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Effect of carbenoxolone on the synthesis of glycoproteins and DNA in rat gastric epithelial cells

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SUMMARY The influence of carbenoxolone on the synthesis of glycoproteins in the surface mucous cells and the production of new cells in the rat gastric mucosa was studied by means of a vascular perfusion system. The rate of incorporation of tritiated galactose, glucosamine, serine, and sulphate in surface mucous cells, studied by autoradiography, was not affected by the addition of carbenoxolone to the drinking water. The sugar composition (determined by gas-liquid chromatography) of the gastric glycoproteins (isolated by centrifugation in CsCl), was not changed in carbenoxolone-treated rats. Compared with untreated animals, the number of [^{3}H]-thymidine labelled nuclei per fundic pit increased by 38% to 76% in carbenoxolone-treated rats, implying a higher number of mitotically active cells. This results in an increased supply of young mucous cells; if this also proves to be true in human gastric mucosa, it may be relevant to the therapeutic effect of carbenoxolone.

Carbenoxolone accelerates the healing of gastric ulcers[1 2] and prevents erosions caused by stress,[3] but not those caused by aspirin.[4] The mechanism by which carbenoxolone exerts its beneficial action is unknown. The reported increase in the amount of PAS-stainable material in the remaining cells and on top of them in the ulcerated stomach of man[5] and animals[3 4] suggests an increased rate of synthesis and secretion of the glycoproteins of the protective mucin coat. However, the same effect might result from a decreased rate of mucin breakdown or removal. The protective effect might also be explained by an altered carbohydrate composition of the glycoprotein.[6–8] These studies, however, did not exclude changes in synthesis and release of connective tissue elements of the lamina propria, or contribution from swallowed salivary and respiratory mucins. Therefore, the amount of solubilised glycoproteins in the gastric juice is a poor estimate of the amount of glycoprotein synthesised and secreted by the mucous cells of the gastric epithelium and glands. Moreover, differences in the amount of fluid, its ion concentration, or its pepsin content might affect the solubilisation of the coat glycoproteins, without affecting their rate of synthesis and secretion. Although a clinical dose of carbenoxolone has no effect on HCl and pepsin secretion,[9] its precipitation of glycoproteins from the gastric lumen[10] is also a complicating factor.

When, after several weeks of carbenoxolone administration, a new steady state has been reached in long-term experiments, an increased rate of secretion is impossible without a higher rate of glycoprotein synthesis in the mucin-producing gland cells of the stomach. We therefore compared the rate of radioactive sugar and amino acid incorporation in the cells of the superficial gastric epithelium of rats treated with carbenoxolone (three weeks) and of control rats by measuring the radioactivity per cell in autoradiographs. The radioactive precursors were administered in a vascular perfusion system of the rat stomach *ex vivo*.[11 12]

Lipkin's report[13] suggests a longer life of the epithelial cells. This implies a greater number of cells (unless the drug should have lowered the number of dividing cells). In that case, a larger amount of glycoproteins may be found in a whole stomach, in spite of an unchanged rate of synthesis, because of an increased number of synthetically active cells. We therefore also studied the effect of carbenoxolone on the number of dividing cells.

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Methods

PERFUSION EXPERIMENTS

Male Wistar rats (TNO, Zeist, The Netherlands) were trained to drink 20 ml of water from 10.00–11.00 hours. After a week one half of this group received 2 mg carbenoxolone sodium (Biogastron, Biorex, London, UK) in 20 ml drinking water (=0·154 mM=10 mg/kg body weight), a common experimental dose,[3 4 9 13] although it is twice the daily clinical dose.[2] Carbenoxolone lowered the pH of the drinking water from 7·7 to 7·6. A difference in osmolality could not be demonstrated (it should be 0·6 mOsm/kg). The other half of the experimental group received 20 ml of normal drinking water.

The body weight of the treated and control animals increased at the same rate as their non-experimental litter mates. The mean weight (± SEM), before they were killed after a fasting period of 19–23 hours, of treated animals was 226 ±5 g (n=18), and the mean weight of the controls was 235±7 g (n=18).

After three weeks of carbenoxolone addition, stomachs of rats from both the treated and the parallel control group were vascularly perfused for 30 minutes with a medium containing fluorocarbon (FC–75) and radioactive precursors. This perfusion time ensures that none of the radioactive molecules is secreted, as secretion of radioactivity labelled macromolecules does not occur within two hours after the start of the perfusion.[11] This synthetic medium and the vascular perfusion system were described previously.[12] The perfusion medium contained 10 μCi/ml of either [^{3}H]-thymidine (41 Ci/mmol) and [^{3}H]-galactose (22 Ci/mmol) together, or [^{3}H]-glucosamine (23 Ci/mmol), or [^{3}H]-serine (17 Ci/mmol) or [^{3}H]-sulphate (carrier-free). All radiochemicals were obtained from the Radiochemical Centre (Amersham, Bucks, UK). The perfusions were started between 11.00 and 15.00 hours. After perfusion during 30 minutes with radioactive precursor, the stomachs were fixed by subsequent perfusion with 1% glutaraldehyde, 1% formaldehyde, and 1% saccharose in 0·1 M phosphate buffer. Four cubes of fundic tissue were taken from each stomach and embedded in glycolmethacrylate. Five micrometre sections from two cubes of both a treated and a control animal were mounted together on one slide, so that possible deviations due to differences in processing could be detected and, if necessary, compensated for. The sections were covered with Kodak AR-10 stripping film and exposed at 4°C. After four days (sulphate), or one (serine), two (galactose+thymidine), or four weeks (glucosamine) the autoradiographs were developed and stained with PAS-haemalum. In three sections from each of the four cubes of stomachs from treated and non-treated rats, the number of nuclei labelled by [^{3}H]-thymidine was determined in 100 pits. In the same number of sections from fundic tissue perfused with [^{3}H]-galactose, [^{3}H]-glucosamine, [^{3}H]-serine, or [^{35}S]-sulphate, the mean number of silver grains per mean cellular area of each cube of tissue was determined over about 50 surface mucous cells at the free surface (SSMC). This value was obtained by counting the number of silver grains over areas of epithelium at the gastric surface and dividing that number by the number of nuclei in those areas, as described earlier.[11] Non-specific label, 10% in our experiments, was subtracted. The amount of radioactive thymidine incorporated in the nuclei of cells in mitotic cycle was estimated by counting the silver grains over labelled nuclei after one day of exposure. Galactose labelling was determined by counting silver grains over superficial mucous cells. After this labelling the grains are situated in the cytoplasm between the nucleus and the secre-

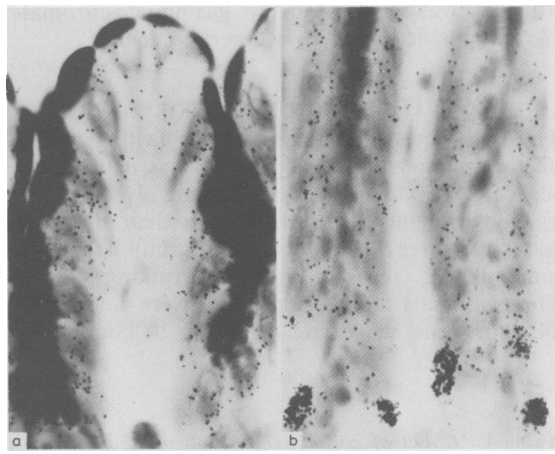

Figure *Autoradiographs of gastric mucosa after labelling with galactose and thymidine. Exposure time 14 days. PAS-haemalum stain. (a) silver grains arising from galactose over surface mucous cells. The isthmus ends where the darkly stained mass of PAS-positive mucous granules starts. (b) silver grains arising from thymidine can be seen over five heavily labelled nuclei of isthmic cells. It can easily be distinguished from the galactose label present in the cytoplasm of these cells. (Original magnification ×1000.)*

tory granules.[14] The large number of silver grains that arise from radioactive thymidine after two weeks of exposure are exclusively located over the nucleus. The labelled nuclei are situated in the cells of the neck of the gland, and never in the most superficial mucous cells (Figure). Moreover, the former cells incorporate a very small amount of galactose.[15] All countings were done on coded slides to guarantee blind quantification.

QUANTITATIVE SUGAR ANALYSIS

Four male Wistar rats were given 10 mg/kg body weight carbenoxolone in their drinking water for three weeks. The control group (from the same batch) received ordinary drinking water. Animals of both groups were fasted during 18 hours and killed by decapitation. The stomachs were washed in ice-cold saline and the mucosal surface was scraped with a razor-blade. Scrapings of the 10 stomachs of the carbenoxolone and control groups were homogenised separately in 1 mM DTT (dithiothreitol) and 1 mM EDTA in saline with a Polytron PCU-2 tissue homogeniser. The gastric glycoproteins were isolated by centrifugation in CsCl (60 hours, 150 000 *g*) of homogenate fractions as described by Spee-Brand *et al.*[16] Quantitative analysis of the sugars in the glycoproteins obtained from both carbenoxolone-treated and non-treated animals was performed by gas-liquid chromatography as described by Kamerling *et al.*[17]

IN VIVO [^{3}H]-THYMIDINE INCORPORATION

The influence of the length of the fasting period on the number of labelled nuclei was determined separately. Six male Wistar rats were given 10 mg/kg body weight carbenoxolone in their drinking water for three weeks. A parallel group, from the same batch, received normal drinking water. Three animals from each group were fasted for 24 hours, the remaining three for 20 hours. Immediately after those periods all animals were injected with 1 μCi/g body weight [^{3}H]-thymidine. All animals were killed by decapitation 30 minutes after injection. The stomachs were taken out and four cubes per stomach were fixed and prepared for autoradiography. The number of labelled nuclei per pit was determined as described for the perfused stomachs.

Results

The influence of carbenoxolone on the number of thymidine labelled nuclei per pit is shown in Tables 1 and 2. The thymidine index increases significantly in both the perfusion experiments and those *in vivo*. Moreover it appears that the thymidine index does not vary with time of fasting at the intervals studied.

The amount of radioactive thymidine incorporated per nucleus was also determined (Table 3).

Table 2 *Influence of fasting during 20 or 24 hours and of carbenoxolone on number of nuclei per pit labelled by [^{3}H]-thymidine in vivo*

	Fasting				
	20 hours			*24 hours*	
Rat no.	*Number of pits*	*Thymidine index*	*Rat no.*	*Number of pits*	*Thymidine index*
Controls					
30	442	1·96±0·44	36	411	1·81±0·30
31	409	0·88±0·11	37	408	1·68±0·18
32	411	1·21±0·24	38	409	1·59±0·25
Mean		1·35±0·32	Mean		1·69±0·06
Carbenoxolone					
33	412	2·75±0·63	39	409	2·33±0·54
34	465	2·02±0·77	40	416	2·13±0·25
35	399	2·34±0·72	41	419	2·58±0·44
Mean		2·37±0·21	Mean		2·34±0·13
Difference		+76%; P<0·05			+38%; P<0·01

Means ±SEM. P: Student's *t*-test.

Table 1 *Effect of carbenoxolone on number of pits per mm mucosa, and number of nuclei per pit labelled by [^{3}H]-thymidine in perfusion experiments*

Controls				*Carbenoxolone*			
Rat no.	*Number of pits*	*Pits/mm*	*Thymidine index*	*Rat no.*	*Number of pits*	*Pits/mm*	*Thymidine index*
1	1113	38	1·55±0·14	4	1232	39	2·25±0·31
2	920	36	1·52±0·13	5	1194	34	2·15±0·37
3	1227	34	1·22±0·26	6	1227	34	2·30±0·37
Mean		36±1	1·42±0·11	Mean		36±1	2·24±0·04
Difference				+58%; P<0·01			

Means ± SEM. The controls were fasted for about 21 hours, the carbenoxolone treated animals about 19 hours. P: Student's *t*-test.

The mean number of silver grains per nucleus varies considerably between the animals, and does not permit a conclusion about an effect of carbenoxolone on the rate of thymidine incorporation.

Carbenoxolone did not affect the incorporation of galactose, glucosamine, serine, and sulphate (Table 4).

Because the rate of incorporation does not always reflect the actual amount of sugars present in the glycoproteins of the stomach (the rate may have been different before perfusion, or sugars may have been metabolised or changed into other sugars) a sugar analysis was also performed. The ratio between the sugars galactosamine : glucosamine : galactose : fucose in the glycoproteins of both the four controls and the four carbenoxolone-treated animals was 1 : 2·5 : 2·5 : 1·3, with no greater deviation from the ratios than 0·1.

Table 3 *Influence of carbenoxolone treatment on incorporation of [^3H]-thymidine in perfusion experiments (mean numbers of silver grains per labelled nucleus)*

	Controls			*Carbenoxolone*	
Rat no.	*Number of nuclei*	*Silver grains per cell*	*Rat no.*	*Number of nuclei*	*Silver grains per cell*
1	283	13·1 ±0·9	4	303	27·5 ±1·2
2	203	12·4 ±1·1	5	229	13·4 ±1·3
3	310	12·1 ±0·7	6	321	14·1 ±0·4
7	213	28·6 ±2·3	8	203	37·2 ±1·6
Mean		16·55±4·02			23·05±5·72

Table 4 *Influence of carbenoxolone treatment on incorporation of [^3H]-galactose, [^3H]-glucosamine, [^3H]-serine, and [^{35}S]-sulphate in perfusion experiments (mean numbers of silver grains per mean cellular area±SEM)*

	Controls			*Carbenoxolone*	
Rat no.	*Number of nuclei*	*Silver grains*	*Rat no.*	*Number of nuclei*	*Silver grains*
[^3H] *galactose*					
1	674	4·12±0·27	4	603	5·05±0·15
2	464	6·42±0·34	5	632	5·00±0·28
3	620	5·29±0·38	6	653	5·36±0·26
Mean		5·28±0·66	Mean		5·14±0·11
[^3H]-*glucosamine*					
9	628	5·79±0·45			
10	621	6·48±0·47	13	496	5·03±0·48
11	608	6·24±0·36	14	625	6·13±0·34
12	1210	6·43±0·19	15	1206	5·27±0·22
Mean		6·23±0·16	Mean		5·48±0·33
[^3H]-*serine*					
16	623	3·61±0·36	19	635	9·33±0·62
17	606	8·00±0·39	20	617	3·98±0·24
18	608	7·97±0·40	21	626	5·00±0·27
Mean		6·53±1·46	Mean		6·11±1·64
[^{35}S]-*sulphate*					
22	607	6·14±0·37	26	608	7·16±0·37
23	604	6·24±0·65	27	623	5·88±0·59
24	670	3·60±0·30	28	664	3·17±0·27
25	623	7·49±0·47	29	629	7·32±0·89
Mean		5·87±0·82	Mean		5·88±0·96

Discussion

The rate of incorporation of galactose, glucosamine, serine, and sulphate in the surface mucous cells of animals treated with carbenoxolone during three weeks did not vary much from that of controls. These results conflict with those of Shillingford *et al*,[6] who observed an increase in glucosamine incorporation and a decrease in galactose incorporation. In their experiments serine showed no variation. A difference in galactose and glucosamine incorporation would also conflict with what is known about the structure of epithelial glycoproteins, because these two sugars are present there as disaccharide units.[18] Their results were obtained by the method of incubating gastric fragments. We determined the biosynthetic effects of carbenoxolone in a vascular perfusion system. In this system (radioactive) precursors reach the gastric mucosal cells along the natural route, and are incorporated in a reproducible way.[11] In incubated cubes of mucosal tissue, however, only a varying number of cells from the superficial layer of the fragment incorporate (radioactive) precursors.[14] The dose of carbenoxolone in their[6] experiments was five times higher than used clinically.[2] We used the common experimental dose to make our results comparable with those of others.[3 4 9 13]

The ratio of the sugars in the glycoproteins of the treated stomachs was not different from that of controls, and did not vary from an earlier analysis.[16] The glycoproteins of the rat stomach contain a very small amount of sialic acid, both in treated and in control animals (galactosamine : sialic acids=1 : 0·05). An increase in the amount of sialic acid, as found after carbenoxolone treatment in human stomach,[7 8] was not observed in the rat.

The unchanged rate of precursor incorporation and the unchanged composition of the sugar moiety of glycoproteins point to an unchanged glycoprotein synthesis *per cell* after carbenoxolone-treatment. The amount of radioactively labelled glycoproteins in the stomach has not been lowered by secretion, as they do not reach the stage of secretory granules within the 30 minutes of incubation.

The total amount of glycoproteins *per stomach*, however, may have been increased by means of a rise in number of the mucous cells. To study a possible effect of carbenoxolone on the kinetics of the gastric epithelium we determined the [^{3}H]-thymidine incorporation *in vivo* and in perfusion experiments. From these autoradiographic data (Tables 1, 2, and 3) we may conclude that carbenoxolone treatment affects cell kinetics in the gastric pits. Compared with untreated animals, the number of labelled nuclei per fundic pit is increased by 38 to 76% after carbenoxolone. Such an increase in the number of cells synthetising DNA during the 30 minutes' perfusion time could be caused by: (1) an enlargement of the proliferative compartment in the pits, or (2) a relative lengthening of the DNA-synthetic period with respect to the other cell cycle phases of the cells belonging to the proliferative compartment.

We observed that, in treated rats, the labelled cells are found higher in the pits and lower in the glands. This might imply that the proliferative compartment is, indeed, larger in stomachs of treated animals than in controls. However, the exact enlargement of this compartment is difficult to determine in the rat stomach as the number of non-dividing cells is unknown and might also have increased. The number of cells in the proliferative compartment of treated animals must be 58% higher than that of controls in order to explain the increase of the labelling index in the carbenoxolone-treated animals of the perfusion experiments (Table 1). The increase in number should be 38–76% in the animals of the experiments *in vivo* (Table 2).

As the numbers of silver grains per nucleus (Table 3) point to an increase rather than to a decrease in the rate [^{3}H]-thymidine incorporation, a lengthening of the DNA-synthetic period cannot be absolute. So, if we do not assume an enlargement of the proliferative compartment by the treatment, other phases of the cell-cycle than the S-phase must be shortened. It can be calculated from the data in Table 1 that the results in the perfusion experiments are explained by assuming a 37% shorter total cycle time in the carbenoxolone-treated animals. A 28–43% shorter total cycle time explains the results of the *in vivo* experiments (Table 2).

It is clear that both models (and a mixture of them) lead to an increased production of young surface mucous cells. Lipkin[13] reported a slower turnover of [^{3}H]-thymidine incorporated in the gastric mucosa of the carbenoxolone-treated mice. This, together with the larger production of new cells, implies an increased number of non-dividing mucus-producing cells per pit, the more so since the number of pits per mm mucosa does not increase (Table 1).

Willems and coworkers[19] demonstrated a fourfold increase of the thymidine index in the dog between eight and 16 hours after food ingestion, followed by a rapid decrease from 15% to 5% between 16 and 24 hours. Hence, small differences in fasting time between 16 and 24 hours after a meal might result in large differences in thymidine index, suggesting a carbenoxolone effect. In our carbenoxolone-treated rats fasting for 24 hours did not result in a lower number of labelled nuclei than fasting for 20 hours. The carbenoxolone effect itself, however, was obvious after both fasting periods.

In conclusion, we may say that carbenoxolone has no significant effect on the synthesis of glycoproteins per cell. The increased number of young mucous cells, however, points to a trophic effect of carbenoxolone. This view is supported by histological observations[5]: around the borderline of human gastric ulcers treated with carbenoxolone more young regenerating surface mucous cells, containing more (neutral) glycoproteins, can be found than in non-treated ulcers. Because more cells are able to secrete more glycoprotein, the presence of more mucus in carbenoxolone-treated stomachs can be expected, and this, too, might add to its protective action.

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