated rats, 7 (2) (p<0.01) in rats pretreated with unmodified hydrotalcit (750 mg/kg), and 3 (2) (p<0.001) in rats pretreated with acidified hydrotalcit (200 mg/kg).

Treatment with hydrotalcit did not affect the absorption of indomethacin. As shown in Figure 7, serum concentrations of indomethacin were virtually identical in rats pretreated with unmodified hydrotalcit (750 mg/kg) or vehicle over the whole study period. Likewise, indomethacin serum concentrations in rats pretreated with a dose of acidified hydrotalcit causing substantial inhibition of indomethacin induced lesions were not different from those in rats treated with indomethacin only (Fig 7).

Figure 8 shows that treatment with hydrotalcit does not affect the anti-inflammatory action of

Figure 6: Attenuation of indomethacin induced gastric mucosal lesions by hydrotalcit administered after the ulcerogen. Rats were treated with unmodified hydrotalcit (750 mg/kg, orally) 0.5, 1, or 2 hours after dosing of indomethacin (20 mg/kg, orally). Five hours after indomethacin administration gastric damage was assessed and mucosal fragments were incubated to determine formation of 6-keto-PGF_{1 α} and PGE₂. Each bar represents the mean (SEM) of six experiments. *p<0.001 v control rats treated with vehicle only; [†]p<0.05, ^{††}p<0.001 v rats treated with indomethacin but receiving methyl cellulose instead of hydrotalcit.

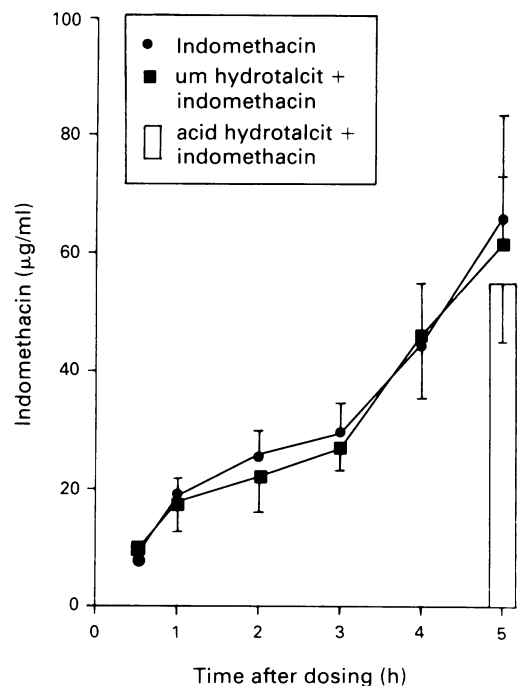
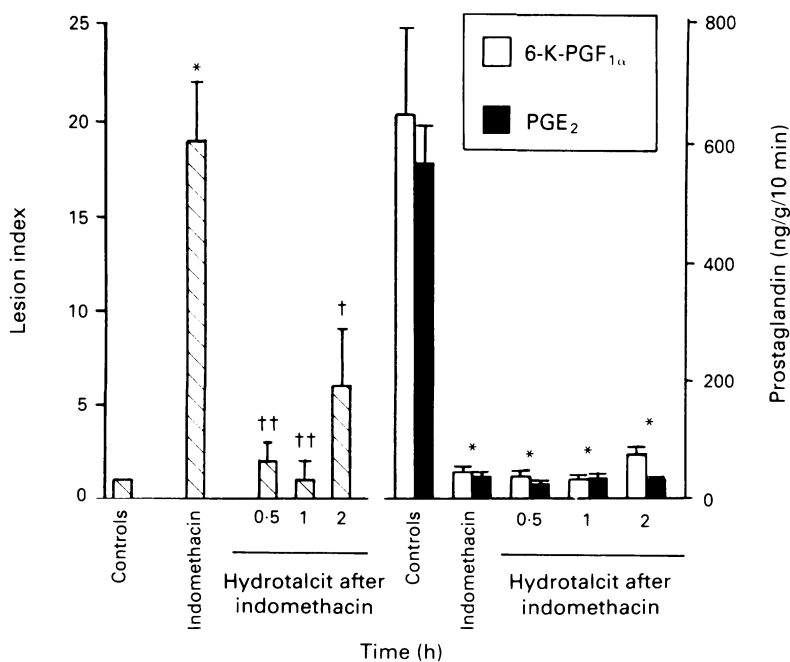


Figure 7: Effect of hydrotalcit on serum concentrations of indomethacin. Rats were treated orally with hydrotalcit or vehicle 60 minutes before administration of indomethacin (20 mg/kg, orally). Indomethacin concentrations in serum were determined 0.5–5 hours after dosing of indomethacin in rats pretreated with methyl cellulose or unmodified (um, 750 mg/kg) hydrotalcit as well as after 5 hours in rats pretreated with acidified (acid, 160 mg/kg) hydrotalcit. Each point represents the mean (SEM) of five rats. The bar represents the mean (SEM) of 10 rats pretreated with acidified hydrotalcit.

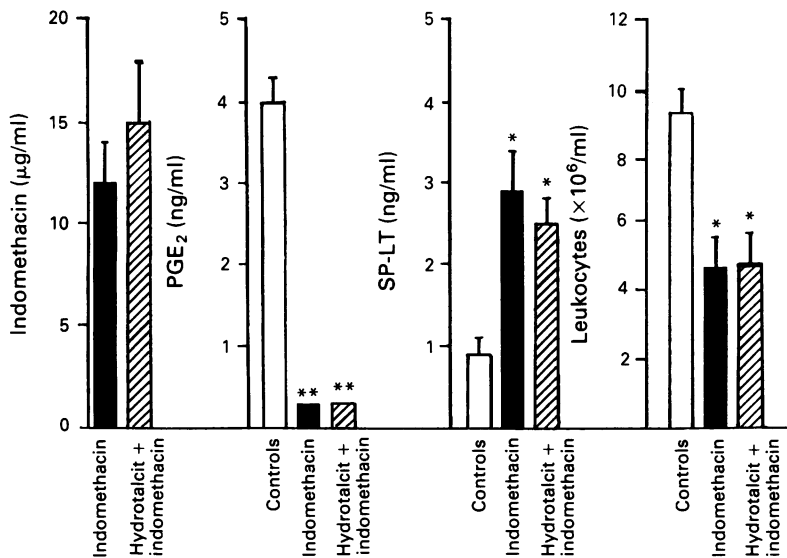


Figure 8: Effect of hydrotalcit on concentrations of indomethacin, PGE₂, sulphidopeptide leukotrienes (SP-LT), and leukocytes in inflammatory exudates in indomethacin treated rats. Unmodified hydrotalcit or vehicle was administered orally 60 minutes before implantation of carrageenin soaked sponges followed by oral administration of indomethacin (20 mg/kg). Five hours after implantation, concentrations of indomethacin, PGE₂ and sulphidopeptide leukotrienes were measured in the supernatant and leukocytes were counted in the sediment of centrifuged exudates. Bars represent the mean (SEM) of six rats. **p*<0.01, ***p*<0.001 v control rats treated with vehicle only.

indomethacin. Thus, release of PGE₂ into inflammatory exudates elicited by subcutaneous implantation of carrageenin soaked sponges was reduced to the same extent in rats treated with indomethacin only and rats treated additionally with a protective dose of hydrotalcit. Indomethacin treatment increased the release of sulphidopeptide leukotrienes into inflammatory exudates. This effect was not modified by coadministration of hydrotalcit. Furthermore, the inhibition of influx of leukocytes into inflammatory exudates observed after treatment with indomethacin was not attenuated by hydrotalcit. Finally, concentrations of indomethacin measured in inflammatory exudates were not affected by pretreatment with hydrotalcit.

Discussion

Hydrotalcit protected against gastric mucosal damage caused by ethanol or indomethacin in a dose dependent manner as shown previously for other aluminum containing antacids. Acidification noticeably enhanced the protective effect. Furthermore, the duration of protection was considerably longer with acidified than with unmodified hydrotalcit. With maximally effective doses, acidified hydrotalcit significantly reduced ethanol-induced gastric mucosal damage even 16 hours after dosing, while the protection conferred by unmodified hydrotalcit was lost after 8 hours. Studies using unmodified and acidified hydrotalcit in doses causing identical initial protection revealed a significant difference in the decline of protection with time suggesting that in addition to the more pronounced initial protective potency different mechanism(s) of action contribute to the prolonged protection by acidified hydrotalcit.

Increased protective efficacy and prolonged duration of action after acidification have been reported previously for various aluminum containing antacids.^{3-5,9} This effect has been explained by formation of an activated aluminum complex associated with an increased appearance of hexa-aquo-aluminum cation [Al(H₂O)₆³⁺].^{5,9} The precise mechanism underlying the protective effect of unmodified and acidified hydrotalcit,

however, is unknown. As acidification of antacids results in loss of acid neutralising capacity,⁹ elevation of intragastric pH cannot explain the protection. This is supported by the fact that unmodified and acidified hydrotalcit prevent ethanol induced gastric lesions,^{1-3,5,7-9} an experimental model known to be independent of intraluminal acid.

The contribution of endogenous prostaglandins to the mucosal protective effects of antacids is controversial. Since the morphological and functional parameters of antacid induced protection are similar to those elicited by prostaglandins, it has been suggested that stimulation of formation of protective prostaglandins in the gastric mucosa by antacids plays a pivotal role in their protective effect. In man and rat, acute or chronic treatment regimens with aluminum containing antacids have been reported to increase prostaglandin formation in gastric mucosal tissues^{21,22} and/or to enhance secretion of prostaglandins into the gastric lumen,^{3,7,10,23,24} although not all studies could confirm this effect.^{3,25} In vitro, aluminum containing antacids released PGE₂ from cultured macrophages.²⁶ Inhibition of prostaglandin formation was found to fully,^{1,10} partially,²⁴ or not^{3,5} antagonise antacid induced protection. The findings of this study clearly show that in doses causing substantial protection, both unmodified and acidified hydrotalcit do not increase ex vivo gastric mucosal formation of 6-keto-PGF_{1α} and PGE₂ in rats challenged or not challenged with ethanol. After ethanol exposure, mucosal formation of PGE₂ was even decreased in rats treated with unmodified hydrotalcit. Whether this effect is related to tissue preservation as a consequence of protection or results from other, as yet unknown, actions of the compound is not known. Likewise, hydrotalcit did not increase the release of 6-keto-PGF_{1α} and PGE₂ into the gastric lumen in vivo. Furthermore, neither preparation of hydrotalcit attenuated the increase in gastric mucosal generation of pro-ulcerogenic LTC₄ in response to intraluminal ethanol. Further support that gastroprotective effects of hydrotalcit are independent of the endogenous eicosanoid system comes from the finding that pretreatment with indomethacin in a dose causing near maximal inhibition of gastric mucosal prostaglandin formation did not reduce the protective effect of unmodified or acidified hydrotalcit.

In man, aluminum containing antacids given in therapeutic doses for 4 weeks significantly reduced the rate of gastric microbleeding induced by aspirin.² As the protective effect was short lived only, it was suggested that if reduction of gastrointestinal side effects because of non-steroidal anti-inflammatory drugs is intended, antacids should be taken regularly 1 hour before the noxious compounds.² In our study, hydrotalcit protected against gastric mucosal lesions caused by indomethacin even when the drug was administered up to 2 hours after the ulcerogen indicating that for therapeutic efficacy pre-dosing of antacids is not necessary. Protection in the presence of full blockade of prostaglandin formation is of major importance for the clinical use of antacids to prevent or treat gastropathy

induced by non-steroidal anti-inflammatory drugs.

The present results also show that unmodified and acidified hydrotalcit confer protection against indomethacin induced gastric damage and do not only delay the development of lesions, since 16 hours after indomethacin administration significantly less gastric mucosal injury was observed in hydrotalcit pretreated rats.

The effect of antacids on the absorption of non-steroidal anti-inflammatory drugs is not completely understood and depends on the particular compound as well as on the antacid preparation used.²⁷ Although in man absorption and disposition of a number of non-steroidal anti-inflammatory drugs was unaffected by combined treatment with antacids (for review see²⁸), reduced bioavailability of indomethacin during coadministration of aluminum and magnesium containing antacids has been described.²⁹ Most non-steroidal anti-inflammatory drugs are weak acids. In an acidic milieu they exist in the unionised form, which is highly lipophilic and easily absorbed into mucosal cells. Increasing the intraluminal pH value by antacids with buffering capacity could possibly interfere with the local absorption of non-steroidal anti-inflammatory drugs from the stomach and thus reduce their gastric toxicity and anti-inflammatory potency. Our results provide evidence that the protective effect of acid neutralising unmodified hydrotalcit is not associated with less marked inhibition of gastric mucosal prostaglandin formation. Furthermore, concentrations of indomethacin in serum and/or inflammatory exudates were not different in rats pretreated with unmodified or acidified hydrotalcit compared with rats treated with indomethacin only. As shown previously,²⁰ treatment with indomethacin substantially inhibited release of PGE₂, increased secretion of sulphidopeptide leukotrienes and reduced influx of leukocytes into inflammatory exudates produced after subcutaneous implantation of carrageenin induced sponges. These effects of indomethacin were not attenuated by concurrent antacid administration. Taken together, our findings indicate that the protection induced by hydrotalcit against indomethacin induced mucosal injury is not the result of reduced gastric or systemic absorption of the non-steroidal anti-inflammatory compound and is not associated with diminished anti-inflammatory activity.

Antacids have been found to elicit gastric mucosal hyperaemia.⁷ Furthermore, aluminum containing antacids prevent ethanol induced injury to endothelial cells suggesting the microvasculature as a possible target of their protective effect. Recently, NO, which is formed from the amino acid L-arginine by various cells including endothelial cells has been described as potent vasodilator.¹¹ The biosynthesis of NO is selectively inhibited by analogues of L-arginine such as N^G-monomethyl-L-arginine (L-NMMA), L-NNA, or L-NAME.¹¹ In rats, inhibition of endogenous NO formation induced a fall in resting gastric mucosal blood flow.¹³ While administration of L-NMMA did not induce acute mucosal injury in the rat stomach, capsaicin pretreatment causing functional ablation of

afferent neurons and/or concurrent treatment with indomethacin induced severe haemorrhagic necrotic damage suggesting that NO acts in concert with sensory neuropeptides and prostaglandins in the modulation of gastric mucosal integrity.¹²

Pretreatment with L-NNA increased the extent of gastric injury induced by ethanol suggesting that endogenous NO represents a compensatory mechanism partly limiting damage to the mucosa. Inhibition of NO biosynthesis by L-NNA and L-NAME partially antagonised the prevention of ethanol induced gastric lesions by unmodified hydrotalcit. The attenuating effect of L-NNA was reversed by concurrent administration of the substrate of NO-synthase, L-arginine, indicating the specificity of the L-NNA effect. Likewise, functional ablation of afferent neurons induced by pretreatment with a neurotoxic dose of capsaicin partially reduced the protective effect of unmodified hydrotalcit. Intragastric administration of capsaicin protects against mucosal injury caused by various noxious agents including ethanol.³⁰ This protection involves stimulation of afferent neurons in the gastric mucosa and is abolished by functional ablation of these neurons induced by a neurotoxic dose of capsaicin.³⁰ Gastroprotection induced by intragastric capsaicin is not associated with increased formation of PGE₂ or 6-keto-PGF_{1α} and is not attenuated by pretreatment with indomethacin, indicating that it is not prostaglandin mediated.³¹ In contrast, treatment with the inhibitor of NO biosynthesis, L-NNA, fully abolished capsaicin induced protection.¹⁴ Aluminum containing antacids have been found to produce distinct morphological changes in normal rat gastric mucosa, including disruption of surface epithelial cells.⁷ Whether such changes are sufficient to elicit stimulation of afferent neurons in the gastric mucosa or whether the increased NO formation is mediated by other, possibly indirect, mechanisms remains to be investigated. Although the dose of L-NNA necessary to completely block capsaicin induced protection (5 mg/kg)¹⁴ is considerably lower than that partially attenuating the protective effect of unmodified hydrotalcit, the parallel attenuation of the protective effect of unmodified hydrotalcit by inhibition of NO formation and functional ablation of afferent neurons is of interest and warrants further investigations. We have previously shown that NO is not an essential mediator of the gastroprotection induced by prostaglandins such as 16,16-dimethyl-PGE₂, the PGI₂ analogue iloprost, and the PGE₂ analogue nocloprost.³² This is further support of the concept that prostaglandins are not crucially involved in the protective property of unmodified hydrotalcit.

Unlike unmodified hydrotalcit, the protection conferred by acidified hydrotalcit is completely independent of the endogenous NO system and is not attenuated by functional ablation of afferent neurons. The failure of inhibition of NO formation and capsaicin pretreatment to attenuate the protection by acidified hydrotalcit is not due to the near maximal protection elicited by this hydrotalcit preparation. It was also observed with doses causing only partial prevention of

ethanol induced gastric damage comparable with the protection conferred by unmodified hydrotalcit. Taken together, the findings of the study indicate that endogenous eicosanoids are not essential mediators of the protective effects of unmodified and acidified hydrotalcit. Protection by hydrotalcit against indomethacin induced gastric lesions is not associated with reduced absorption and diminished anti-inflammatory activity of the non-steroidal anti-inflammatory drug. The mechanism of protection by unmodified hydrotalcit is partly related to endogenous NO and afferent neurons and partly independent of these systems. In contrast, endogenous NO and afferent neurons do not contribute to the protection by acidified hydrotalcit suggesting that different mechanisms mediate the mucosal protective effects of acid neutralising and non-buffering antacids.

Results have been presented in part at meetings of the American Gastroenterological Association in 1990 (San Antonio, USA). (Trautmann M, Peskar BM. Gastroprotection in rats by the antacids hydrotalcit and maalox does not involve the endogenous prostaglandin and leukotriene system. *Gastroenterology* 1990; **98**: A139) and in 1992 (San Francisco, USA) (Lambrecht N, Korolkiewicz R, Peskar BM. Effects of endogenous nitric oxide (NO) inhibition of the gastroprotective activity of the antacid hydrotalcit in rats. *Gastroenterology* 1992; **102**: A105).

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