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INVESTIGATION OF THE VASODILATOR AND ANTISECRETORY ROLE OF PROSTAGLANDINS IN THE RAT GASTRIC MUCOSA BY USE OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

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1 The effects of non-steroidal anti-inflammatory drugs on gastric acid secretion and mucosal blood flow were studied in the rat.

2 Indomethacin, in ulcerogenic doses, caused a dose-dependent rise in pentagastrin-stimulated acid secretion, but decreased mucosal blood flow per unit acid secretion.

3 During resting conditions, indomethacin had no significant effect on acid output, but reduced mucosal blood flow.

4 Pretreatment with indomethacin, phenylbutazone or meclofenamate potentiated the secretory response to dibutyryl cyclic adenosine 3′,5′-monophosphate.

5 Indomethacin markedly reduced the mucosal prostaglandin-like activity at a time when mucosal erosion formation had reached steady levels.

6 These results provide evidence that prostaglandins have a local role in the regulation of blood flow and acid secretion in the rat gastric mucosa, and suggest that non-steroidal anti-inflammatory drugs cause mucosal erosions by disrupting these processes.

Introduction

Prostaglandins of the E and A series, which inhibit gastric acid secretion, have a direct vasodilator action on the rat gastric mucosa (Main & Whittle, 1973a). These findings, together with the increased release of mucosal prostaglandins during secretory stimulation (Shaw & Ramwell, 1968) raised the possibility that prostaglandins have a physiological role, not only as negative feed-back inhibitors of acid secretion, but also as mediators of functional vasodilatation in the gastric mucosa.

In the present study, these hypotheses have been tested by the use of indomethacin and other non-steroidal anti-inflammatory drugs which inhibit prostaglandin synthesis (Vane, 1971). We have also investigated the relationship between inhibition of gastric mucosal prostaglandin synthesis and the formation of mucosal erosions by these drugs.

Preliminary accounts of this work have been presented to the British Pharmacological Society (Main & Whittle, 1973b, c).

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Methods

Measurement of gastric acid secretion and mucosal blood flow

Acid output into the perfused gastric lumen of the urethane-anaesthetized rat was measured, and mucosal blood flow was determined simultaneously by the [^{14}C]-aniline clearance technique (Main & Whittle, 1973d).

Assessment of mucosal erosions

Female Wistar rats (200-250 g), starved for 18 h but allowed water, were killed at various time intervals following drug treatment and the stomachs removed. After being opened along the lesser curvature, the stomachs were rinsed under a stream of water and pinned flat on a cork-board. The stomachs were coded to prevent observer bias and studied with the aid of a binocular microscope (x10). Erosions, which formed only in the glandular mucosa, were counted and each one given a severity rating on a 1 to 3 scale (1 = less than 1 mm; 2 = 1 to 2 mm; 3 = greater than 2 mm). The overall total, divided by a factor of 10, was designated the 'ulcer index' for that stomach.

Extraction and estimation of prostaglandin-like material from rat gastric perfusate and mucosae

Gastric perfusate was collected directly into glass cylinders stored on ice. An aliquot (0.5 ml) was taken for estimation of the acid content, whilst the remainder (acidified to pH 4 with citric acid if necessary) was extracted twice with ethyl acetate and the pooled organic phase evaporated to dryness under reduced pressure at 45° C. The residue was either immediately bioassayed or stored at −15° C until required.

Gastric mucosae, from rapidly dissected stomachs washed in ice-cold saline (0.9% w/v NaCl solution), were scraped from the underlying muscle layers with a chilled glass slide and placed immediately in 96% aqueous ethanol at 0° C containing 100 nCi [^{3}H]-prostaglandin E_1 (0.4 ng). This procedure lasted about two minutes. The tissue was rapidly homogenized, extracted into ethyl acetate, pH 8 phosphate buffer and 67% aqueous ethanol as described by Horton & Main (1967), and the biological activity determined on the rat isolated stomach strip. The efficiency of extraction, as estimated by the radioactivity in the final residue, was 68 ± 2% (n = 7). Thin-layer chromatography of mucosal extracts was carried out on aluminium-backed plates (0.2 mm layer of silica gel F_{254}, fast-running; Merck). Silver nitrate impregnated plates were prepared by immersing in an alcoholic solution of silver nitrate B.P. (approximately 4% in ethanol) for 1 h and drying at room temperature. The AI and AII solvent systems of Gréen & Samuelsson (1964) were used.

Measurement of binding of indomethacin and dibutyryl cyclic adenosine 3',5'-monophosphate (dbcAMP) to plasma protein

Five ml of bovine serum albumin (4% in pH 7.4 sodium phosphate buffer), containing indomethacin (50-300 μg/ml), dbcAMP (50 μg/ml) or a mixture of both, was pipetted into Visking tubing and dialysed against 10 ml pH 7.4 buffer maintained at 37° C in a shaking water bath. After 24 h, the concentration of indomethacin (absorbance measured at 265 nm) and dbcAMP (measured at 273 nm) in the dialysate was determined with a u.v.-spectrophotometer (Unicam SP.800 B).

Statistical analysis

Results are shown as the mean ± standard error of the mean, where (n) is the number of values in the group. The significance of the data was evaluated using Student's t test for unpaired or paired data where appropriate. $P < 0.05$ was taken as significant.

Drugs

Pentagastrin (Peptavlon, I.C.I.); N^6-2'-*O*-dibutyryl cyclic adenosine 3',5'-monophosphate (Boehringer); prostaglandin E_1-[5,6-^{3}H] (New England Nuclear). Indomethacin (Merck, Sharp & Dohme, Ltd) was added to saline containing a stoichiometrically-equivalent quantity of sodium bicarbonate, and dissolved with the aid of the dropwise addition of 0.1 N NaOH (taking care not to exceed pH 8) to give a final concentration of 4-5 mg/ml. Before use, the concentration and spectrum of the solution was determined by spectrophotometry (between 200 and 400 nm). Phenylbutazone (Geigy Pharmaceuticals) was suspended in carboxy-methyl cellulose (1% in distilled water), to give a final concentration of 100 mg/ml. In control experiments, the appropriate vehicle was administered. Sodium meclofenamate (Parke-Davis & Co.) was dissolved in saline. Prostaglandins were supplied by the Upjohn Co., Kalamazoo, U.S.A.

Results

Effects of indomethacin during basal and pentagastrin-stimulated acid secretion

During resting conditions, indomethacin (30 mg/kg) injected intravenously over a period of 5-10 min, did not significantly alter acid output ($P > 0.1$) but caused a gradual fall in mucosal blood flow (Figure 1). Mucosal blood flow had fallen significantly ($P < 0.02$) by a mean of 49.2 ± 9.3% (n = 4) 2 h after administration as shown in Figure 2.

Indomethacin (20-40 mg/kg i.v.) had no significant effect on systemic arterial blood pressure (eight observations). During the gastric perfusion of acidic saline (0.01 N HCl) under basal conditions, indomethacin (30 mg/kg i.v.) did not increase the back-diffusion of hydrogen-ions (two observations).

During the steady submaximal acid secretory response to an infusion of pentagastrin (0.20-0.33 μg kg^{-1} min^{-1}), indomethacin (20 and 40 mg/kg i.v.) caused a gradual increase in acid output during the following 2 to 3 hours. The acid output measured after 2 h had increased significantly from the control values ($P < 0.02$ for both series) in a dose-dependent manner (Figure 2). Although an increase in mucosal blood flow accompanied the rise in acid, the ratio of mucosal blood flow to acid output was decreased

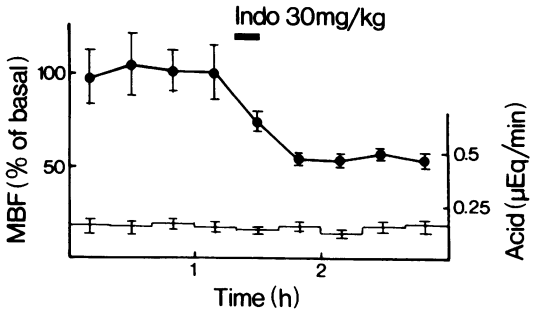

Figure 1 Effects of intravenously-administered indomethacin (Indo) on resting gastric mucosal blood flow (MBF) (•) and acid secretion (⌐¬) in the rat. The results are the mean of four experiments. Vertical bars show s.e. mean.

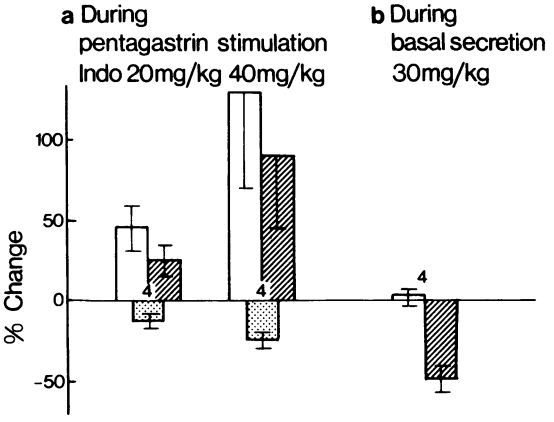

Figure 2 Effects of intravenously-administered indomethacin on gastric acid secretion (open columns), mucosal blood flow (hatched columns) and mucosal blood flow per unit acid secretion (dotted columns) during (a) pentagastrin stimulation, and (b) basal secretion. Results, expressed as % change from control value, are given as the mean of four experiments in each series. Vertical bars show s.e. mean.

($P < 0.05$), as shown in Figure 2. These effects were usually followed by the appearance of blood in the gastric perfusate and mucosal erosions were observed post mortem.

Effects of indomethacin on dbcAMP-induced acid secretion

Intravenous injection of dbcAMP during basal conditions caused a small but reproducible increase in acid output lasting approximately 80 min (Main & Whittle, 1974a). After control responses to dbcAMP (20 mg/kg i.v.) had been obtained, indomethacin (10-30 mg/kg) was injected intravenously. Injection of dbcAMP 1 h later caused a marked increase in acid output. The results, expressed as the increase in acid output (μEq) from basal levels during the 80 min following injection of dbcAMP, are shown in Figure 3. The dbcAMP-induced secretory responses following indomethacin administration (10-30 mg/kg) were significantly greater than the control responses ($P < 0.001$ for each group). The response following a 30 mg/kg dose of indomethacin was significantly greater than that with 10 mg/kg ($P < 0.001$).

Pretreatment of rats with indomethacin (15 mg/kg) injected subcutaneously 6 or 24 h before stimulation (that is, 4 or 22 h before surgery), in a dose which produced gastric mucosal erosions, significantly ($P < 0.001$) potentiated the secretory response to dbcAMP (Figure 3b).

Theophylline (20 mg/kg i.v., 1 h prior to dbcAMP-stimulation) significantly ($P < 0.001$) augmented the potentiating effect of indomethacin (Figure 3b) and gastric bleeding occurred in each experiment during these very high secretory rates. This dose of theophylline, which had no consistent effect on basal or pentagastrin-stimulated secretion (Main & Whittle, 1974a) did not significantly alter the response to dbcAMP alone, in three experiments. In another experiment, where theophylline increased basal secretion three-fold, the dbcAMP response was increased four-fold.

Effects of sodium meclofenamate and phenylbutazone

Intravenous administration of sodium meclofenamate (30 mg/kg) caused a significant ($P < 0.001$) increase of the secretory response to dbcAMP (20 mg/kg) injected 1 h later (Figure 3a).

Pretreatment of rats with phenylbutazone (100 mg/kg s.c.) 6 h prior to stimulation, significantly ($P < 0.001$) increased the dbcAMP-induced secretory response (Figure 3b). This dose of phenylbutazone was found to produce gastric mucosal erosions within 6 h of subcutaneous administration.

Production of gastric erosions by indomethacin

Indomethacin (5-30 mg/kg) was administered subcutaneously to rats (eight per group), and after a predetermined time interval the ulcer index of each rat stomach was assessed. Indomethacin in a dose of 15 mg/kg produced a submaximal degree

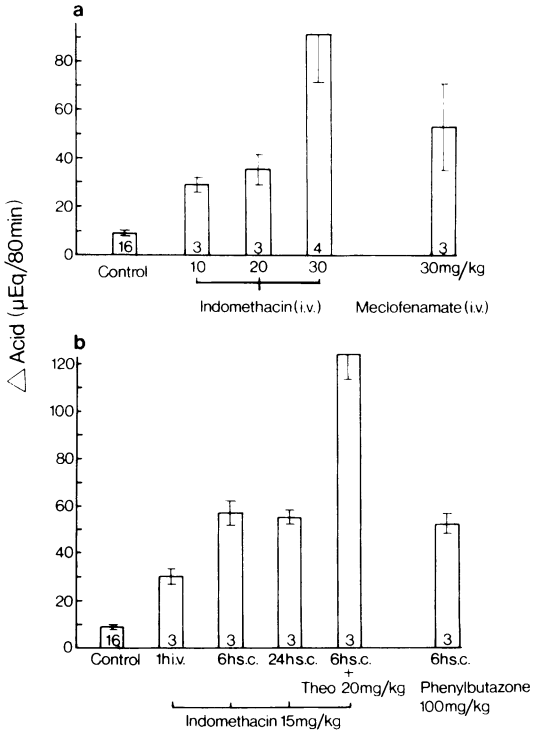

Figure 3 Effects of anti-inflammatory drugs on the secretory response to intravenous dibutyryl cyclic adenosine 3′,5′-monophosphate (dbcAMP) (20 mg/kg) during basal secretion in the rat. Acid output (μEq) is the increase from basal during the 80 min following dbcAMP. All results are expressed as mean with s.e. (vertical bars) where *n* is the number of experiments.

of gastric ulceration after 6 h, which remained almost constant during the following 12 to 24 h (Figure 4).

Prostaglandin-like activity in the rat gastric perfusate and mucosa

No prostaglandin-like activity (less than 0.06 ng/min in terms of prostaglandin E_2) could be detected in extracts of gastric perfusate collected over periods of 20 to 120 min during either basal, pentagastrin- or histamine-stimulated acid secretion (14 determinations from six experiments). This lack of activity was not due to inadequate extraction, since [^{3}H]-prostaglandin E_1 or E_2 added to the acidic perfusate (to give a final concentration of 1 to 10 ng/ml), was completely recovered (99 ± 2%, $n = 9$). Further, no loss of biological activity was observed when prostaglandin E_2 (10 ng/ml) was incubated in gastric perfusate (pH 2) for 40 min at room temperature (three experiments).

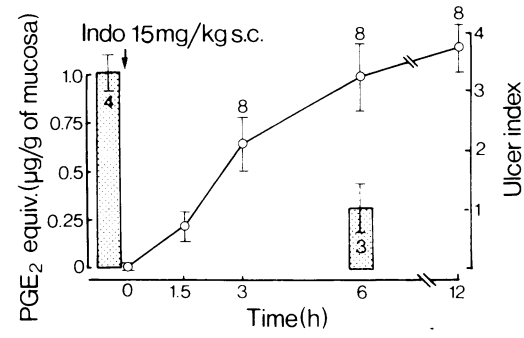

Figure 4 Effects of indomethacin on prostaglandin-like activity (dotted columns) and erosion formation (○) in the rat gastric mucosa. Results are expressed as the mean with s.e. (vertical bars) where *n* is the number of values in the group.

In three experiments, where [^{3}H]-prostaglandin E_1 (4nCi/ml, 10 ng/ml; 0.2 ml/min) was perfused through the gastric lumen for up to 200 min during basal or stimulated secretion, 86 ± 5% ($n = 14$) of the biological activity, and 87 ± 1% ($n = 23$) of the radioactivity was recovered in the samples of perfusate. In a further two experiments during pentagastrin-stimulated acid secretion, [^{3}H]-prostaglandin E_1 (2 μg kg^{-1} min^{-1}; 0.2 μCi kg^{-1} min^{-1}) was infused intravenously for 40 minutes. The inhibition of acid output was not accompanied by an output of biologically active prostaglandin-like material into the perfusate (<0.1 ng/min), and less than 1% of the administered radioactivity appeared in the perfusate during the following 2 hours.

Gastric mucosal tissue from three rats was used in each determination of prostaglandin-like activity. After chromatographic separation on the AI system, 85% of the biological activity was located in the prostaglandin E zone. When chromatographed on the AII system, 70% of this biological activity was located in the prostaglandin E_2 zone. The prostaglandin-like activity of the mucosa, expressed in terms of prostaglandin E_2-equivalents (after correction for efficiency of extraction) was 1.03 ± 0.09 μg/g of mucosa ($n = 4$). This was significantly ($P < 0.01$) reduced to 0.32 ± 0.13 μg/g of mucosa, ($n = 3$) 6 h following indomethacin (15 mg/kg s.c.), as shown in Figure 4.

Binding of indomethacin and dbcAMP to plasma protein

Indomethacin in concentrations of 50 to 300 μg/ml was 96 ± 1% ($n = 6$) bound to bovine

serum albumin as determined by equilibrium dialysis, whereas dbcAMP (50 μg/ml) was not bound (less than 3% bound, $n = 4$).

Discussion

Parenteral administration of indomethacin, which inhibits prostaglandin synthesis (Vane, 1971), caused a fall in rat gastric mucosal blood flow during resting conditions and a reduction in mucosal blood flow per unit acid secretion during pentagastrin stimulation. This supports our concept that prostaglandins have a physiological role as vasodilators in the gastric mucosa of this species.

However, the mucosa still retained much of its ability to vasodilate during stimulation of acid secretion. This could be due to incomplete inhibition of prostaglandin synthesis in the mucosa. Alternatively, other vasodilators such as histamine, adenosine metabolites or CO_2 play an important role or may be capable of doing so under conditions of reduced prostaglandin synthesis, although the mucosal blood flow which they maintain may be inadequate. Comparable studies in other species have not yet been reported. In the dog, a decrease in resting mucosal blood flow following intragastric administration of aspirin was observed (O'Brien & Silen, 1973) although in an earlier study, no such effect was found (Augur, 1970).

Indomethacin, in ulcerogenic doses, had no significant effect on basal acid output. This agrees with the results of previous studies in the pylorus-ligated rat (Catanese, Lisciani & Silvestrini, 1970; Lee, Mollison & Cheng, 1971), though pylorus ligation may not be equatable with basal conditions in the gastric perfusion preparation which yields more precise and meaningful information on secretory processes. Indomethacin significantly increased pentagastrin-stimulated secretion, which is compatible with a negative feed-back inhibitory role of prostaglandins in the mucosa, operative during stimulated secretion. An increase in basal and pentagastrin-stimulated secretion has been observed in pylorus-ligated rats, following chronic administration of 5, 8, 11, 14-eicosa tetraynoic acid (Shaw, Jessup & Ramwell, 1972) which inhibits prostaglandin synthesis. In contrast, indomethacin failed to augment pentagastrin- or histamine-stimulated acid secretion in man (Winship & Bernard, 1970; Bennett, Stamford & Unger, 1973) and decreased secretion in the dog (Nicoloff, 1968). However, before these results can be attributed to species differences in the effects of indomethacin and in the role of mucosal prostaglandins, other factors such as the dose and route of administration must be considered. Although back diffusion of hydrogen-ions did not occur with the gastric perfusion technique used in our experiments, this phenomenon has often been observed following administration of aspirin-like drugs (Chvasta & Cooke, 1972), and may have masked significant changes in acid secretion in dog and man.

Since cyclic adenosine 3′,5′-monophosphate (cyclic AMP) may be the intracellular mediator of gastric acid secretion, it was considered of value to investigate the effects of indomethacin on the secretory response induced by the dibutyryl analogue of cyclic AMP (dbcAMP). When injected during basal secretion, dbcAMP gives a small but reproducible acid secretory response and hence any changes can be readily detected. Indomethacin or the other potent non-steroidal anti-inflammatory drugs, sodium meclofenamate or phenylbutazone, caused a marked potentiation of the dbcAMP-induced responses. These effects were not caused by simple displacement of dbcAMP by these highly plasma protein-bound drugs.

Prostaglandins may depress the formation of cyclic AMP in the gastric mucosa by an action on the adenyl cyclase system (Way & Durbin, 1969; Main & Whittle, 1974a). Thus, a reduction in mucosal prostaglandin activity by aspirin-like drugs would allow the endogenous level or turnover of cyclic AMP to increase and hence augment the acid secretory response. This is supported by recent findings that aspirin stimulates rat mucosal adenyl cyclase activity (Mangla, Kim & Rubulis, 1974). Whether the potentiation of the dbcAMP-induced acid secretion can likewise be attributed to such an action, awaits further investigation into the nature of the secretory response to dbcAMP (Main & Whittle, 1974a). The mechanism by which theophylline, a phosphodiesterase inhibitor, further augments the indomethacin-potentiated secretory response to dbcAMP is not clear, since dbcAMP is resistant to this enzyme (Cehovic, Posternak & Charollais, 1972).

The observed effects of the non-steroidal anti-inflammatory drugs may be attributable to properties other than their ability to inhibit prostaglandin synthesis. Indomethacin and several similar drugs inhibit phosphodiesterases *in vitro* (Flores & Sharp, 1972; Karppanen & Puurunen, 1974; Newcombe, Thanassi & Ciosek, 1974), though the concentrations required are much higher than those which inhibit prostaglandin synthesis, and may be of limited significance *in vivo*. Furthermore, the potent phosphodiesterase inhibitor, theophylline, had no consistent effect on the secretory response to either pentagastrin or dbcAMP in the rat. The fall in mucosal blood flow

is unlikely to result from increased cyclic AMP levels since cyclic AMP causes vasodilatation of the mucosa (Main & Whittle, 1974a). It has been suggested that the mucosal damage which follows gastric instillation of salicylic acid in the pylorus-ligated rat is due to histamine release from the mucosa (Johnson, 1966). This release could not explain the reduced mucosal blood flow observed in the present experiments, and we have found that parenterally-administered indomethacin had no significant effect on the histamine output into the rat gastric lumen during basal or pentagastrin-stimulated acid secretion (Main & Whittle, unpublished results).

If non-steroidal anti-inflammatory drugs are acting by reducing the formation of mucosal prostaglandins, then the output of prostaglandin-like material into the rat gastric perfusate, observed by Shaw & Ramwell (1968), might be expected to decrease prior to the changes in acid secretion and mucosal blood flow. However, this has remained untested since, in the current investigation, no prostaglandin-like activity could be detected in gastric perfusate extracts. Likewise, very little prostaglandin-like material could be detected in human gastric juice (Horton, Main, Thompson & Wright, 1968; Bennett *et al.*, 1973). This may reflect low permeability of the gastric mucosa to prostaglandins, since, following intravenous injection of [^{3}H]-prostaglandin E_1, very little radioactivity could be detected in the gastric perfusate. Inactivation or loss of prostaglandins following their release from the mucosa into the lumen is unlikely to account for our results, since when low concentrations of [^{3}H]-prostaglandin E_1 or E_2 were perfused through the gastric lumen, approximately 90% of the biological activity and radioactivity were recovered from the perfusate. In contrast, Shaw & Ramwell (1968), using a similar technique, detected prostaglandin-like activity in the gastric perfusate despite a 75% loss of perfused prostaglandins. We cannot exclude the possibility that A-prostaglandins or metabolites of E-prostaglandins which are undetectable by biological assay, were present in the gastric perfusate in our experiments. Both A and E prostaglandins may be present in dog gastric secretion (Dozois & Thompson, 1974) though the amounts are very low and, as with prostaglandin E_2 in man (Bennett *et al.*, 1973), no significant change in the concentration and output has been detected during stimulation of acid secretion. Nevertheless, lack of prostaglandin release into the lumen cannot be taken as conclusive evidence against a local role in the mucosa, since prostaglandins may be rapidly metabolized at their site of action, or may pass rapidly into the mucosal capillaries and be carried away by the increased mucosal blood flow.

The failure to detect prostaglandin output necessitated a study of the effects of indomethacin on mucosal prostaglandin activity, though it is not known how extractable prostaglandin reflects the activity of the prostaglandin system *in vivo*. Indomethacin markedly reduced the prostaglandin-like activity of mucosal extracts. Although some of the prostaglandin-like activity may have been formed *in vitro* prior to homogenization, the results indicate that the concentration of indomethacin in mucosal tissue *in vivo* was sufficient to inhibit prostaglandin synthesis. Inhibition of prostaglandin synthesis in human gastric mucosa *in vitro* by indomethacin has recently been reported (Bennett *et al.*, 1973).

We have confirmed that non-steroidal anti-inflammatory drugs such as indomethacin and phenylbutazone produce gastric mucosal erosions in the rat when administered parenterally. We have also shown that, at a time when the incidence and severity of mucosal erosions had reached a steady level, gastric mucosal prostaglandin levels were much reduced. The precise mechanism underlying the formation of erosions by aspirin-like drugs is unknown. Our results in the rat raise the possibility that a reduction of mucosal blood flow per unit acid secretion, possibly resulting in mucosal ischaemia, coupled with an increased sensitivity of the parietal cells to secretory stimuli, may contribute to the production of erosions. An increase in acid output is itself unlikely to cause erosions, but would promote their formation, as suggested by the increased incidence of mucosal bleeding following indomethacin at high rates of acid secretion. Thus, the prevention of indomethacin-induced mucosal erosions by prostaglandin E_2 and its (15S) methyl analogue (Main & Whittle, unpublished results), which are potent inhibitors of rat gastric acid secretion (Main & Whittle, 1974b), may reflect an antisecretory action rather than the role of endogenous prostaglandins. Whether the changes in mucosal permeability to ions, water and protein which often characterize mucosal damage by aspirin-like drugs (Davenport, 1964) can also be attributed to changes in mucosal prostaglandins has yet to be determined.

To establish causal relationships will require careful investigation not only of the relative potency of non-steroidal anti-inflammatory drugs in inhibiting gastric mucosal prostaglandin synthetase and in causing erosions, but of the simultaneous effects of these and other drugs which affect the prostaglandin system on gastric secretion, mucosal blood flow and mucosal permeability. In view of the species differences in susceptibility to erosion formation (Wilhelmi, 1974) and the possibility that differences exist

between prostaglandin synthetase in different tissues (Flower & Vane, 1972) or in the same tissue from different species, it may be necessary to correlate these parameters in a single species.

Although the nature of the relationship between the intake of non-steroidal anti-inflammatory drugs and the incidence of peptic ulcer (Alp, Hislop & Kerr Grant, 1970) has not been clearly established, other factors which alter prostaglandin turnover or the relative amounts of different prostaglandins, may also be implicated in the pathogenesis of both acute erosions and peptic ulcer. It is also possible that drugs which are known to cause or exacerbate ulcers, for example the steroidal anti-inflammatory agents, may act by altering the prostaglandin system.

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