Biochemical Pharmacology, Vol. 21, pp. 2161–2167. Pergamon Press, 1972. Printed in Great Britain.

CORTICOSTEROID-INDUCED INHIBITION OF THE BIOSYNTHESIS OF HUMAN SKIN COLLAGEN*

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(*Received* 1 *November* 1971; *accepted* 4 *April* 1972)

Abstract—The effects of hydrocortisone acetate, fluocinolone acetonide, fluclorolone acetonide, betamethasone-17-valerate, fluprednyliden-21-acetate and flumethasone pivalate on the biosynthesis of human skin collagen were studied *in vitro*. Skin specimens were incubated in a medium containing a test substance and radioactive proline, and the formation of radioactive hydroxyproline in nondialysable proteins was taken as an index of the rate of collagen biosynthesis.

Hydroxyproline formation was inhibited by all the corticosteroids tested in concentrations of 30 μg/ml or higher. The effect on hydroxyproline formation was smallest with hydrocortisone acetate and most pronounced with betamethasone-17-valerate in all concentrations used. In general, the corticosteroid-induced inhibition of collagen biosynthesis was found to be dose-dependent. The differences in the degree to which collagen formation is inhibited by the different corticosteroids may have relevance to the extent of the local side-effects, such as atrophy of skin, reported to be produced by fluorinated corticosteroids.

IN RECENT years, dermatologists have become increasingly aware of the undesirable side-effects of topically applied corticosteroids on connective tissue. In addition to systemic manifestations, attention has been drawn to local ones. Especially, prolonged use of fluorinated corticosteroids has appeared to produce adverse effects, such as atrophy of the skin and formation of telangiectases.[2–4] The latter conditions may reflect disturbances of the connective tissue in skin caused by the steroid preparation used.

In the present investigation we studied the effects of hydrocortisone acetate and various fluorinated corticosteroids commonly used in the treatment of dermatological disorders on collagen biosynthesis in human skin *in vitro*. The skin specimens were incubated in a medium containing the substance to be tested and radioactive proline. The formation of radioactive hydroxyproline in the non-dialysable protein fraction was taken as an index of the rate of collagen biosynthesis. Furthermore, dialysable ^{14}C-hydroxyproline as well as total ^{14}C-radioactivity in the non-dialysable fraction was assayed in the same skin specimens.

This study has been supported by grants from the Finnish Anti-Tuberculosis Association and the National Research Council for Medical Sciences, Finland.

* Some of the results were preliminarily reported at the 8th Acta Endocrinologica Congress, Copenhagen, July, 1971.[1]

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MATERIALS AND METHODS

Test substances. The effects of the following corticosteroids on collagen biosynthesis were studied: hydrocortisone acetate, fluocinolone acetonide, fluclorolone acetonide, betamethasone-17-valerate, fluprednyliden-21-acetate and flumethasone pivalate. These substances were dissolved in physiological saline containing ethyl alcohol in sufficient amounts to keep the steroid in solution. In control incubations, physiological saline containing the corresponding proportion of ethyl alcohol was used. All solutions were freshly prepared prior to the incubations, and the concentrations were arranged so that 0·1 ml of a steroid solution could be added to the medium to bring the final concentration to the desired level.

Preparation of skin specimens. Skin specimens, weighing up to 5 g, were obtained from surgical wounds immediately after the start of the operation. The samples were carefully trimmed of subcutaneous fat, cut and sliced at maximum thickness of 0·5 mm with a Stadie–Riggs microtome. Before incubation the slices were weighed on a torsion balance. These manipulations were performed at 4°. In a single experiment each flask contained skin from the same person, and the amount of skin tissue, about 100 mg per flask, in an experiment varied maximally by 10 per cent.

Incubation conditions. The incubations were carried out under conditions previously reported to be suitable for human skin.[5] The incubation medium contained 20 mM glucose, 20 mM N-2-hydroxy-ethylpiperazine-N′-2-ethanesulphonic acid (HEPES) buffer (Calbiochem) of pH 7·4, 50 μg/ml ampicillin (Pentrexyl®, Lundbeck), all in a final volume of 3·0 ml of phosphate-free Krebs–Ringer solution. At the beginning of the experiment, 0·1 ml of test substance solution was added, and the skin slices were incubated at 37° in a metabolic shaker.[5] After 30 min pre-incubation, 2 μC of ^{14}C-proline, specific activity 180 mC/mmole (New England Nuclear Corp.), was added to the incubation medium. Incubation was continued for an additional 10 hr and the reaction was stopped by chilling the samples to below 0°.

Determination of non-dialysable hydroxyproline. After incubation, the skin specimens were homogenized with an Ultra-turrax homogenizer five times for 5 sec. The homogenates were dialysed against cold running tap water for 24 hr and then hydrolysed in 6 M HCl at 135° for 6 hr. The amount[6] and radioactivity[7] of the hydroxyproline as well as the total ^{14}C radioactivity[8] were then determined in the hydrolysate.

Determination of dialysable hydroxyproline. The dialysable fraction of hydroxyproline was recovered from tissue samples homogenized after incubation as above. The homogenate was dialysed against a known volume of distilled water for 24 hr. The dialysable fraction was hydrolysed with an equal volume of concentrated HCl at 135° for 6 hr and then evaporated to dryness. The residue was dissolved in distilled water and an aliquot of the solution was used for assay[7] of ^{14}C-hydroxyproline after a preliminary separation of radioactive hydroxyproline and proline on a Dowex 50-X8 column with 2 N HCl as the eluting agent. The recovery of radioactive hydroxyproline was checked by using 2 mg of ^{12}C-hydroxyproline as carrier in the column and assaying[6] the hydroxyproline content before and after separation.

For comparison, two series of incubations were performed. First, at the beginning of the pre-incubation, the skin samples were heated up to 65° for 15 min, the incubation was then continued as above. Secondly, sliced skin specimens were incubated in a medium containing 100 μg/ml cycloheximide and 1 mM α,α′-dipyridyl. In both experiments, ^{14}C-hydroxyproline in dialysable fraction was assayed as above.

Presentation of the results. In the experiments five parallel incubations were used for each determination. The values for radioactive hydroxyproline were expressed as dpm per μg hydroxyproline or per mg wet wt. of the tissue. The results were evaluated statistically by Student's *t*-test and the differences at the $P < 0.05$ level were regarded as significant.

RESULTS

When the skin specimens were incubated in a medium containing one of the test substances in a concentration of 100 μg/ml, all the steroid preparations tested (hydrocortisone acetate, fluocinolone acetonide, fluclorolone acetonide, betamethasone-17-valerate, fluprednyliden-21-acetate and flumethasone pivalate) markedly inhibited ^{14}C-hydroxyproline formation in the non-dialysable fraction. The inhibition was smallest with hydrocortisone acetate and proved most pronounced with betamethasone-17-valerate (Table 1). Significant inhibition of ^{14}C-hydroxyproline formation

TABLE 1. EFFECTS OF VARIOUS CORTICOSTEROIDS ON COLLAGEN BIOSYNTHESIS IN HUMAN SKIN *in vitro*

Corticosteroid tested	Concentration μg/ml	^{14}C-hypro* dis./min/μg hypro	P†	^{14}C-hypro* dis./min/mg wet wt. skin	P†
Control		2·44 ± 0·33		45·5 ± 4·9	
Hydrocortisone acetate	100	1·69 ± 0·13	<0·005	34·9 ± 10·6	<0·05
Fluocinolone acetonide	100	1·44 ± 0·41	<0·005	35·3 ± 7·9	<0·05
Fluclorolone acetonide	100	1·15 ± 0·08	<0·001	21·3 ± 2·8	<0·001
Betamethasone-17-valerate	100	0·36 ± 0·16	<0·001	12·1 ± 4·3	<0·001
Fluprednyliden-21-acetate	100	0·71 ± 0·38	<0·001	15·1 ± 4·7	<0·001
Flumethasone pivalate	100	1·00 ± 0·66	<0·001	29·9 ± 2·7	<0·05

Sliced skin specimens were incubated at 37° in a medium containing the test substance. After 30 min preincubation, 2 μC of ^{14}C-proline was added, and incubation was continued for another 10 hr. Thereafter, the amount and radioactivity of hydroxyproline were assayed, as described in Materials and Methods.

* Each value is the mean ±S.D. of five parallel samples.

† The significance of the differences from the control values were calculated by Student's *t*-test.

could also be observed with all the test substances at concentrations of 30 μg/ml (Table 2). When skin specimens were incubated in a medium containing 10 μg/ml of

TABLE 2. EFFECTS OF VARIOUS CORTICOSTEROIDS ON COLLAGEN BIOSYNTHESIS IN HUMAN SKIN *in vitro*

Corticosteroid tested	Concentration μg/ml	^{14}C-hypro* dis./min/μg hypro	P†	^{14}C-hypro* dis./min/mg wet wt. skin	P†
Control		1·88 ± 0·32		47·6 ± 6·2	
Hydrocortisone acetate	30	1·27 ± 0·15	<0·005	35·6 ± 3·3	<0·01
Fluocinolone acetonide	30	1·36 ± 0·46	<0·05	31·0 ± 6·6	<0·01
Fluclorolone acetonide	30	1·40 ± 0·11	<0·01	32·1 ± 5·1	<0·005
Betamethasone-17-valerate	30	1·21 ± 0·24	<0·005	27·4 ± 1·8	<0·001
Fluprednyliden-21-acetate	30	1·50 ± 0·34	NS	26·3 ± 7·0	<0·005
Flumethasone pivalate	30	1·44 ± 0·16	<0·05	29·4 ± 7·5	<0·001

For experimental conditions, see legend to Table 1.

* Each value is the mean ±S.D. of five parallel samples.

† The significance of the differences from the control values were calculated by Student's *t*-test; NS = statistically not significant.

corticosteroids, a significant decrease was observed with betamethasone-17-valerate and fluclorolone acetonide as compared to the controls (Table 3). When the skin

TABLE 3. EFFECTS OF VARIOUS CORTICOSTEROIDS ON COLLAGEN BIOSYNTHESIS IN HUMAN SKIN *in vitro*

Corticosteroid tested	Concentration μg/ml	^{14}C-hypro* dis./min/μg hypro	P†	^{14}C-hypro* dis./min/mg wet wt. skin	P†
Control		3·12 ± 0·67		51·8 ± 10·8	
Hydrocortisone acetate	10	2·72 ± 0·74	NS	46·4 ± 9·1	NS
Fluocinolone acetonide	10	2·73 ± 0·92	NS	39·0 ± 10·4	NS
Fluclorolone acetonide	10	2·20 ± 0·56	<0·05	37·0 ± 8·3	<0·05
Betamethasone-17-valerate	10	2·09 ± 0·73	<0·05	36·1 ± 7·8	<0·05
Fluprednyliden-21-acetate	10	3·09 ± 0·29	NS	46·2 ± 2·8	NS
Flumethasone pivalate	10	3·00 ± 0·67	NS	44·6 ± 8·1	NS

For experimental conditions, see legend to Table 1.
* Each value is the mean ±S.D. of five parallel samples.
† The significance of the differences from the control values were calculated by Student's *t*-test; NS = statistically not significant.

specimens were incubated in a medium containing test substances at concentration of 300 μg/ml, hydrocortisone acetate inhibited ^{14}C-hydroxyproline formation to about 60 per cent of the controls, whereas with other corticosteroids the values for radioactive hydroxyproline were about one-tenth of the control value (Table 4). In all

TABLE 4. EFFECTS OF VARIOUS CORTICOSTEROIDS ON COLLAGEN BIOSYNTHESIS IN HUMAN SKIN *in vitro*

Corticosteroid tested	Concentration μg/ml	^{14}C-hypro* dis./min/μg hypro	P†	^{14}C-hypro* dis./min/mg wet wt. skin	P†
Control		1·00 ± 0·17		10·4 ± 1·1	
Hydrocortisone acetate	300	0·57 ± 0·35	<0·05	7·1 ± 2·1	<0·05
Fluocinolone acetonide	300	0·11 ± 0·04	<0·001	1·5 ± 1·1	<0·001
Fluclorolone acetonide	300	0·05 ± 0·01	<0·001	1·6 ± 0·1	<0·001
Betamethasone-17-valerate	300	0·04 ± 0·01	<0·001	0·7 ± 0·4	<0·001
Fluprednyliden-21-acetate	300	0·16 ± 0·08	<0·001	2·0 ± 0·5	<0·001
Flumethasone pivalate	300	0·14 ± 0·03	<0·001	3·1 ± 1·0	<0·001

For experimental conditions, see legend to Table 1.
* Each value is the mean ±S.D. of five parallel samples.
† The significance of the differences from the control values were calculated by Student's *t*-test.

these experiments, the incorporation of total ^{14}C into non-dialysable skin fraction was inhibited by all the test substances to the same extent as the ^{14}C-hydroxyproline formation.

The possibility that the decreased radioactivity of hydroxyproline in the non-dialysable fraction could be explained by increased degradation of newly-synthesized radioactive hydroxyproline containing collagen molecules into dialysable form was studied by assaying the radioactivity of ^{14}C-hydroxyproline in the dialysable fraction, after the skin specimens had been incubated with the test substances at concentrations

of 100 μg/ml. No significant differences in the amount of dialysable ^{14}C-hydroxyproline could be observed in specimens incubated with any of the various test substances as compared with the controls, if the values were expressed per wet wt. of skin (Table 5). However, if the values of dialysable ^{14}C-hydroxyproline were related to

TABLE 5. DIALYSABLE ^{14}C-HYDROXYPROLINE IN SKIN AFTER 10 HR INCUBATION IN MEDIA CONTAINING VARIOUS CORTICOSTEROIDS AND RADIOACTIVE PROLINE*

Corticosteroid tested	Concentration μg/ml	^{14}C-hypro† dis./min/mg wet wt. skin
Control		14·7 ± 1·3‡
Hydrocortisone acetate	100	13·9 ± 2·7
Fluocinolone acetonide	100	14·9 ± 4·9
Flucorolone acetonide	100	13·7 ± 1·3
Betamethasone-17-valerate	100	13·5 ± 3·7
Fluprednyliden-21-acetate	100	15·9 ± 5·7
Flumethasone pivalate	100	11·6 ± 4·4

For incubation conditions, see legend to Table 1. After incubation, the radioactivity of the ^{14}C-hydroxyproline in the dialysable fraction was assayed, as described in Materials and Methods.

* The skin specimens are the same as in Table 1.

† Each value is the mean ±S.D. of five parallel samples.

‡ The difference between the controls and the various corticosteroids tested was not significant when evaluated by Student's *t*-test.

those of non-dialysable ^{14}C-hydroxyproline in the same tissue samples (Table 1), all the test substances seemed to affect the dialysable fraction of radioactive hydroxyproline. The assays revealed that in controls about one-fourth of the ^{14}C-hydroxyproline synthesized during *in vitro* incubation existed in dialysable form. The corticosteroids increased the ratio dialysable/non-dialysable ^{14}C-hydroxyproline, and in specimens incubated with betamethasone-17-valerate or with fluprednyliden-21-acetate about half of the radioactive hydroxyproline was in dialysable form.

When the skin specimens were incubated in a medium containing cycloheximide and α,α′-dipyridyl, or the tissue samples were inactivated by heating prior to incubation, the values for ^{14}C-hydroxyproline in dialysable fraction were less than 10 per cent of the control values presented in Table 5.

DISCUSSION

The effect of corticosteroids on the metabolism of connective tissue has received considerable attention, because these drugs are widely used as therapeutic agents in various clinical disorders involving connective tissue (Refs. 9 and 10). The mode of action of corticosteroids on the metabolism of connective tissue components is mainly anti-anabolic. The biosynthesis of collagen, the main fibrillar protein of the connective tissue, has been shown to be inhibited by various corticosteroids (Ref. 11). In animal skin, cortisone and hydrocortisone have been demonstrated to decrease the rate of collagen formation,[12–14] and direct application of betamethasone-17-valerate on a psoriatic lesion has been shown to suppress collagen biosynthesis.[15]

In recent years, new corticosteroid preparations have been introduced, which are reported to differ in their clinical efficacy and in the extent of their side-effects. In the

present investigation we studied the effects of hydrocortisone acetate, fluocinolone acetonide, fluclorolone acetonide, betamethasone-17-valerate, flupredniliden-21-acetate and flumethasone pivalate on collagen biosynthesis in human skin *in vitro*.

Collagen is characterized by a high content of hydroxyproline, an amino acid found almost exclusively in this protein. The hydroxyproline in collagen is not derived from the free amino acid pool but is formed through hydroxylation of proline residues already incorporated into a polypeptide precursor of collagen, called protocollagen (Refs. 16 and 17). Consequently, the conversion of radioactive proline to radioactive hydroxyproline can be taken as a measure of the rate of collagen formation. Furthermore, the incorporation of total ^{14}C radioactivity into non-dialysable fraction of the same skin specimen can be taken as a parameter reflecting the overall protein synthesis.

In larger, non-physiologic concentrations, all the test substances inhibited the rate of collagen formation. Hydrocortisone acetate seemed to suppress collagen biosynthesis to a smaller extent than did other corticosteroids tested. These results are in agreement with our previous publication on the effect of various corticosteroids on collagen biosynthesis in chick embryo bones.[11] The finding that total ^{14}C incorporation is suppressed to the same extent as the ^{14}C-hydroxyproline formation seems to suggest that the inhibition is not specific to collagen but that the inhibition is part of a more general depression of the protein synthesis.

In the present study, the possibility that the decrease of ^{14}C-hydroxyproline in non-dialysable fraction could be due to increased degradation of the non-dialysable proteins into dialysable form was studied by assaying the radioactivity of ^{14}C-hydroxyproline in the dialysable fraction. Because contaminating radioactive hydroxyproline in the proline used for labelling contributes to the values for dialysable ^{14}C-hydroxyproline, two control series were performed. In these experiments, either heat-inactivated tissue was used for incubations, or collagen formation was inhibited by adding cycloheximide and α,α'-dipyridyl to the incubation medium. In both these experiments the amounts of dialysable ^{14}C-hydroxyproline recovered after incubations were insignificant, when 2 μC of radioactive proline per incubation was used. Consequently, the results of these control experiments support the view that the ^{14}C-hydroxyproline in dialysable form is, indeed, derived from the degradation of newly-synthesized ^{14}C-hydroxyproline-containing molecules. Incubation with corticosteroids caused no pronounced change in the amount of dialysable ^{14}C-hydroxyproline. However, incubation with corticosteroids increased the ratio dialysable/non-dialysable ^{14}C-hydroxyproline, a finding indicating a shift towards dialysable forms of ^{14}C-hydroxyproline containing molecules. This finding is in agreement with earlier statements[18,19] that corticosteroids may increase the degradation of some forms of collagen, even though, at least in pharmacological doses, the mode of action is mainly anti-anabolic.[13,14]

Topical application of corticosteroid preparations has been demonstrated to lead to atrophy of the skin, in both clinical[2–4] and experimental conditions.[20] This may well be a consequence of the decreased collagen content of the skin. A decrease in the amount of tissue collagen may be thought to be due to decreased biosynthesis of collagen, increased degradation of collagen, or both. In the light of the present study, it seems likely that a decrease in the rate of collagen biosynthesis contributes to the atrophy of skin produced by topically applied corticosteroids. In addition to that, increased collagenolysis may play a role in that process. In the present study, the

corticosteroid-induced inhibition of collagen biosynthesis seemed to be roughly dose-dependent, even though the individual incubations are not strictly comparable. Consequently, there may be expected to be a correspondence between the anti-anabolic action and the severity of the atrophy produced. On the other hand, the occurrence of these side-effects should be related to the dose of the corresponding corticosteroid preparation used to achieve the topical anti-inflammatory effect desired.

In conclusion, all the corticosteroids tested in this study, i.e. hydrocortisone acetate, fluocinolone acetonide, fluclorolone acetonide, betamethasone-17-valerate, fluprednyliden-21-acetate and flumethasone pivalate, were found to reduce the rate of collagen biosynthesis in human skin *in vitro*. Consequently, the present findings may have relevance to the appearance of local side-effects on connective tissue produced by topical application of various corticosteroids on skin in the treatment of dermatological disorders. This possibility seems real in the light of reports indicating that various corticosteroids applied to the skin are penetrated.[21–23] The effect on the skin may be expected to be especially strong if occlusive dressings are used and in sites affected by disease, where the conditions for the penetration of the drugs are likely to be more favourable than in uninvolved skin.

Acknowledgements—The fluorinated corticosteroids used in this study were kindly supplied in pure form by Syntex Pharmaceuticals Ltd., Berkshire, (fluocinolone acetonide, fluclorolone acetonide and betamethasone-17-valerate), E. Merck, Darmstadt (fluprednyliden-21-acetate) and Ciba Pharmaceuticals, Basel (flumethasone pivalate).

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