Biochemical Pharmacology, Vol. 26, pp. 875–879. Pergamon Press, 1977. Printed in Great Britain.

EFFECT OF BETAMETHASONE-17-VALERATE ON SYNTHESIS OF COLLAGEN AND PROLYL HYDROXYLASE ACTIVITY IN CHICK EMBRYO TENDON CELLS

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(*Received* 1 *October* 1976; *accepted* 23 *November* 1976)

Abstract—The effect of betamethasone-17-valerate on the biosynthesis of collagen was studied in matrix-free chick embryo tendon cells *in vitro* and the activity of prolyl hydroxylase was assayed in the cells after incubation with the steroid *in vitro* and after injection of the steroid on to chorioallantoic membrane of embryonated eggs. Tendon cells synthesized collagen at a rapid rate and the synthesis was essentially linear for up to about 7 hr when studied by labelling with [^{14}C]proline. The secretion of collagen [^{14}C]hydroxyproline was also almost linear for up to 7 hr. Betamethasone-17-valerate decreased the total incorporation of [^{14}C]proline and the decrease in the synthesis of [^{14}C]hydroxyproline was larger than the decrease in the total incorporation, indicating that the collagen synthesis was affected more than other protein synthesis. Betamethasone-17-valerate had no effect on the activity of prolyl hydroxylase, when the cells were incubated with this steroid *in vitro* or, when the activity of the purified enzyme was assayed in the presence of this steroid. When the steroid was injected on to chorioallantoic membrane of embryonated eggs, the activity of prolyl hydroxylase decreased markedly in the tendon cells, indicating that in the system studied the amount of prolyl hydroxylase is decreased or that the enzyme is inactivated by this steroid *in vivo*.

The administration of anti-inflammatory steroids produces profound effects on collagen metabolism both in humans [1] and in experimental animals [2, 3, 4]. There is general agreement that steroids have mainly an anti-anabolic effect on collagen metabolism [3, 4, 5]. Recently it has been reported that the anti-anabolic effect of anti-inflammatory steroids may result from a decrease in the activity of prolyl hydroxylase [6], the enzyme catalyzing the hydroxylation of certain prolyl residues during collagen biosynthesis (for reviews on collagen biosynthesis, see [7–10]. Triamcinolone diacetate decreased markedly the activity of prolyl hydroxylase in rat tissue and the effect was dependent on the dosage of steroid [6]. The decrease in the activity of prolyl hydroxylase correlated to a decrease in the synthesis of collagen [11].

Matrix-free chick-embryo tendon cells have been used to study collagen biosynthesis *in vitro* [12, 13]. The major protein synthesized by the cells is collagen [12] and so these cells are suitable for studying collagen synthesis *in vitro*.

In the present work the effect of betamethasone-17-valerate on collagen biosynthesis was studied *in vitro* in freshly isolated chick-embryo tendon cells. In addition the activity of prolyl hydroxylase was assayed in tendon cells *in vitro* and after administration of this steroid on to chorio-allantoic membranes of chick embryos.

MATERIALS AND METHODS

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

[^{14}C]Proline was purchased from New England Nuclear Corp. (Boston, MA, U.S.A.), purified bacterial collagenase from Sigma Chemical Co. (Kingston-upon-Thames, U.K.), and trypsin, 2.5% solution in physiological saline from Gibco Corp. (Grand Island, NY). Eagle's minimum essential medium with glutamine for monolayer cultures was purchased from Orion Yhtymä (Helsinki, Finland) and foetal calf serum from Flow Laboratories (Irvine, Ayrshire, U.K.).

Betamethasone-17-valerate was provided from Glaxo Laboratories (Greenford, Middlesex, U.K.).

Isolation and incubation of chick-embryo tendon cells. Cells were isolated from leg tendons of 17-day-old chick embyos by controlled digestion with trypsin and purified bacterial collagenase, as described previously [12, 13]. After the digestion for 45–60 min the cells were filtered through lens paper and washed three times with modified Krebs medium [12, 13] containing 10% foetal calf serum, then resuspended in Eagle's minimum essential medium. The incubations were carried out in siliconized flasks with shaking at 37° under 95% O_2 and 5% CO_2. In most experiments the incubation medium contained 1–1.5 μCi [^{14}C]proline and 7.5–10 μg of unlabelled proline in one ml of Eagle's medium. Betamethasone-17-valerate was dissolved in physiological saline containing 80% (w/v) ethylalcohol *in vitro* incubations. In control incubations, physiological saline containing 80% ethyl alcohol was used [4].

At each time point 2.0 ml aliquots of the incubation system (cells plus medium) were quickly pipetted into test tubes contining a one-tenth vol. of modified Krebs medium with sufficient cycloheximide and α,α'-bipyridyl to provide a final concentration of 100 μg of cycloheximide and 1 μmole of α,α'-bipyridyl per ml

[12]. The samples were centrifuged at 1200 *g* for 10 min at room temperature in order to separate the medium from the cells and the cell pellet was washed with 1.5 ml of modified Krebs medium containing 100 μg of cycloheximide and 1 μmole of α,α'-bipyridyl per ml. The sample was then re-centrifuged and the wash solution discarded [12].

Preparation of [^{14}C]proline-labelled protocollagen. Isolated cells obtained from leg tendons of 100 17-day-old chick embryos were incubated with 60 μCi [^{14}C]proline in the presence of 0.3 mM α,α'-bipyridyl for 4 hr [13] and the protocollagen was extracted with 0.1 *M*-acetic acid [14]. After centrifugation at 20,000 *g* for 30 min, the supernatant was dialyzed against a solution containing 0.2 *M*-NaCl and 0.05 *M*-Tris-HCl buffer adjusted to pH 7.8 at 4° with several changes. The preparation was then heated to 100° for 10 min, centrifuged at 1000 *g* for 10 min to remove the precipitate formed during heating, and the supernatant was stored frozen in aliquots of 60,000 d.p.m. [15].

Assay of prolyl hydroxylase activity in tendon cells. The cells were incubated with the substances for the times indicated and after incubation the cells were separated by centrifugation at 600 *g* at room temperature for 5 min. The supernatant was discarded and the cells in the pellet were gently suspended in 2 ml of homogenizing medium (without addition of Triton X-100) and recentrifuged. The cells were then homogenized in 1–2 ml of 0.2 M NaCl, 0.1 M glycine, 50 μM dithiothreitol, 20 mM Tris-HCl buffer (pH 7.5 at 4°) containing 0.1% of Triton X-100 [16]. Cells were homogenized either with sonicator (30–60 sec) or with a Teflon and glass homogenizer for 60 strokes [17]. Because the latter led to better recovery of enzyme activity, this was used further. After homogenizing the samples were centrifuged at 15,000 *g* for 20 min and the protein content was determined [18]. Aliquots of the supernatant, 10–20 μg protein (see Fig. 1), were incubated with 60,000 d.p.m., [^{14}C]proline labelled protocollagen substrate, 50 mM Tris-HCl buffer (pH 7.8 at 25°), 2 mM ascorbic acid, 0.5 mM α-ketoglutarate, bovine serum albumin 2 mg/ml, 0.05 mM $FeSo_4$, 0.1 mM dithiothreitol and 0.1 mg/ml catalase, all in a final vol. of 2.0 ml [19]. After incubation for 30 min at 37° the samples were hydrolyzed in 6 M HCl overnight at 120°. Thereafter the total ^{14}C-radioactivity and [^{14}C]hydroxyproline were determined in the hydrolysates [20].

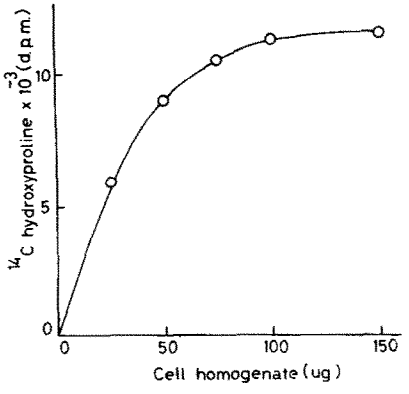

Fig. 1. Effect of enzyme concentration on product formation in the prolyl hydroxylase reaction. The enzyme concentration is expressed as μg of the 15,000 *g* supernatant of the cell homogenate in the standard incubation vol.

When the effect of betamethasone-17-valerate on the activity of prolyl hydroxylase was studied *in vivo*, test substances were injected on the chorioallantoic membrane of chick embryos in the amounts indicated and after the incubation times indicated, tendons were dissected and the tendon cells were isolated as described above. The cells were homogenized as described above. Protein content was determined in the 15,000 *g* supernatant of the cell homogenates and aliquots of supernatant were used to assay the activity of prolyl hydroxylase as described above.

For the experiments in which the effect of betamethasone-17-valerate on the activity of purified prolyl hydroxylase was tested the enzyme was purified by an affinity column procedure using poly (*L*-proline) [21]. During this investigation substrates having various specific activities were used in the enzyme assay. As a result comparisons of enzyme activities can only be made within each table or figure.

Assays. After incubation with [^{14}C]proline the medium and cell fractions were dialyzed separately against running tap water for 24 hr and the dialysates were hydrolysed in sealed tubes in 6 M HCl at 120° overnight. The hydrolysed samples were evaporated on a steam bath and dissolved in 4 ml of water. The total radioactivity was determined and the [^{14}C]hydroxyproline content was assayed by a specific radiochemical procedure [20].

All ^{14}C-radioactivity counting was performed in a Wallac liquid-scintillation spectrometer with an efficiency of 80% and a background of 25 c.p.m.

RESULTS

Incorporation of proline and synthesis of hydroxyproline by isolated tendon cells. Tendon cells were isolated as described in Materials and Methods and usually about $10 \cdot 10^6$ cells were obtained from one chick embryo and over 90 per cent of the cells were alive when stained with Trypan Blue. In initial experiments the cells were incubated as described by Dehm and Prockop [12] in a modified Krebs medium not containing added unlabelled proline. In these experiments the amount of free [^{14}C]proline in the medium decreased markedly after incubation for 3 hr, resulting in nonlinear incorporation curves (data not shown). The modified Krebs medium was therefore replaced by Eagle's minimum essential medium containing a small amount of unlabelled proline in order to supply steady incorporation of [^{14}C]proline. In continuous labelling experiments with this medium the cell fractions contained more radioactivity than the medium for up to 90 min. Thereafter the medium fractions contained most of the radioactivity and the appearance of radioactivity in the medium was linear for up to 7 hr (Fig. 2a). The appearance of [^{14}C]hydroxyproline in the medium, which measures the collagen secretion, was also linear for up to 7 hr (Fig. 2b).

The capacity of the isolated tendon cells to synthesize and secrete collagen was further studied with pulse experiments. The cells were pulsed after incubation intervals indicated with [^{14}C]proline and the

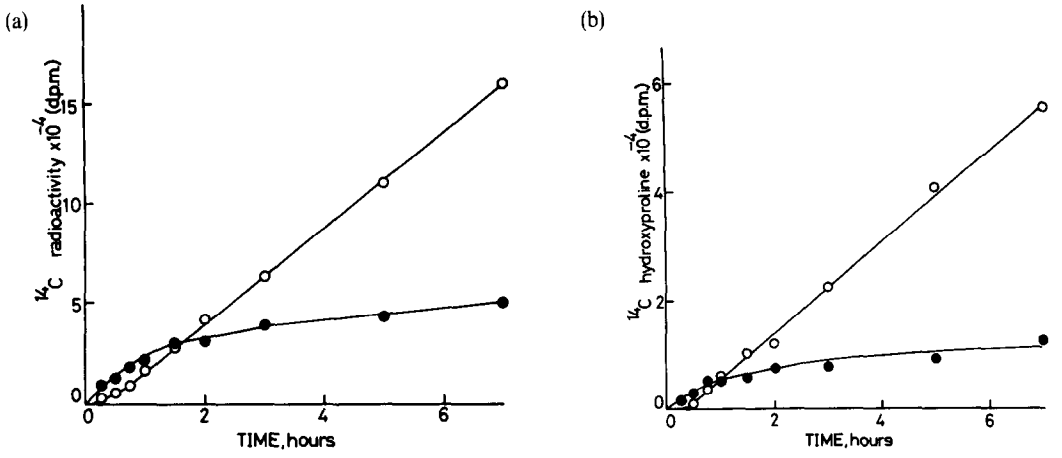

Fig. 2. Incorporation of [^{14}C]proline and synthesis of [^{14}C]hydroxyproline by isolated tendon cells. Tendon cells, $1 \cdot 10^8$, were incubated in 23 ml of Eagle's medium with 30 μCi [^{14}C]proline and 200 μg unlabelled proline. At each time point an aliquot of 2.0 ml was taken and assayed for total radioactivity and [^{14}C]hydroxyproline in the cells and medium. Symbols: (2a) total radioactivity in the medium, O——O; total radioactivity in the cells, ●——●. (2b) [^{14}C]hydroxyproline in the medium, O——O; [^{14}C]hydroxyproline in the cells, ●——●.

total radioactivity and [^{14}C]hydroxyproline were assayed in the cell and medium fractions. There was a slight decrease in the total radioactivity, in the synthesis of [^{14}C]hydroxyproline and in the secretion of collagen [^{14}C]hydroxyproline in this experiment when cells were incubated for 6 hr (Fig. 3).

Effect of betamethasone-17-valerate on the incorporation of proline and on the synthesis of hydroxyproline in isolated tendon cells. Tendon cells were incubated with betamethasone-17-valerate or without this com-

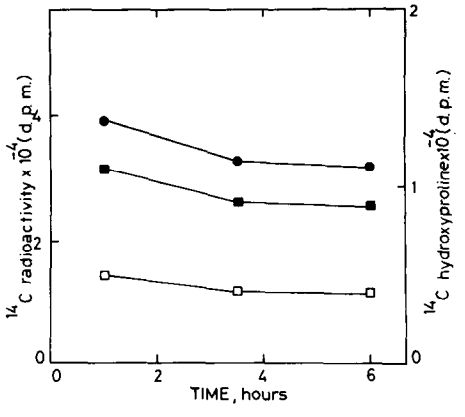

Fig. 3. Effect of incubation interval on the incorporation of [^{14}C]proline, the synthesis of [^{14}C]hydroxyproline and the secretion of collagen [^{14}C]hydroxyproline by isolated tendon cells. Tendon cells, $2 \cdot 10^7$, were incubated for periods indicated in 4 ml of Eagle's medium containing 10 μg/ml unlabelled proline. The cells were pulsed with [^{14}C]proline (1.5 μCi/ml) at the beginning of the last 60 min of each incubation period. Thereafter samples were handled as described in Materials and Methods. Values are the mean of duplicate samples from one experiment. Symbols: total radioactivity in the medium plus cells, ●——●; total [^{14}C]hydroxyproline in the medium plus cells, ■——■; [^{14}C]hydroxyproline in the medium □——□.

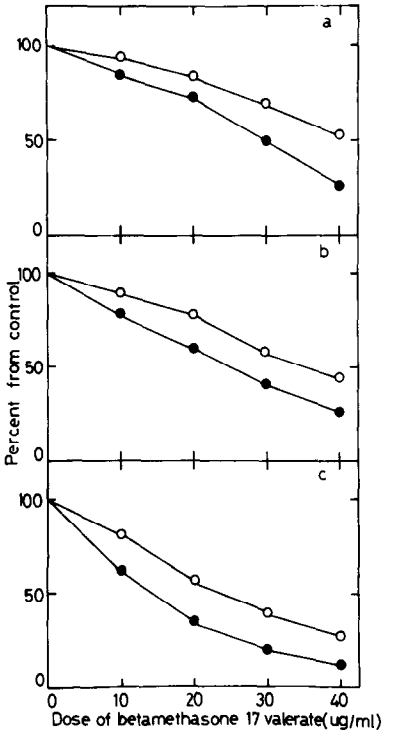

Fig. 4. Effect of different amounts of betamethasone-17-valerate and the incubation time on the incorporation of [^{14}C]proline and on the synthesis of [^{14}C]hydroxyproline in isolated tendon cells. Tendon cells were incubated in Eagle's minimum essential medium containing different amounts of betamethasone-17-valerate and unlabelled proline (7.5 μg/ml). The cells were pulsed with [^{14}C]proline (1 μCi/ml) for 1(4a), 3(4b) and 7(4c) hours and total radioactivity and [^{14}C]hydroxyproline were assayed in the total system (cells plus medium). Values are expressed as a percentage of control values. Symbols: total radioactivity in the cells plus medium, (O——O); total [^{14}C]hydroxyproline in the cells plus medium (●——●). Each point is the mean of duplicate samples from different experiments. The deviation of the values between the duplicate samples was less than 20 per cent in every experiment.

pound. This steroid markedly decreased the total incorporation of [^{14}C]proline (Fig. 4a–c), and the decrease in the synthesis of [^{14}C]hydroxyproline was even larger than the decrease in the total radioactivity (Fig. 4a–c). The decrease in the total protein synthesis and in the collagen synthesis was dependent on the amount of steroid used and on the incorporation time (Fig. 4a–c).

Experiments were continued to study the effect of reversal of betamethasone-17-valerate induced inhibition. The cells were incubated for 150 min with betamethasone-17-valerate (40 μg/ml) or without this compound and thereafter the cells were washed three times with modified Krebs medium [12] and resuspended in Eagle's minimum essential medium and incubated with [^{14}C]proline for a further 3 hr. In this experiment betamethasone-17-valerate decreased the total radioactivity by about 50 per cent and the synthesis of [^{14}C]hydroxyproline by about 80 per cent (data not shown.).

Effect of betamethasone-17-valerate on the activity of prolyl hydroxylase in vitro *and* in vivo *in tendon cells and on the activity of purified enzyme.* Tendon cells were incubated in Eagle's minimum essential medium for the times indicated and the cells were separated. The cells were homogenized with a Teflon and glass homogenizer in a medium containing 0.1 per cent Triton X-100. This homogenizing system liberated over 90 per cent of the enzyme activity (data not shown). The amount of protein obtained from $1 \cdot 10^6$ tendon cells was about 30 μg in the 15,000 g supernatant of cell homogenate. For the 7 hr of incubation the activity of prolyl hydroxylase did not change significantly and betamethasone-17-valerate had no effect on the enzyme activity (Table 1).

Betamethasone-17-valerate was injected *in vivo* on the chorioallantoic membranes of chick embryos and the activity of prolyl hydroxylase was assayed in tendon cells. The activity of prolyl hydroxylase decreased markedly after administration of betamethasone-17-valerate (Table 1).

The effect of betamethasone-17-valerate on the activity of purified prolyl hydroxylase was tested. A dosage of 100 μg/ml of this steroid had no effect on the activity of this enzyme (data not shown).

DISCUSSION

Freshly isolated chick embryo tendon cells were used to study collagen synthesis *in vitro*. Collagen synthesis was studied by continuous labelling and pulse experiments. These experiments indicated that the incorporation of [^{14}C]proline and the synthesis of [^{14}C]hydroxyproline were almost linear for up to 7 hr and pulse experiments further indicated that the total radioactivity, the synthesis of [^{14}C]hydroxyproline and the secretion of collagen [^{14}C]hydroxyproline were essentially constant and there was only a slight decrease in these parameters in an incubation lasting 6 hr.

Betamethasone-17-valerate decreased both the incorporation of [^{14}C]proline and the synthesis of [^{14}C]hydroxyproline, indicating that the total protein synthesis was decreased by this steroid. Since the decrease in the amount of [^{14}C]hydroxyproline was larger than the decrease in the total incorporation, the collagen synthesis was affected even more than other protein synthesis. This is consistent with experiments with rats [11] and chick-embryo tibias [4].

The effect of betamethasone-17-valerate on the activity of prolyl hydroxylase was studied both in tendon cells and with purified enzyme preparation. The enzyme activity could be measured in the 15,000 g supernatant of the cell homogenate. Using a Teflon and glass homogenizer and adding Triton X-100 to the medium liberated over 90 per cent enzyme activity [17]. The activity of prolyl hydroxylase did not change significantly during incubation for 7 hr *in*

Table 1. Effect of betamethasone-17-valerate on the activity of prolyl hydroxylase in chick embryo tendon cells *in vitro* and *in vivo*

Experimental condition and test substance	Incubation time hr	Prolyl hydroxylase activity (d.p.m. $\cdot 10^{-4}$/mg supernatant protein)	(%)
In vitro			
Control	1	22.8	
Betamethasone (20 μg/ml)	1	21.7	
Control	7	22.5	
Betamethasone (20 μg/ml)	7	21.3	
In vivo			
Control	–	27.7	100
Betamethasone	–	10.3	37

In experiments *in vitro*, tendon cells, $5 \cdot 10^6$ per one ml of Eagle's minimum essential medium, were incubated with test substances for 1 and 7 hr and thereafter the cells were separated and homogenized. In experiments *in vivo*, 200 μg of betamethasone-17-valerate in 0.1 ml of physiological saline containing 80 per cent ethyl alcohol was injected on the chorioallantoic membranes of ten 15-day-old fertilized eggs two times at 24 hr intervals. Control eggs twice received the same amount of physiological saline containing 80 per cent ethyl alcohol. The tendon cells were isolated as described in Materials and Methods after 24 hr incubation and the activity of prolyl hydroxylase was assayed in the 15,000 g supernatant of the cell homogenates.

The values are expressed as dis./min [^{14}C]hydroxyproline formed in an aliquot of 50,000 dis./min [^{14}C]proline labelled protocollagen substrate per 30 min. Values are the mean of duplicate samples.

vitro. Betamethasone-17-valerate had no effect on the enzyme activity *in vitro* under the conditions studied. This finding is consistent with experiments in cell culture [6]. The activity of purified prolyl hydroxylase did not change when betamethasone-17-valerate was added in the incubation mixture, indicating that this steroid has no direct effect on the activity of formed prolyl hydroxylase *in vitro*. When betamethasone-17-valerate was injected on to the chorioallantoic membrane of chick embryos and the activity of prolyl hydroxylase was assayed in the tendon cells there was a marked decrease in enzyme activity. This is in contrast to findings in chick embryo tibias [4], but it should be noted that in the present study the incubation time with the steroid was longer, and that in the present study prolyl hydroxylase activity was measured directly from the cells that synthesize collagen. Other experiments with rats have indicated that prolyl hydroxylase activity decreases after the treatment with anti-inflammatory steroid [6]. The decrease observed in the present study in the activity of prolyl hydroxylase may be due to a decrease in the amount of enzyme protein in chick embryo tendon cells or due to the possibility that this steroid or some of its metabolites have some effect on the activation mechanism of prolyl hydroxylase *in vivo*. However, the decrease in the activity of prolyl hydroxylase cannot be due to the decrease in the number of cells synthesizing collagen, because enzyme activity was measured directly in the cells.

Since in the present study collagen synthesis was decreased more than other protein synthesis by betamethasone-17-valerate *in vitro* and prolyl hydroxylase activity remained unchanged in these experiments, it seems that this steroid has a specific effect on collagen synthesis which is not mediated by a decrease in prolyl hydroxylase activity [11].

Acknowledgements—This work was supported in part by a grant from the Medical Research Council of the Academy of Finland. The author gratefully acknowledges the valuable suggestions and comments of Professor Kari I. Kivirikko M.D. and the expert technical assistance of Mrs. Ritva Savilaakso.

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