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Early and Delayed Effects of Hydrocortisone and Onapristone on Intestinal Brush-border Enzymes and their Sialylation and on Thymus Growth in Suckling Rats

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The antigestagen–antiglucocorticoid onapristone (ZK 98.299) was tested on three glucocorticoid-sensitive systems after hydrocortisone (HC) administration to suckling male rats, by determining onapristone (ZK)-induced inhibition of HC-provoked (1) increase of activities of intestinal brush-border enzymes, (2) desialylation of brush-border components and (3) thymolysis. HC acetate (75 mg/kg body weight (b.w.)) was injected s.c. on postnatal days 9 and 10, and ZK (150 mg/kg b.w.) on days 9, 10 and 11. The animals were killed on day 12 for assessing the early effect, or on days 15–17 for determining the delayed effect of HC and ZK. In all three systems the glucocorticoid effects were antagonized by ZK. The most sensitive to HC were systems 1 and 3, which exhibited both the early and the delayed effects. The most sensitive to the counteraction of ZK against administered HC was system 1, where HC was antagonized in both its early and delayed effects, whereas only delayed antagonistic action against administered HC was found in system 2. ZK alone had an early inhibitory effect on the activities of several brush-border enzymes and produced an early increase in thymus weight, accompanied by an increased DNA-protein ratio. No delayed effects of ZK alone on the three systems were observed.

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INTRODUCTION

Onapristone (ZK 98.299 Schering AG, Berlin, Germany) (ZK) is an 11β-aryl-substituted 4,9-estradiene compound with high affinity for progesterone and glucocorticoid (GC) receptors [1]. It is structurally related to the compound mifepristone (RU-38486 Roussel-Uclaf, Romainville, France) (RU) and possesses similar antigestagenic, but reduced antiglucocorticoid activities [2, 3]. The antiglucocorticoid activity of steroid analogues *in vivo* is usually tested by their effect on corticoid-induced thymolysis in adult adrenalectomized male rats [3]. Another organ sensitive to GC action is the small-intestinal mucosa of infant rats. Administration of hydrocortisone (HC) to suckling rats causes a precocious induction of biosynthesis of intestinal brush-border enzymes, especially α-glycosidases [4], and a precocious decrease of membrane-bound sialic acid, resulting in the increase of pI of all investigated brush-border glycosidases and peptidases [5]. This corresponds to a decrease of sialyltransferase and an increase in membrane-bound fucose and fucosyltransferase activity in the membrane fraction of intestinal mucosa in the infant animals after cortisone injection [6, 7].

The regulation of thymus involution and of changes in the activities of intestinal brush-border α-glycosidases and glycosyltransferases during ontogenic development resembles the pattern observed after GC injection. In suckling rats the activities of intestinal brush-border hydrolases change dramatically

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Abbreviations: HC, hydrocortisone; GC, glucocorticoid; C, control(s); RU, RU-38486—mifepristone Roussel-Uclaf, Romainville, France; ZK, ZK 98.299—onapristone Schering AG, Berlin, Germany; IEF, isoelectric focusing; b.w., body weight; Gly-Pro-MNA, glycyl-L-proline (4-methoxy-2-naphthylamide); FBB, Fast Blue B.

during the period of weaning. α-Glycosidases, which are virtually absent or very low at birth, increase rapidly during the third and fourth postnatal weeks while lactase is very high in newborns and low in adult animals [8]. The pI of intestinal brush-border enzymes also stabilizes between days 20–30, when it reaches adult values [9]. The latter changes run parallel with the corresponding decrease of sialic acid and sialyltransferase and increase in fucose and fucosyltransferase [10, 7], although the desialylation and fucosylation of intestinal microvillar proteins are established somewhat later than mature levels of hydrolytic enzymes [11].

These systems offer an opportunity for investigating the mechanisms of action of a GC or of an antiglucocorticoid on various organs. Both ZK and RU antagonize the effects of a GC *in vivo* at the level of its receptor [12, 13]. Thus any effect of a GC, which is suppressed by these compounds, can be explained by a direct effect of the hormone via its own receptor. On the other hand, pharmacological doses of the applied GC may only modulate intrinsic timing mechanisms during ontogenic development, which themselves are not regulated by or dependent upon physiological levels of the secreted hormones. In such a case the antiglucocorticoid alone would not exhibit any effect on the physiological phenomena under investigation.

The effect of RU on HC induction of α-glycosidases in the small intestine of suckling rats was investigated by Galand [13]. It has been concluded that only until 14 days of postnatal life does the applied HC induce the activities of α-glycosidases by a direct interaction with its own receptor, while the spontaneous rise in these enzymes during the third postnatal week is not prevented by RU. This compound alone had no significant effect on enzyme activities in suckling rats.

The responsiveness of fucosyltransferase activity in rats to HC during suckling and weaning was examined by Biol *et al.* [14]. During suckling, RU counteracted the effect of HC, suppressing the HC-provoked increase of fucosyltransferase activity, but alone it had no effect. During the weaning period neither RU nor HC exhibited any influence on the developmental rise of fucosyltransferase.

The aim of our study was to assess the action of ZK alone and in combination with HC on the early postnatal activities of intestinal brush-border enzymes and on the sialylation of intestinal brush-border membranes and enzymes in suckling rats and to compare the early and delayed effects of these compounds. Under similar conditions we have also tested the effects of HC and ZK on thymus growth in suckling rats. No effects of ZK on the small-intestinal enzymes have so far been reported and no data on the effect of ZK or RU on the sialylation of the intestinal brush border are available. To the best of our knowledge our report is the first to deal with the effect of an antiglucocorticoid on thymus growth in suckling rats.

MATERIALS AND METHODS

Products

Onapristone (ZK 98.299) was a gift from Schering AG (Berlin, Germany); hydrocortisone acetate was from Sigma Chemical Company (St Louis, MO, U.S.A.); sucrose, soluble starch, lactose, glucose oxidase, peroxidase, crystalline papain and *N*-acetylneuraminic acid were from Koch-Light Laboratories (Colnbrook, England); glycyl-L-proline-4-nitroanilide was synthesized by Dr E. Kasafírek, Research Institute for Pharmacy and Biochemistry (Prague, Czech Republic); L-glutamic acid-5-(4-nitroanilide), glycylglycine, Fast Blue B, 4-(chloromercuri)benzoate, Lachema (Brno, Czech Republic); glycyl-L-proline-(4-methoxy-2-naphthylamide) were from Bachem Feinchemikalien AG (Bubendorf, Switzerland); neuraminidase from *Clostridium perfringens* was from Boehringer (Mannheim, Germany); AgaroseIEF, Pharmalyte™ 3–10, Isoelectric Focusing Calibration Kits were from Pharmacia LKB Biotechnology (Uppsala, Sweden); Coomassie Brilliant Blue R and 2-thiobarbituric acid were from Serva, Feinbiochemica (Heidelberg, Germany); and bovine serum albumin was from Mann Research Laboratories (New York, NY, U.S.A.). All chemicals were reagent grade.

Animals

Infant 9-day-old male rats of the Wistar strain were used for HC and ZK treatment. The suckling pups remained with their mothers in normal cages. The dams had unrestricted access to food and water.

Administration of HC and ZK. Two sets of littermates of 9-day-old male rats were used, each consisting of four groups. Litters were randomly divided into individual groups, each consisting of 6–8 animals. The first set was tested for the early effects of the injected compounds and the animals were killed on postnatal day 12. The second set served for the evaluation of the delayed effects and the animals were killed on postnatal day 15, 16 or 17. In both sets one group was given s.c. injections of saline suspension of HC acetate in two doses, 1 mg on day 9 and 0.5 mg on day 10 (total dose 75 mg/kg body weight (b.w.)); the second group received a saline suspension of ZK in three doses, 1 mg each, on postnatal days 9, 10 and 11 (total dose 150 mg/kg b.w.); the third group was given both compounds s.c. (HC + ZK) and the fourth group, which served as control (C), received injections of saline only.

Preparation of mucosal homogenates. The animals were killed by decapitation. The entire small intestine was removed and rinsed with cold 0.9% (w/v) NaCl. All the procedures were performed at 4°C. Only the proximal third of the small intestine (jejunum) was used, distal parts were discarded. The intestinal mucosa was scraped off and 10 mg of mucosa from each rat was homogenized with 150 μl of 50 mM potassium

phosphate buffer pH 7.0 at 4°C. The pellet was resuspended in 150 µl of the same buffer for the assay of enzyme activities and protein content.

Preparation of brush-border fraction and solubilization of brush-border proteins. The brush-border fraction was prepared from mixed jejunal homogenates of 5–8 rats from the same group by the method of Schmitz *et al.* [15]. In this fraction enzyme activities, bound sialic acid and protein content were assayed. Part of this fraction was solubilized for analytical isoelectric focusing (IEF) by papain treatment [9].

Thymus weight. After the animals were killed by decapitation, the thymus was extirpated, its wet weight was compared with the body weight of the intact animal and expressed as the relative weight in g/kg b.w.

Methods

Enzyme activities. Glycosidases were assayed by the method of Dahlqvist [16], with soluble starch 12 mg/ml for glucoamylase (EC 3.2.1.20 α-D-glucoside glucohydrolase) and 50 mM sucrose for sucrase (EC 3.2.1.48 sucrose α-D-glucohydrolase), incubated at pH 5.9, and the glucose released was measured with the Tris–glucose oxidase peroxidase reagent [17]. Lactase (3.2.1.108 lactose galactohydrolase) was determined by the same method with 28 mM lactose at pH 5.5 in the presence of 4-(chloromercuri)benzoate as in [18]. Dipeptidyl peptidase IV (EC 3.4.14.5 dipeptidylpeptide hydrolase) was determined with 1.4 mM glycyl-L-proline-4-nitroanilide at pH 8.0 and the released chromogen measured at 405 nm [19]. γ-Glutamyltransferase (EC 2.3.2.2(5-glutamyl)-peptide: amino acid 5-glutamyltransferase) was assayed with 7 mM L-glutamic acid 5-(4-nitroanilide) in 0.1 M glycyl–glycine buffer at pH 8.2 in the presence of 0.1 M NaCl as the activator and the released chromogen measured at 410 nm [20]. All enzyme activities were expressed as nkat/mg protein.

Sialic acid. Sialic acid bound in the brush-border fraction was determined after hydrolysis with 0.05 M H_2SO_4 at 80°C for 60 min by the thiobarbituric acid assay as in [21].

Protein content. Protein content in mucosal homogenates, in the intestinal brush borders and in thymus homogenates was determined by the method of Lowry [22]. Bovine serum albumin was used as standard.

Analytical isoelectric focusing (IEF). Analytical IEF of the papain-solubilized brush-border proteins was performed as in [9] on a thin AgaroseIEF layer. Isoelectric points of the separated fractions were determined by comparison with the IEF Calibration Kit.

Detection of proteins and dipeptidyl peptidase IV in the agarose gel after IEF. Detection methods were the same as in [9] using Coomassie Brilliant Blue solution for proteins and simultaneous azo-coupling reaction with 0.5 mM glycyl-L-proline-(4-methoxy-2-naphthylamide) and Fast Blue B at pH 7.2 for dipeptidyl peptidase IV.

Treatment of solubilized brush-border proteins with neuraminidase [9]. In order to demonstrate the presence of sialic acid bound to dipeptidyl peptidase IV, part of the papain-solubilized brush-border fraction was incubated before IEF separation with neuraminidase from *Clostridium perfringens* and compared after IEF separation on the focusogram with the pI of dipeptidyl peptidase IV in the untreated samples.

Determination of DNA in thymus. The extirpated thymus was homogenized in 10% trichloroacetic acid (w/v) (0.2 g wet tissue per 1 ml trichloroacetic acid solution), following the method of Schneider [23]. After several washings with ethanol and ethanol/ether the nucleic acids were extracted by 10% (w/v) trichloroacetic acid for 15 min at 90°C. After cooling the DNA was determined in a diphenylamine reaction by the method of Burton [24] at 595 nm.

Statistical evaluation

The results are expressed as means ± SEM. Statistical significance was calculated by Student's *t*-test and *F*-test.

RESULTS

Small-intestinal enzymes

HC induced the precocious appearance of sucrase and increased the activities of glucoamylase, lactase and γ-glutamyltransferase in the jejunal mucosa of 12-day-old rats. The increase in dipeptidyl peptidase IV was nonsignificant. ZK suppressed this effect of applied HC on glucoamylase, lactase and γ-glutamyltransferase, for sucrase the suppression was nonsignificant (Fig. 1).

An important finding was the decrease in the activities of glucoamylase, lactase and dipeptidyl peptidase IV after ZK alone (Fig. 1). The effect on sucrase could not be evaluated at this developmental stage due to the virtual absence of this enzyme in the control group of 12-day-old animals.

In order to follow the delayed effect of applied HC and ZK two typical brush-border markers, sucrase and glucoamylase, were determined in the isolated brush borders of 16–17-day-old rats (Table 1). Each value corresponds to the specific activity of the particular enzyme in the brush-border fraction prepared from 6–8 rats of the same experimental group. The increase in sucrase and glucoamylase induced by HC was much more evident than in the early effect and so was the inhibitory action of ZK.

ZK itself had no delayed suppressing effect on sucrase and glucoamylase (Table 1).

Sialic acid in the intestinal brush border

Neither HC nor ZK exhibited any significant early effect on the relative content (nmol/mg protein) of sialic

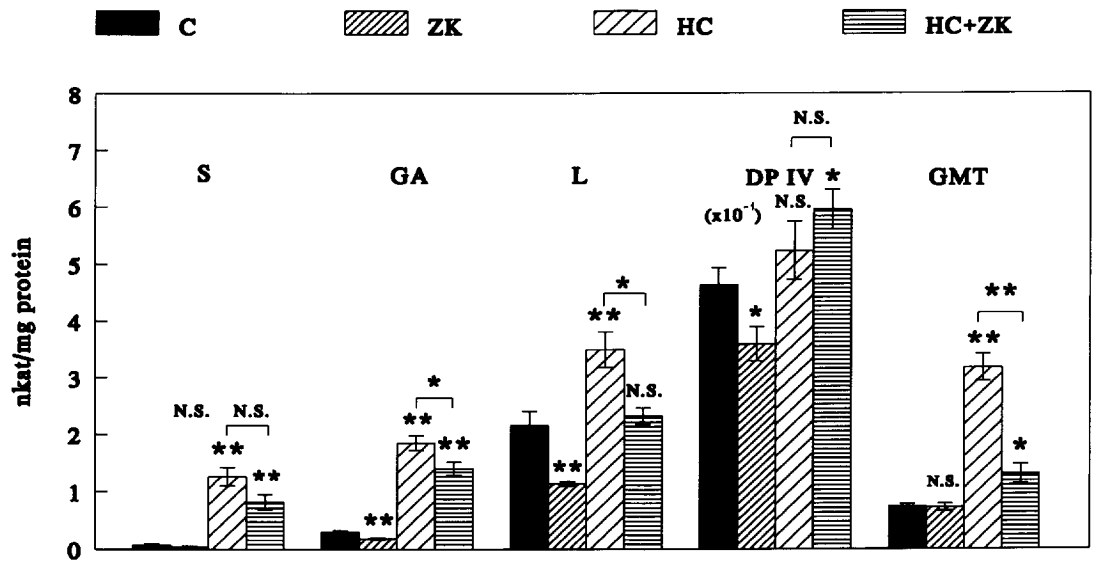

Fig. 1. Specific activities (nkat/mg protein) of enzymes in the jejunal mucosa of 12-day-old male rats as the test for the early effects of hydrocortisone acetate (HC) and onapristone (ZK). S, sucrase; GA, glucoamylase; L, lactase; DP IV, dipeptidyl peptidase IV; GMT, γ-glutamyltransferase. 4 groups of animals were tested, each consisting of 6–8 rats. The bars represent means ± SEM. Statistical significance between the individual experimental groups vs controls (C) or between the denoted groups: *$P < 0.05$; **$P < 0.01$. N.S. not significant. (For dipeptidyl peptidase IV the values are to be multiplied by 10^{-1}.)

acid bound in the brush border (Fig. 2). On the other hand, in 15-day-old rats the suppressing effect of HC is convincing (Fig. 2), and it was also supported by IEF of brush-border enzymes reported in our earlier work [5].

Our previous experiments [25] demonstrated that the primary target of HC in its induction of the premature desialylation (or repression of sialylation) of brush-border enzymes is the differentiating cells of Lieberkuhn crypts. It takes several days before these reprogrammed cells move to the tips of the villi. This explains the lack of the early effect of HC in 12-day-old animals (Fig. 2) on desialylation of the whole brush borders originating both from the crypt and the villus cells. A more sensitive marker of desialylation is the pI shift of typical brush-border enzymes to more basic values [9], where dipeptidyl peptidase IV exhibits larger differences during ontogenic development and after HC than other enzymes, e.g. α-glycosidases. We have therefore chosen this enzyme in the solubilized brush-border fraction of 16- and 17-day-old rats to demonstrate possible differences in pI after HC and ZK (Fig. 3). The dipeptidyl peptidase IV band on the IEF-zymograms from the controls corresponds to a pI of 4.2–5.0; HC treatment causes a pI shift to 4.5–5.6 which is partly inhibited by ZK. The content of sialic acid in the brush borders of 16- and 17-day-old rats was 92 nmol/mg protein in controls, 66.4 nmol/mg protein in HC-treated and 77 nmol/mg protein in HC + ZK-treated animals.

In the dose used, ZK alone had no visible effect on the sialylation of the intestinal brush border (Figs 2

Table 1. Specific activities (nkat/mg protein) of α-glycosidases in jejunal brush borders of 16- and 17-day-old male rats, demonstrating the delayed effects of HC and ZK. The brush borders were prepared from the mucosa of 6–8 animals in a group

Group	Age (days)	Sucrase (nkat/mg protein)	Inhibition of HC by ZK	Glucoamylase (nkat/mg protein)	Inhibition of HC by ZK
Controls	(16)	0.128		0.840	
Controls	(17)	0.224		0.620	
HC	(16)	10.410		4.860	
HC	(17)	9.500		2.210	
ZK	(16)	0.202		1.010	
HC + ZK	(16)	1.084	90%	1.080	94%
HC + ZK	(17)	1.040	91%	1.060	72%

% of inhibition was calculated from the difference between HC and control groups, which was taken as 100% of the HC effect.

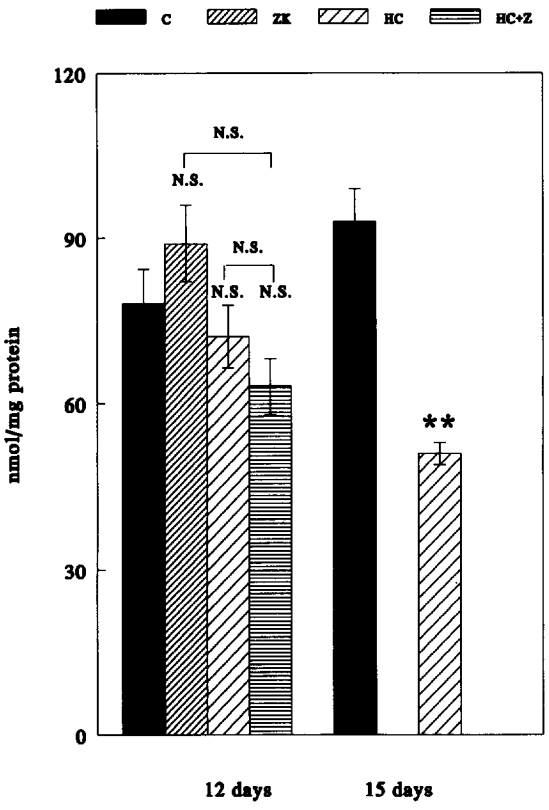

Fig. 2. Sialic acid (nmol/mg protein) bound in the small-intestinal brush border of 12- or 15-day-old rats. Each bar is a mean of three brush-border preparations ± SEM. Each preparation was obtained from homogenates of jejunal mucosa from 5–8 rats in each of the control (C) and experimental groups. Groups of 12-day-old animals represent the early effect of HC and ZK, 15-day-old rats represent the delayed effect of HC. Statistical significance as in Fig. 1.

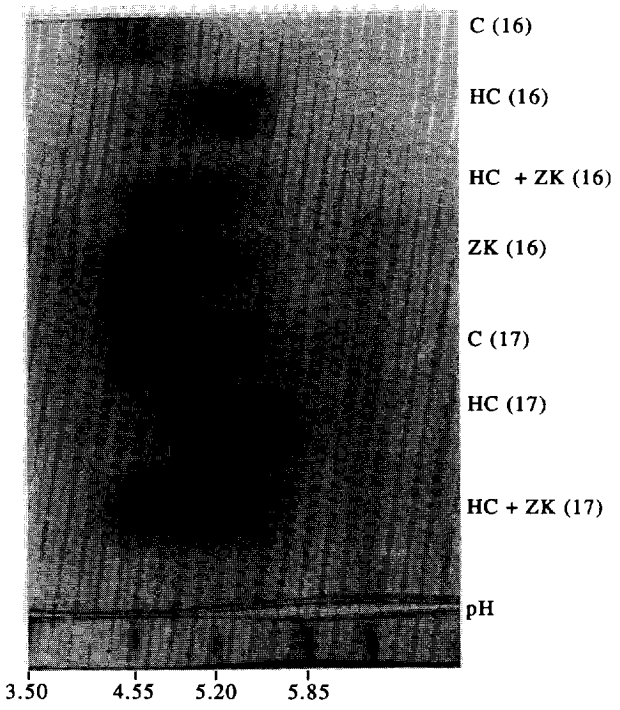

Fig. 3. Analytical isoelectric focusing (IEF) of dipeptidyl peptidase IV from jejunal brush border in a thin layer of AgaroseIEF gel, demonstrating the delayed effects of HC and ZK. Part of the whole pH gradient (3–10) is shown. Experimental conditions (C, HC, ZK) as in Table 1, age given in brackets. After separation the upper part of the gel was stained for enzyme activity. The zymogram bands were compared with pI values of known proteins in the calibration kit, which were run in parallel, fixed and stained for proteins (bottom). The pI values of the known standards indicated below.

and 3). In control experiments a virtually complete desialylation of dipeptidyl peptidase IV was found on the focusogram after a previous treatment of the samples of the papain-solubilized brush-borders with neuraminidase, the enzyme band corresponding to pI 5.4–5.8, which is identical to the zymogram pattern of adult animals [5, 9].

Thymus mass

A significant decrease in the relative thymus weight was induced by HC as both the early and the delayed action (in 12- and 16-day-old rats), the thymus mass dropping to less than 50% of that in the control groups (Fig. 4). No inhibition of HC-induced thymolysis by ZK was observed at the early date and only a partial, nonsignificant inhibition in the delayed effect.

An important finding is the 50% increase in the relative thymus weight early after ZK alone in 12-day-old rats (Fig. 4), which corresponds to a 2-fold increase in the DNA/protein ratio (the right-hand part of Fig. 4). No delayed effect of ZK alone on thymus weight was observed in 16-day-old rats (Fig. 4).

DISCUSSION

Three systems were employed for investigating the effect of HC and ZK in infant male rats: small-intestinal brush-border enzymes, intestinal brush-border sialylation and thymus mass.

Like Galand [13], we used small intestine of suckling rats in our experiments with ZK; however, the total doses of HC and ZK administered were 50% higher than the doses of HC and RU used in [13] and only male animals were tested for the action of ZK. Moreover, the early and delayed effects of the compounds were compared. In addition to α-glucosidases investigated in [13], we have also included lactase, dipeptidyl peptidase IV and γ-glutamyltransferase in our determinations of intestinal enzymes. In contrast to [13], where the whole small-intestinal homogenate was used as the starting material, we worked only with jejunal mucosa (Fig. 1) or brush-border fraction (Table 1).

The early HC-induced increase in the activities of the small-intestinal enzymes in 12-day-old rats

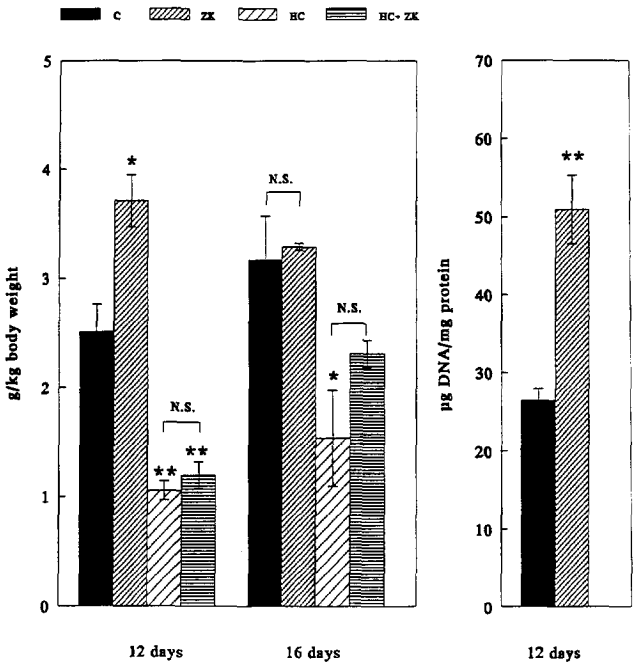

Fig. 4. Relative thymus weight (g/kg body weight) and DNA/protein (μg/mg) ratio (the right-hand part of the figure) in thymus homogenates. Experimental conditions as in Fig. 1. Bars represent means ± SEM. 12-day-old rats correspond to the early effects and 16-day-old rats correspond to the delayed effects of HC and ZK. C = controls. Statistical significance as in Fig. 1.

was suppressed by ZK, although not significantly for sucrase (Fig. 1).

The situation was different with the delayed effects of HC and ZK in 16- and 17-day-old rats (Table 1). ZK caused a 90% inhibition of HC-induction of sucrase and 72–94% inhibition of glucoamylase, apparently due to the reprogramming of the differentiating cells in the Lieberkuhn crypts, which are the primary targets of HC [25–27]. The delayed effects of HC and ZK in 16- or 17-day-old rats result from the movement of these reprogrammed (after HC) or not reprogrammed (after HC + ZK) cells to the tips of the villi, which takes approx. 4 days, and perhaps also from the continuous action of these drugs as the cells migrate along the villi [27].

An interesting new finding was the early suppression of the activities of intestinal enzymes by ZK alone in 12-day-old animals (Fig. 1) representing 39% inhibition of glucoamylase, 47% inhibition of lactase and 22% inhibition of dipeptidyl peptidase IV. Inhibition of sucrase could not be evaluated and no significant inhibition was found for γ-glutamyltransferase. Thus, for the typical markers of the intestinal brush-border fraction, the suppressing effect of ZK seems to occur at the level of receptors for the endogenously secreted GC, which may be regarded as the physiological trigger at this developmental stage. No delayed effect for brush-border sucrase and glucoamylase was found in 16-day-old rats (Table 1).

Whereas the activity of GC-receptors in the small intestine of infant rats reaches its maximum between the postnatal days 5–10 [28, 29], the corticosterone levels in plasma are at their maximum between postnatal days 15–19 [30]. The maximum sucrase activity induced by a single injection of HC in suckling rats is reached within 4–7 days [31]. All these factors should be taken into account when trying to explain the early and delayed effects of HC and ZK on intestinal brush-border enzymes.

Sialylation of the intestinal brush border represents another system sensitive to HC [5]. The inhibitory effect of an antiglucocorticoid on the HC-induced desialylation of brush-border components is reported in this paper for the first time (Fig. 3).

The role of sialic acids bound to glycoproteins and glycolipids of cell membranes is being intensively investigated in many laboratories. Sialic acids are mostly responsible for the net negative charge of plasma membranes, and may influence many vital functions such as the binding of ligands to receptors, bacterial and virus infection, effect of bacterial toxins, membrane transport, cell adhesion, differentiation and cell–cell-recognition. They may serve also as biological masks [32] and onco-developmental antigens [33].

Unlike for brush-border hydrolases, where ZK had an early suppressing effect (Fig. 1), we could not demonstrate any significant increase in sialylation in the whole brush-border fraction after ZK (Fig. 2). A detailed examination will be required to confirm or exclude the role of endogenous GC in the physiological regulation and suppression of the sialylation process in the early postnatal period, especially in relation to the transcription of α2,6-sialyltransferase [25, 34–37].

Similarly, as for the activities of α-glycosidases, ZK alone had no delayed effect on the pI value of dipeptidyl peptidase IV in 16-day-old rats (Fig. 3). Biol [14] could not demonstrate any effect of HC and RU on fucosyltransferase in the membrane fraction of the small-intestinal mucosa of weaned rats and no decrease of fucosyltransferase after RU application in suckling rats.

HC application was found to cause both early and delayed thymus mass reductions by more than 50% (Fig. 4). The antiglucocorticoid action of ZK in this classical system, however, was very weak compared with its effect on brush-border hydrolases, only a nonsignificant ZK antagonism against delayed HC-induced thymolysis being found on the 16th postnatal day. This accounted for about 53% inhibition of the HC-effect (where 100% HC-effect is given by the difference between the thymus weight of HC-treated and control groups). In contrast to [3] we did not work with adrenalectomized adult male rats, but with nonadrenalectomized male sucklings.

A very important new finding was the early ZK-induced 50% increase in the relative thymus weight accompanied by the corresponding 2-fold increase in

the DNA/protein ratio on postnatal day 12 (Fig. 4). Analogously to the suppression of intestinal enzymes, the early effect of ZK again disappeared within 5 days after the administration of ZK was stopped and there was no delayed effect of ZK on the relative thymus mass in 16-day-old rats. The early effect of ZK again demonstrates the role of endogenously secreted GC as the physiological trigger of thymus involution during the early postnatal development.

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