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Cortisol decreases the concentration of translatable type-I procollagen mRNA species in the developing chick-embryo calvaria

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The calvarial mRNA species of chick embryos were translated in the rabbit reticulocyte-lysate cell-free translation system. The amount of procollagen type-I mRNA species was determined by digestion with bacterial collagenase and by fluorography of the cell-free translation products. Administration of cortisol resulted in a specific decrease in the cellular concentration of translatable procollagen type-I mRNA species in the calvaria of developing chick embryos. There was a lag period of up to 12h before the response, which was dose-dependent. The data suggest that the decrease in amounts of procollagen mRNA species is the main reason for the lower amount of tissue collagen after topical or systemic administration of glucocorticoids, although other factors may contribute to the response.

Topical or systemic administration of pharmacological doses of glucocorticoids elicits several connective-tissue manifestations, such as striae, thin skin and impaired wound healing, and these steroids also inhibit normal growth and developmental processes. All these phenomena can, at least partly, be explained by an effect of the steroid on collagen metabolism, leading to a lower amount of tissue collagen.

Several mechanisms for the decrease in tissue collagen by glucocorticoids have been proposed. The most widely accepted theory is that the glucocorticoids exert an anti-anabolic effect on fibroblasts (Kivirikko *et al.*, 1965; Manthorpe *et al.*, 1974), which can be either a generalized one (Thompson & Lippman, 1974; Kruse *et al.*, 1978) or selective in decreasing collagen synthesis (Oikarinen, 1977*a*; McNelis & Cutroneo, 1978; Ponec *et al.*, 1979). The basis for the generalized depression in protein synthesis may be a direct result of the hormone on the transcriptional or translational activities of the cell (Thompson & Lippman, 1974) or an indirect result of substrate depletion secondary to decreased transport of amino acids (Murota *et al.*, 1976) or glucose (Hallahan *et al.*, 1973). One mechanism proposed that would lead to a selective decrease in collagen synthesis is that the glucocorticoids may decrease the activity of some rate-limiting enzyme involved in post-translational reactions in procollagen biosynthesis, and there is evidence for depression of the synthesis of prolyl 4-hydroxylase and a decrease in its activity (Cutroneo *et al.*, 1975; Oikarinen, 1977*b*; Risteli, 1977) and for a decrease in the activities of other intracellular enzymes of collagen biosynthesis (Oikarinen, 1977*b*; Risteli, 1977). These changes in enzyme activities may, however, be contemporaneous with a decreased rate of procollagen polypeptide-chain synthesis, and may in themselves be of no significance in decreasing collagen synthesis (McNelis & Cutroneo, 1978).

In addition to their inhibitory effects on protein synthesis, the glucocorticoids also inhibit fibroblast proliferation (Kruse *et al.*, 1978; Ponec *et al.*, 1979), which contributes to a decrease in the amount of tissue collagen. Under certain conditions, however, stimulation of collagen synthesis has been observed after the administration of glucocorticoids (Saarni & Tammi, 1978), and thus inhibition of collagen synthesis may prove to be a manifestation of the toxic effects of pharmacological doses of these steroids.

Methods have been developed during the last few years for measuring the amounts of translatable mRNA species (Monson & Goodman, 1978; Rowe *et al.*, 1978; Cheah *et al.*, 1979), offering for the first time an opportunity to quantify the cellular concentration of procollagen mRNA species after glucocorticoid administration. We have thus measured here the effect of pharmacological doses of cortisol on the concentration of procollagen mRNA species in developing chick embryos by extracting

Abbreviation used: SDS, sodium dodecyl sulphate.

the total RNA from calvaria and assaying the procollagen-synthesizing activity in the cell-free translation system derived from the rabbit reticulocyte lysate. It is observed that the administration of cortisol results in a decrease in the cellular concentration of translatable procollagen type-I mRNA species. There is a lag period of up to 12h before the response.

Experimental

Materials

Fertilized eggs of White Leghorn chickens were obtained from Siipikarjanhoitajien liitto r.y. (Hämeenlinna, Finland) and incubated in a moist atmosphere at 37°C until used.

L-[2,3,4,5-^{3}H]Proline (117Ci/mmol) and rabbit reticulocyte lysate (nuclease-treated, mRNA-dependent) were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). Purified bacterial collagenase and guanidine hydrochloride were from Sigma (London) Chemical Co. (Kingston-upon-Thames, Surrey, U.K.) and cortisol acetate was from Lääke Oy (Turku, Finland). Proteinase K was obtained from E. Merck (Darmstadt, Germany).

Treatment of chick embryos

Cortisol acetate dissolved in 0.9% (w/v) NaCl to a concentration of 1mg/ml was pipetted on to the chorioallantoic membranes of fertilized eggs. The days of administration and amounts of cortisol acetate pipetted are indicated in the legends to the Figures and Tables. Control eggs received the same amount of 0.9% (w/v) NaCl. The embryos (five for each separate assay) were decapitated after the incubation times indicated in the legends to the Figures and Tables. The calvaria were removed immediately and frozen in liquid nitrogen.

Isolation of total RNA for cell-free translation assay

RNA was isolated by a combination and slight modification of the methods of Rowe *et al.* (1978) and Monson & Goodman (1978). Frozen calvaria were powdered in a steel mortar cooled in liquid nitrogen and the powder was homogenized in 1% (w/v) SDS/5mM-EDTA/10mM-Tris/HCl (pH7.5) containing 65μg of proteinase K/ml with an Ultra-Turrax homogenizer, further disruption being obtained with a loose- and a tight-fitting Dounce homogenizer. The homogenate was incubated for 1h at 40°C. Total nucleic acid was isolated by two consecutive extractions with 1vol. of phenol/chloroform/3-methylbutan-1-ol (25:25:1, by vol.), followed by precipitation with 66% (v/v) ethanol/0.1M-NaCl. RNA was purified from the total nucleic acid by extraction with 6M-guanidine hydrochloride followed by precipitation with 66% (v/v) ethanol/0.1M-NaCl. Further elimination of contaminating DNA was obtained by washing with 3M-sodium acetate (pH6) and shearing with a glass rod. The RNA isolated was then washed with 66% (v/v) ethanol/0.1M-NaCl, freeze-dried and dissolved in water at approx. 1mg/ml. About 0.8mg of RNA was obtained per g of tissue. Portions were stored at −70°C and used for the translation assay of protein-synthetic activity.

Cell-free translation assay for procollagen-synthesis activity

Various concentrations of total RNA and 8μl of nuclease-treated reticulocyte lysate (Pelham & Jackson, 1976), in a total volume of 11μl, were incubated for 90min at 30°C in the presence of freeze-dried [^{3}H]proline, as indicated in the legends to the Figures and Tables. After incubation, two 4μl samples were taken from each reaction mixture and assayed in a total volume of 100μl for bacterial-collagenase-resistant material and for the total incorporation of radioactivity under conditions described by Peterkofsky & Diegelmann (1971). The samples were incubated for 1h at 37°C. Subsequently, 200μl of 1M-NaOH, 100μl of 40mM-proline and 50μl of 30% (v/v) H_2O_2 were added. After an additional incubation for 15min at 37°C, the samples were precipitated with 10% (w/v) trichloroacetic acid on ice and filtered through a 0.45μm Millipore filter. The dried filters were dissolved in 5ml of 2-methoxyethanol, and counted for radioactivity in a Wallac liquid-scintillation spectrometer with an efficiency of 25% and a background of 60d.p.m., with 10ml of 2,5-diphenyloxazole (6g/litre, in toluene) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (20mg/litre) as scintillant.

Determination of RNA and DNA contents

Calvaria were weighed and powdered in a steel mortar cooled in liquid nitrogen. Further disruption was achieved in 6% (w/v) trichloroacetic acid with an Ultra-Turrax homogenizer. DNA and RNA were isolated from the homogenate by the Schmidt & Thannhäuser (1945) extraction procedure. The DNA content was determined by the indole procedure (Hubbard *et al.*, 1970), with calf thymus DNA as the standard, and the RNA content was measured by the orcinol method (Schneider, 1957).

Additional methods

The cell-free translation products were also analysed electrophoretically in SDS/polyacrylamide slab gels (5% acrylamide) by the method of Laemmli (1970). Fluorography of the gels was performed as described by Bonner & Laskey (1974) and Laskey & Mills (1975). The films were scanned with a K-495000 densitometer.

Results

Effect of cortisol administration on the concentration of translatable procollagen mRNA species

The response was studied after the application of 50μg of the steroid to the chorioallantoic membranes of fertilized eggs on 3 consecutive days. The embryos were decapitated 12h after the last treatment, when they were 18 days old. The protein-synthesizing activity was studied by incubating samples of calvarial RNA in the reticulocyte-lysate cell-free translation system in the presence of [^{3}H]proline. The incorporation of radioactivity into trichloroacetic acid-precipitable proteins and bacterial-collagenase-resistant material was determined from the linear part of the RNA concentration curve (Fig. 1). The amount of procollagenous material synthesized in the cell-free translation system has been shown to correlate with the amount of procollagen mRNA species existing in an RNA preparation from chick calvaria (Moen *et al.*, 1979; Kream *et al.*, 1980). The amount of bacterial-collagenase-resistant material may have some correlation with the concentration of mRNA species for non-collagenous proteins, but no direct conclusions can be drawn because of heterogeneity of the pool and the present lack of knowledge about possible translational control.

The amount of bacterial-collagenase-sensitive material synthesized per unit of calvarial RNA assayed in the cell-free translation system was about 50% lower with the RNA obtained from the cortisol-treated embryos than with that from the untreated controls (Fig. 1), whereas the synthesis of bacterial-collagenase-resistant material remained almost unchanged. When the results are expressed per unit of DNA, there is an even greater decrease in the concentration of translatable procollagen mRNA species, because of the decrease in the RNA/DNA ratio after the administration of cortisol acetate (Table 1).

Polyacrylamide-gel electrophoresis of the cell-free translation products revealed two major bacterial-collagenase-sensitive polypeptide chains, which are designated prepro-α1(I) and prepro-α2(I). The mobility of the chains was slightly greater than that of the standard pro-α1(I) and pro-α2(I) chains. The amounts of prepro-α1(I) and prepro-α2(I) chains synthesized were estimated from the areas of the relevant peaks in the scanning fluorograms of the cell-free translation products. The incorporation of radioactive proline into the prepro-α1(I) and prepro-α2(I) chains decreased by 32% and 38% respectively after the administration of cortisol, as compared with the untreated controls (Fig. 2).

Time course of the response

In order to determine the time course of the response, 50μg of cortisol acetate was pipetted on

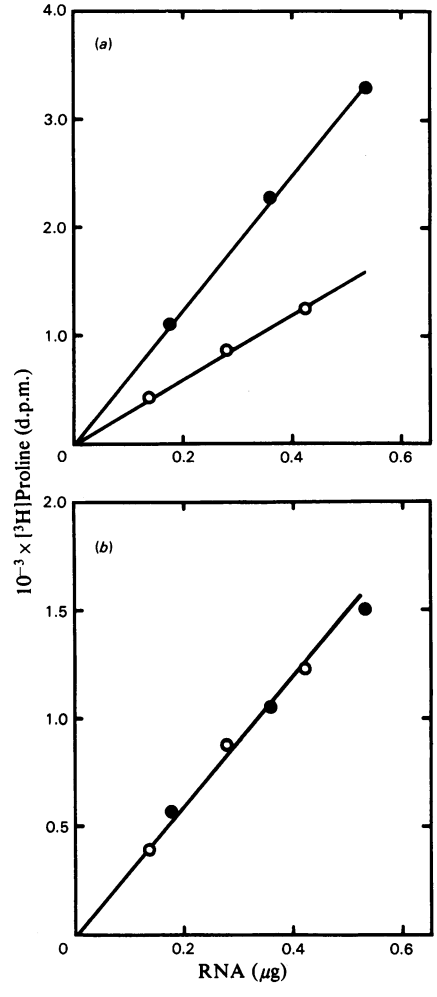

Fig. 1. *Effect of cortisol acetate treatment on the mRNA in the reticulocyte-lysate cell-free translation system with calvarial RNA from chick embryos*

Cortisol acetate (50μg) was pipetted daily on to the chorioallantoic membranes of fertilized eggs on days 15, 16 and 17 of embryonic development. The embryos were dissected 12h after the last treatment and weighed. The calvaria were removed immediately and frozen in liquid nitrogen. Total RNA was isolated from control (●) and cortisol-treated embryos (○), and translated in the rabbit reticulocyte-lysate cell-free translation system in the presence of 3μCi of freeze-dried [^{3}H]proline. The total incorporation of radioactivity and radioactivity in bacterial-collagenase-resistant material were assayed by the method of Peterkofsky & Diegelmann (1971). The dependency of the amount of radioactivity incorporated into the bacterial-collagenase-sensitive (*a*) and -resistant material (*b*) on the RNA concentration is shown. For further details of RNA isolation and the translation assay, see the Experimental section. The RNA concentration was estimated by u.v. absorbance at 260nm, 20 A_{260} units corresponding to 1mg of RNA/ml.

Table 1. *Effect of administration of cortisol acetate on the concentration of translatable procollagen and non-procollagen mRNA species in calvaria of developing chick embryos, as determined by bacterial-collagenase digestion of the cell-free translation products*

Experimental conditions were as in Fig. 1. The radioactivity per unit of DNA in the prepro-α chains is estimated according to the principles described by Breul *et al.* (1980), by using the formula $(S+yS)r$, where S is the radioactivity in the bacterial-collagenase-sensitive material per unit of RNA, r is the RNA/DNA ratio estimated as described in the Experimental section, and y is the mean of the proportions of imino acids present in the bacterial-collagenase-resistant parts of the prepro-α chains. The coefficient y (0.13) is calculated by supposing that prepro-α1(I) and prepro-α2(I) chains are synthesized in the ratio 2:1, that the collagenous α chain contains 81% of the imino acids in prepro-α1(I) and 98% in prepro-α2(I) chains, and that the collagenous region of the aminopeptide is not susceptible to bacterial collagenase. The radioactivity in non-procollagenous proteins is estimated by using the formula $(R-yS)r$, where R is the radioactivity in the bacterial-collagenase-resistant material per unit of RNA.

	Prepro-α chains (d.p.m./μg of DNA)	Difference (%)	Non-procollagenous proteins (d.p.m./μg of DNA)	Difference (%)
Control	15680	—	4773	—
Cortisol-treated	6233	−60	4403	−8

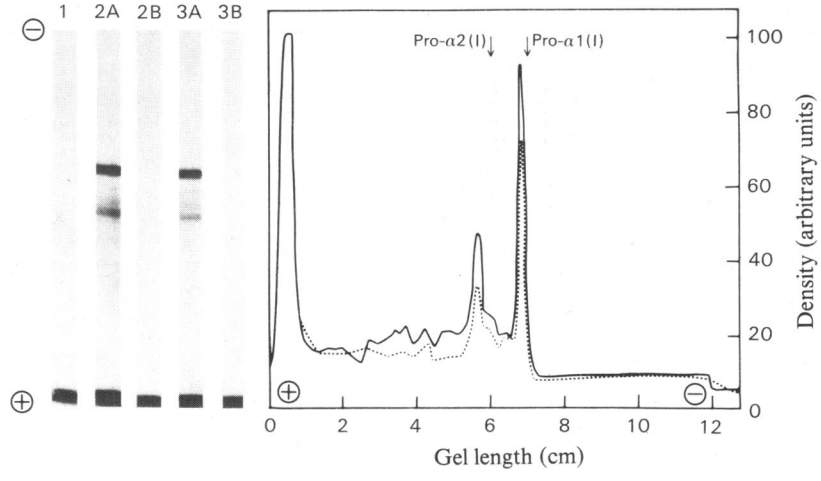

Fig. 2. *Fluorography of proteins synthesized by a reticulocyte lysate with calvarial RNA obtained from controls and cortisol-treated embryos*

Experimental conditions were as in Fig. 1, and the RNA species were isolated and translated as described in Fig. 1. The samples were subjected to electrophoresis in SDS/polyacrylamide slab gels (5% acrylamide) after reduction with dithiothreitol. (1) Translation products in the absence of exogenous RNA, (2) translation products with 0.3 μg of calvarial RNA from control embryos and (3) translation products with 0.3 μg of calvarial RNA from cortisol-treated embryos. A represents the total translation products and B the same after digestion with bacterial collagenase. The density profiles of the prepro-α chains from gels 2A (control, ——) and 3A (cortisol-treated, ----) are shown. The arrows indicate the positions of the pro-α1(I) and pro-α2(I) standards. The direction of electrophoresis is from the cathode to the anode.

day 15 of embryonic development. The embryos were decapitated after an exposure period of variable length, and the concentration of procollagen-mRNA species was measured in the cell-free translation system. There was a lag of some hours before the concentration of translatable procollagen-mRNA species began to decrease, in comparison with the controls of the same age (Fig. 3). The decrease was evident whether the amount of procollagen mRNA was estimated by digestion with bacterial collagenase or from the fluorograms, the latter method suggesting a slightly greater decrease than the former. This difference might be due to the synthesis of incomplete bacterial-collagenase-sensitive polypeptide chains. Since a decrease was again observed in the RNA/DNA ratio, the changes were even greater when expressed per unit of DNA.

Effect of the dose of cortisol acetate

The dose-dependence of the response was studied after an exposure period of 24 h. The decrease in the amount of translatable procollagen mRNA species,

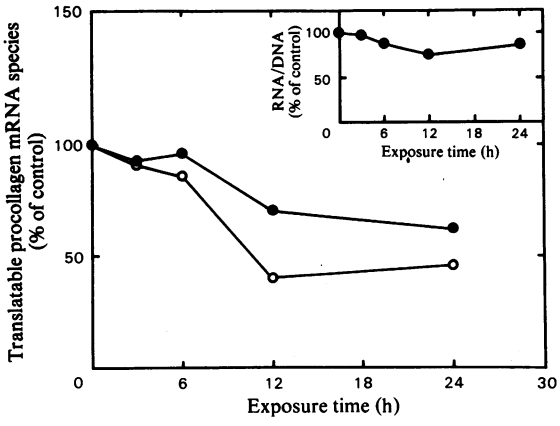

Fig. 3. *Time course of the changes in the amount of translatable procollagen mRNA species after the administration of cortisol acetate*

Cortisol acetate (50 μg) was pipetted on to the choriollantoic membranes of fertilized eggs on day 15 of embryonic development. The calvarial RNA species were isolated after the exposure times indicated in the Figure and translated in the cell-free translation system as in Fig. 1. The amount of prepro-α chains synthesized was quantified as described in Figs. 1 and 2. The results are expressed as percentages of the control value for the same age, as determined by digestion with bacterial collagenase (●) or fluorography (○). The insert shows the changes in the RNA/DNA ratio, determined as described in the Experimental section.

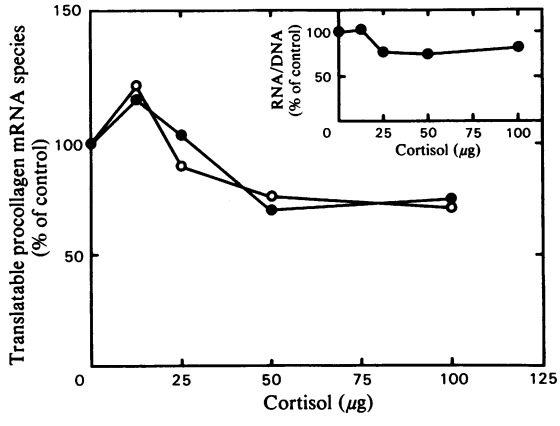

Fig. 4. *Effect of administration of various amounts of cortisol acetate on the response in the concentration of translatable procollagen mRNA species*

Various amounts of cortisol acetate were administered as described in Fig. 3. The calvarial RNA was isolated after an exposure time of 24 h and translated in the cell-free translation system, as in Fig. 1. The amount of prepro-α chains synthesized was quantified as described in Figs. 1 and 2. The results are expressed as described in Fig. 3.

and also that observed in the RNA/DNA ratio, reached its maximum with 50 μg of cortisol acetate. There was a slight increase in the concentration of translatable procollagen mRNA with 12.5 μg of cortisol acetate, whether expressed per unit of RNA or DNA (Fig. 4). In 6 h no change, or even a slight increase, was observed, even with 200 μg of cortisol acetate, when expressed per unit of RNA, and the RNA/DNA ratio decreased only slightly (results not shown).

Effect of addition of exogenous calf liver tRNA

The changes in the incorporation of radioactivity into proteins in the cell-free translation system were not due to variations in $tRNA^{Pro}$ concentrations between the various RNA preparations, since addition of 1 μg of calf liver tRNA did not affect the incorporation, nor did it restore the procollagen-synthesizing activity of the RNA species obtained from the cortisol-treated embryos to the control value.

Effect of cortisol administration on chick-embryo growth

The daily dose of 50 μg of cortisol acetate given on days 15, 16 and 17 of embryonic development decreased the weight gain of the embryos, the mean weight of the 18-day chick embryos being 30% less than that of the controls, and the calvaria being more membrane-like and resilient. The inhibition of growth after cortisol administration in a single dose on day 15 of embryonic development was not statistically significant, not even with 200 μg of the steroid.

Discussion

Glucocorticoids modulate the expression of several gene products, the amounts of which may be either increased or decreased. It is currently thought (Baxter, 1976; Thompson & Lippman, 1974) that both the inhibitory and the stimulatory effects of these hormones involve a binding of the steroids to specific cytosolic receptors, followed by transport of a transformed steroid–receptor complex to the nucleus and binding of the complex with an acceptor protein to chromatin. This interaction results in modulation of the synthesis of specific mRNA molecules, whose translation products then mediate the glucocorticoid response. Such responses can be highly selective in each target tissue, i.e. the expression of only a few genes is affected.

The connective-tissue manifestations of glucocorticoid administration are thought to be due to inhibitory effects on the anabolism of fibroblasts. In a number of studies the response has been a selective decrease in collagen synthesis rather than a general inhibition of protein synthesis. It is not known,

however, at which level these hormones exert their primary influence.

The present results indicate that cortisol decreases the amount of translatable procollagen mRNA in the calvaria of developing chick embryos, a response that can be seen only after a lag period of up to about 12h. The decrease in the cell-free synthesis of the procollagenous material is not likely to be due to a depletion in the overall amount of tRNA or in any specific tRNA species, since the addition of exogenous tRNA had no effect on the results. It seems evident, therefore, that the glucocorticoid response in the fibroblasts involves a specific decrease of the cellular concentration of procollagen mRNA species, this probably being the main reason for the lower amount of tissue collagen. In intact cells, other factors, e.g. depletion of amino acids, glucose or tRNA species, decreased activities of the intracellular enzymes of post-translational reactions of procollagen biosynthesis (see the introduction) and overall decrease in the translational capacity of the cell, may contribute to this response. It is noteworthy that parathyrin has been shown to have a very similar effect on the concentration of translatable procollagen mRNA species in rat calvaria (Kream *et al.*, 1980).

It has been postulated that the glucocorticoid response may be mediated through some other extracellular factor, such as tri-iodothyronine (Martial *et al.*, 1977) or fibroblast growth factor (Gospodarowicz & Moran, 1974), thus being an indirect effect. It is also possible that collagen metabolism may be controlled by a specific derivative of the glucocorticoid hormones. The present study used whole animals, and therefore the modulation of other hormonal systems cannot be excluded. On the other hand, several studies have demonstrated that even cultured fibroblasts respond to glucocorticoid administration (Kruse *et al.*, 1978; Saarni & Tammi, 1978; Ponec *et al.*, 1979), and hence a direct effect of the hormone is quite possible.

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