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EFFECTS OF FASTING, STRESS AND DRUGS ON GASTRIC GLYCOPROTEIN SYNTHESIS IN THE RAT

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1 The relationship between gastric mucosal damage and synthesis of gastric glycoproteins, as measured by the rate of incorporation of N-acetyl-[^{3}H]glucosamine, was investigated in rats after fasting and restraint stress and a single administration of aspirin (200 mg/kg, orally), phenylbutazone (200 mg/kg, orally), prednisolone (200 mg/kg, orally), or adrenaline (2 mg/kg, i.p.). In one experiment, the effects of aspirin and phenylbutazone on carbohydrate content of the glycoproteins were also determined.

2 Restraint stress, phenylbutazone and aspirin resulted in acute gastric mucosal erosions in some of the rats. Adrenaline produced severe sub-mucosal haemorrhage, but no erosions or ulceration, while prednisolone and fasting gave no gross pathology.

3 The rate of incorporation of N-acetyl-[^{3}H]glucosamine into glycoproteins was decreased after all treatments except adrenaline. In the groups receiving restraint stress, aspirin or phenylbutazone, the decreases were more marked in rats which developed erosions than in those with no gastric pathology.

4 Aspirin and phenylbutazone also produced changes in the carbohydrate content of the glycoproteins, the effects again being greater in the rats which developed erosions.

5 The results are discussed in the context of a possible association between erosion formation and glycoprotein synthesis and it is proposed that inhibition of mucus glycoprotein biosynthesis may be one mode of action of stress and drugs in causing gastric mucosal damage.

Introduction

In studies of the pathogenesis of peptic ulcer, much attention has been concentrated on the aggressive forces of acid and pepsin and perhaps too little attention on the natural defence of the mucosa. The concept of a gastric mucosal barrier, protecting the stomach against its own secretions, was developed by Code and by Davenport (Code, Higgins, Moll, Orvis & Scholer, 1963; Davenport, 1964; Davenport, Warner & Code, 1964) and, although the precise location and nature of this barrier has not been fully defined, its function may be performed in part by the mucus coating the surface of the stomach and in part by the apical plasma membrane of the surface epithelial cells (Avery Jones, 1975). It is now generally accepted that the integrity of the mucus cells of the stomach and their ability to secrete mucus continuously are essential protective mechanisms (Kirsner, 1966) but the precise role of mucus in the aetiology of gastric ulcers and erosions is unknown.

The present study was undertaken to investigate the effects on rat gastric mucosa and mucus, of stress, phenylbutazone, aspirin, prednisolone and adrenaline, all of which are capable of producing gastric mucosal damage in the rat. As glycoproteins are the principal constituents of mucus (Kent, 1967; Spiro, 1970) the effects of stress and these drugs on the synthesis of gastric glycoproteins in the rat were studied as an index of mucus production and integrity. It is believed that, in mucus producing cells, the protein moiety of the mucus is produced by ribosomes associated with the endoplasmic reticulum and that membrane bound transferases probably attach one sugar at a time to the protein

chain as it moves through the channels of the endoplasmic reticulum (Spiro, 1970). In the present study, the biosynthesis of the glycoproteins was assessed by measurement of the rate of incorporation of a radioactively-labelled sugar into rat gastric glycoproteins and by measurement of the carbohydrate content of the glycoprotein. In a previous study, N-acetyl-[^{3}H]glucosamine was found to give the best incorporation rates of a series of labelled hexoses and amino-sugars (Shillingford, Lindup & Parke, 1974) and was thus chosen for the present work. Some of these results have previously been communicated to the British and Italian Pharmacological Societies (Dekanski, MacDonald & Parke, 1974).

Methods

Female Biorex Wistar rats, 140-160 g, were used in all the experiments. Restraint was carried out according to the method of Brodie & Hanson (1960) in which the rats are wrapped in wire screening while under light ether anaesthesia and then allowed to recover from anaesthesia. The rats were restrained for 24 h at normal room temperature of 22°C, or for 24 or 6 h at a lower temperature (17°C). The rats were not fasted prior to the restraint period but had no access to food during restraint and therefore groups of rats fasted for the same length of time and at the same temperature as the restrained rats were included to determine the effects of fasting alone.

Assessment of mucosal damage

After the restraint period, or 6 h after drug administration, the rats were killed by cervical dislocation and the stomachs excised and washed in ice-cold 0.9% w/v NaCl solution (saline). The stomachs were examined and rated for pathology according to the following arbitrary scale: 0 = no damage; 1 = blood in lumen; 2 = pin point erosions; 3 = 1-5 small erosions (<2 mm); 4 = >5 small erosions; 5 = 1-3 large erosions (>2 mm); 6 = >3 large erosions.

Glycoprotein synthesis

The rate of incorporation of N-acetyl-[^{3}H]glucosamine into gastric glycoproteins was measured by minor modifications of the method of Lukie & Forstner (1972). Circular pieces of stomach from the corpus were removed using a cork borer, diameter 14 mm, to ensure similar sample sizes. Incubations were carried out at 37°C in a Mickle shaking incubator (60 rev/min). The samples were pre-incubated for 10 min in a modified Krebs Medium I (Lukie & Forstner, 1972) before the addition of 1 μCi of N-acetyl-O-[1-^{3}H]glucosamine (4 Ci/mmol, Radiochemical Centre, Amersham) and incubations were carried out for 2.5 h, gassing with 95% O_2 and 5% CO_2 every 20 minutes. Incubations were terminated by draining the medium and washing the samples twice with 5.0 ml of ice-cold saline. The tissue samples were then homogenized in 20 ml of 5 mM disodium ethylenediamine tetraacetate (EDTA; pH 7.4). Glycoproteins were precipitated overnight at 4°C with 10% trichloroacetic acid and 1% phosphotungstic acid. The acid-soluble supernatant was discarded and the precipitate washed twice with distilled water followed by two lipid extractions with chloroform/methanol (1 : 1, v/v). After air-drying, the precipitate was weighed and solubilised overnight in 2 ml of 0.5M NaOH. The samples were acidified before adding a 1 ml aliquot to 10 ml scintillant consisting of 3.3 g/l 2,5-diphenyloxazole and 0.2 g/l 1, 4-di-2-(4 methyl-5-phenyloxazolyl) benzene in Triton X-100/toluene (1 : 2, v/v). The tritium content was determined in a Packard Tricarb Model 3320 liquid scintillation spectrometer.

The carbohydrate content of the precipitated glycoprotein was determined in one experiment as follows: approximately 20 mg of each sample was weighed accurately and hydrolysed overnight in 2 ml of 2M HCl at 90°C. After hydrolysis, the samples were filtered and made up to 5 ml with distilled water. One ml of the filtrate was used for the combined estimation of fucose and hexoses by the method of Dische & Shettles (1948). A 3 ml aliquot of the filtrate was used for the assay of hexosamines by the method of Boas (1953) modified by the omission of the resin separation as similar values were obtained in preliminary experiments without this step. For sialic acid estimation 20-40 mg samples were hydrolysed for 1 h in 2 ml 0.025 M H_2SO_4 at 80°C. A 0.2 ml aliquot of the hydrolysate was assayed according to the method of Warren (1959).

Drugs

Phenylbutazone, aspirin and prednisolone were suspended in 0.1% aqueous Tween 80 and administered orally to rats via a stainless steel stomach tube at a dose of 200 mg/kg (0.5 ml/100 g bodyweight). (-)-Adrenaline bitartrate (BDH) was dissolved in saline and administered intraperitoneally to rats at a dose of 2 mg/kg (0.5 ml/100 g bodyweight). The animals were killed 6 h after a single administration.

Results

The effects of fasting, restraint, cold and combinations of these treatments are given in Table 1. It was found that temperature markedly affected the degree of mucosal damage in the restraint experiments, the incidence and severity of erosions being far greater in the restraint plus cold group than with restraint alone for 24 hours. A reduction in the rate of incorporation of

Table 1 The effect of fasting, restraint and cold on the rate of incorporation of N-acetyl-[^{3}H] glucosamine into rat gastric glycoproteins

Group	*n*	*% With erosions*	*Mean erosion index*	*Incorporation of N-acetyl-[^{3}H] glucosamine d/min per mg glycoprotein*	*% Change from control value*
Control	9	0	0	1520 ± 200	–
Fasted 24 h	4	0	0	720 ± 240[1]	−53
Restrained 24 h	4	75	1.5	540 ± 170[2]	−65
Cold 24 h	10	0	0	1870 ± 320 NS	+24
Fasted plus cold 24 h	4	0	0	510 ± 130[2]	−67
Restrained plus cold 24 h	4	100	5.3	730 ± 300[1]	−52
Cold 6 h	6	0	0	1410 ± 420 NS	−8
Fasted plus cold 6 h	6	0	0	1110 ± 210 NS	−27
Restrained plus cold 6 h	6	50	1.8	640 ± 150[2]	−58

Portions of corpus tissue were incubated with 1.0 μCi N-acetyl-[^{3}H] glucosamine for 2.5 hours. Values shown are means for the number of rats (*n*) ± s.e. mean. Significance of difference from control value, Student's *t* test; (*P*), [1] <0.05; [2] <0.01; NS, not significant.

Table 2 The effect of various procedures on the rate of incorporation of N-acetyl-[^{3}H] glucosamine into gastric glycoproteins

Treatment	*n*	*% With erosions*	*Mean erosion index*	*Incorporation of N-acetyl-[^{3}H] glucosamine d/min per mg glycoprotein*	*% Change from control value*
Control	22	0	0	1470 ± 150	–
Restraint plus cold 6 h	11	55	1.9	890 ± 150[1]	−39
* Phenylbutazone (200 mg/kg orally)	11	37	0.9	910 ± 120[2]	−38
* Aspirin (200 mg/kg orally)	10	60	1.4	1180 ± 220 NS	−20
* Adrenaline (2 mg/kg i.p.)	6	0	0	1600 ± 320 NS	+9
* Prednisolone (200 mg/kg orally)	5	0	0	830 ± 310 NS	−43

* Rats were killed 6 h after single administration.
Values shown are means for the number of rats (*n*) ± s.e. mean. Significance of difference from control value, Student's *t* test; (*P*), [1] <0.05; [2] <0.01; NS, not significant.

N-acetyl-[^{3}H]glucosamine was also observed in the restrained rats but this was similar for the restraint and the restraint plus cold groups with no correlation between the extent of mucosal damage and the reduction in rate of incorporation. Fasting for 24 h produced a reduction in the rate of incorporation of N-acetyl-[^{3}H]glucosamine similar to that observed with 24 h restraint but without causing gastric mucosal damage. The effect of fasting plus cold for 24 h on the rate of incorporation of N-acetyl-[^{3}H]glucosamine was slightly greater than that of fasting alone but not significantly so and again, no mucosal damage was observed. With a shorter experimental period of 6 h, cold alone or fasting plus cold produced no mucosal damage, whereas restraint plus cold for 6 h resulted in erosions in 3 out of 6 rats. The rate of incorporation of N-acetyl-[^{3}H]glucosamine was not significantly reduced after cold or fasting plus cold for 6 h, but was reduced after restraint plus cold for 6 hours.

In a separate series of experiments, the results of which are given in Table 2, restraint plus cold for 6 h, aspirin (200 mg/kg orally) and phenylbutazone (200 mg/kg orally), all resulted in acute gastric mucosal erosions in 37-60% of the treated rats. Adrenaline (2 mg/kg i.p.) produced severe sub-mucosal haemorrhage with no sign of discrete erosion or ulceration and the rats receiving prednisolone (200 mg/kg orally) showed no gross gastric pathology. The rate of incorporation of N-acetyl-[^{3}H]glucosamine was significantly reduced after restraint plus cold for 6 h and after phenylbutazone administration, but not after aspirin, adrenaline or prednisolone. In fact, in the prednisolone treated group, 3 out of 5 rats had markedly lowered rates of incorporation with 2 animals giving values in the normal range. The reduction failed to attain statistical significance owing to the large variation within the group.

If separate consideration is given to the rats which developed erosions (Table 3) then restraint

Table 3 Inhibition of the rate of incorporation of N-acetyl-[^{3}H]glucosamine into gastric glycoproteins in rats with and without gastric erosions

Treatment	*Erosions present*	*n*	*Incorporation of N-acetyl-[^{3}H]glucosamine d/min per mg glycoprotein*	*% Change from control value*
Control	NO	22	1470 ± 150	–
Restraint plus	NO	5	1130 ± 270 NS	−23
cold 6 h	YES	6	690 ± 150^3	−53
* Phenylbutazone	NO	7	960 ± 170^1	−34
(200 mg/kg orally)	YES	4	810 ± 160^2	−45
* Aspirin	NO	4	1790 ± 320 NS	+22
(200 mg/kg orally)	YES	6	770 ± 150^2	−48

* Rats were killed 6 h after a single administration.
Values shown are means for the number of rats (*n*) ± s.e. mean. Significance of difference from control value, Student's *t* test; (*P*), ^{1} <0.05; ^{2} <0.01; ^{3} <0.001; NS, not significant.

Table 4 Effect of aspirin and phenylbutazone on the carbohydrate content of rat gastric glycoproteins

			Carbohydrate content nmol/mg glycoprotein			
Treatment	*Erosions present*	*n*	*Hexosamines*	*Hexoses*	*Fucose*	*Sialic acid*
Control	NO	8	33 ± 2	61 ± 12	14 ± 3	0.40 ± 0.03
* Aspirin	NO	3	30 ± 6 NS	46 ± 17 NS	20 ± 4 NS	0.42 ± 0.03 NS
(200 mg/kg orally)	YES	5	35 ± 3 NS	19 ± 4^2	26 ± 2^2	0.39 ± 0.01 NS
* Phenylbutazone	NO	4	41 ± 1^2	21 ± 6^1	21 ± 2 NS	0.39 ± 0.01 NS
(200 mg/kg orally)	YES	4	34 ± 3 NS	15 ± 3^2	21 ± 3 NS	0.38 ± 0.01 NS

* Rats killed 6 h after single administration.
Values are means for the number of rats (*n*) ± s.e. mean. Significance of difference from control values, Student's *t* test; (*P*), ^{1}<0.05; ^{2}<0.01; NS, not significant.

plus cold for 6 h, phenylbutazone and aspirin all gave significant reductions in the rate of incorporation of N-acetyl-[^{3}H]glucosamine in rats with erosions. In the treated rats which failed to develop erosions, restraint plus cold for 6 h produced a small but non-significant reduction in N-acetyl-[^{3}H]glucosamine incorporation, while aspirin increased the incorporation but again not significantly so. A significant reduction in the rate of incorporation of the labelled hexosamine in rats without erosions was seen only in the group receiving phenylbutazone.

The results of a single experiment to determine the effects of aspirin and phenylbutazone on the carbohydrate content of rat gastric glycoproteins are given in Table 4. The changes in hexose and fucose content were greater in aspirin-treated rats with erosions than in those without. In the phenylbutazone-treated group the reduction in hexose content was greater in rats with erosions than in those without, but the reduction was significant for both groups. The fucose content of phenylbutazone-treated rats appeared similar in rats with or without erosions and, although perhaps slightly raised, failed to differ significantly from the control value. A significant rise in hexosamine content was also observed in the phenylbutazone treated group but only in the rats in which no erosions had formed. No changes in the content of sialic acids were observed.

Discussion

Decreases in the quantity, and carbohydrate content, of secreted mucus in rat and dog have previously been reported after administration of aspirin (Menguy & Masters, 1965), phenylbutazone (Menguy & Desbaillets, 1967) and prednisolone (Robert & Nezamis, 1963). Decreases in the glycoprotein content of rat and guinea pig gastric mucosa have also been shown after restraint stress (Ludwig & Lipkin, 1969; Lambert, Truchot, Andre & Chayvialle, 1971). These effects have led to the hypothesis that stress or drugs may cause gastric mucosal injury by inhibition of the biosynthesis of mucus components. In the present study, there are three theoretical possibilities regarding a relationship between erosion formation and the observed inhibition of glycoprotein synthesis. First, erosion formation and inhibition of glycoprotein synthesis are not related; second, impaired glycoprotein synthesis may be a direct result of mucosal cell damage; and third, mucosal pathology may be a consequence of impaired glycoprotein synthesis.

The first possibility is seen in fasting for 24 h which causes an inhibition in the rate of incorporation of N-acetyl-[^{3}H]glucosamine into gastric glycoproteins without causing gastric mucosal erosions. The incorporation also appeared to be lowered in some rats after prednisolone treatment, although no mucosal damage occurred, while adrenaline resulted in severe gastric pathology without any reduction in glycoprotein synthesis. However, it is known that more prolonged fasting (Robert, Bayer & Nezamis, 1963) or prednisolone treatment (Robert & Nezamis, 1963) does lead to mucosal damage and the observed reduction of glycoprotein synthesis may be a necessary prerequisite or even the cause of the damage. Also, the nature of the adrenaline-induced gastric damage was markedly different from that after treatment with the other agents used in this study. No discrete erosions or ulcerations were present but diffuse sub-mucosal haemorrhage affecting large areas of the stomach was observed. Thus, the primary effect of adrenaline appears to be on the blood vessels, causing vasoconstriction with no immediate effect on glycoprotein biosynthesis, although more prolonged treatment may lead to an inhibition of synthesis possibly through reduction of the mucosal blood supply.

The second possibility, that impaired glycoprotein synthesis is a result of mucosal damage is unlikely as first, the reduction in N-acetyl-[^{3}H]glucosamine incorporation can occur when no mucosal damage is present (e.g. after 24 h fasting and in phenylbutazone-treated rats which failed to develop erosions) and second, when mucosal damage does occur, the reduction in the rate of incorporation of N-acetyl-[^{3}H] glucosamine is not related to the intensity of the damage (e.g. restraint and restaint plus cold for 24 h, Table 1).

The third possibility, that mucosal damage is a consequence of impaired glycoprotein synthesis, may apply. We have shown that erosion formation after restraint plus cold stress, aspirin or phenylbutazone is associated with a reduction in the rate of incorporation of N-acetyl-[^{3}H] glucosamine into rat gastric glycoproteins. Changes in the carbohydrate content of the glycoproteins, also associated with erosion formation after treatment with aspirin or phenylbutazone, were also found. Several of the changes in the rate of incorporation of N-acetyl-[^{3}H]glucosamine and carbohydrate content occurred in treated rats which had not developed erosions suggesting that they not only accompany, but actually precede, erosion formation.

Thus it is possible that one mode of action of stress and drugs in causing gastric erosions is inhibition of the glycosylation of the mucus glycoprotein and hence a decreased synthesis of

glycoprotein or the synthesis of a modified glycoprotein, or both. The changes in fucose and hexose content after treatment with aspirin and phenylbutazone suggest synthesis of a modified glycoprotein. A decreased synthesis of glycoproteins as measured by radioactive glucose uptake by sheep colonic mucosal scrapings in the presence of aspirin *in vitro* has previously been found (Kent & Allen, 1968) whereas the drug carbenoxolone has been shown to increase the rate of incorporation of radioactively labelled carbohydrates into glycoproteins of rat, ferret and human gastric mucosa (Shillingford, Lindup & Parke, 1974; Johnson, Lindup, Shillingford, Smith & Parke, 1975), an effect which may explain its beneficial effects on mucus production (Goodier, Horwich & Galloway, 1967) and its proven efficacy in the treatment of gastric ulcer (Sircus, 1972). Studies of the effects of carbenoxolone and analogues on the rate of incorporation of labelled carbohydrates into glycoproteins of rat gastric mucosa and their correlation with the prevention or healing of experimental ulcers or erosions are in progress, using a modified *in vitro* technique.

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