Biochemical Pharmacology, Vol. 27, pp. 1029–1032. Pergamon Press, 1978. Printed in Great Britain.

CORTISOL EFFECTS ON THE GLYCOSAMINOGLYCAN SYNTHESIS AND MOLECULAR WEIGHT DISTRIBUTION *IN VITRO*

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(*Received* 17 *June* 1977; *accepted* 21 *September* 1977)

Abstract—The effect of cortisol at different concentrations on the incorporation rate of [^{3}H]glucosamine and [^{35}S]sulphate into glycosaminoglycans (GAGs) in human fibroblast culture medium was studied. The mol. wt distribution of the synthesized GAGs was determined by Sepharose 2B chromatography. Two sensitivity levels of GAGs to cortisol were observed: at a low cortisol concentration (1×10^{-7} M) only the hyaluronic acid synthesis decreased and no changes were observed in the synthesis of sulphated GAGs or glycoproteins. At a high steroid concentration (1×10^{-3} M) both the synthesis of hyaluronic acid and sulphated GAGs drastically decreased. The mol. wt distribution of medium GAGs did not change at cortisol concentrations 1×10^{-9} M–1×10^{-5} M. The possible role of cortisol in the metabolism of hyaluronic acid *in vivo* is discussed.

Anti-inflammatory steroids have many different effects on connective tissue [1]. They decrease the metabolic activity of the fibroblast, inhibit cell division and decrease the synthesis of collagen and glycosaminoglycans (GAGs) [2–10]. Karzel and Domenjoz [11] studied all these parameters under the same experimental conditions and found that the most sensitive response to prednisolone in cultured fibroblasts was the decrease of the synthesis of GAGs.

Anti-inflammatory steroids have also been shown to effect the physicochemical properties of extracellular components, e.g. collagen and GAGs. It has been suggested that the half life of collagen and both sulphated and non-sulphated GAGs have increased during steroid treatment [12, 13]. Cortisol and other anti-inflammatory steroids increase the viscosity of synovial fluid hyaluronic acid and decrease the relative amount of small mol. wt fractions *in vivo* of it [14]. The increase in the relative viscosity of GAGs by steroids is also found in cell culture media [15]. Cortisol has been shown to increase the viscosity of purified hyaluronic acid *in vitro*, even in the absence of cells [16].

The purpose of this study is to characterize the effect of cortisol on GAG synthesis in cultured human skin fibroblasts and to demonstrate the possible effects of the glucocorticoid on the mol. wt distribution of synthesized GAGs.

MATERIALS AND METHODS

Cells. Early passage (7–11) human foetal skin fibroblasts maintained in a monolayer culture in Dulbecco's modification of Eagle's minimal essential medium (Flow Laboratories, Irwine, U.K.) were used. The medium contained 10% foetal calf serum (Flow Laboratories), 100 U/ml penicillin, 50 μg/ml streptomycin sulphate, 20 mM HEPES (Gibco Biocult Ltd., Paisley, U.K.) and 23 mM sodium bicarbonate. For all experiments the cells were trypzined and suspended in a medium to a concentration of 2.5×10^5 cells per ml. This suspension was added to tissue culture flasks. After 3–5 days, when the cells had formed a dense confluent monolayer, a fresh medium was changed containing the isotope and different concentrations of cortisol (hydrocortisone sodium succinate, Orion, Mankkaa, Finland).

Experimental. The effect of different concentrations of cortisol on the synthesis of GAGs was studied by using 5.5 cm^2 cell culture flasks with 2 ml of the medium. Different concentrations of cortisol (1×10^{-10} M–1×10^{-3} M) and either 1.5 μCi/ml of [^{3}H]glucosamine (D- 1[^{3}H]glucosamine hydrochloride, 3.2 Ci/m-mole, The Radiochemical Centre, Amersham) or 3 μCi/ml of [^{35}S]sulphate (Sulphur-35, carrier free, The Radiochemical Centre) were added with the fresh medium and allowed to incorporate for 30 hr at 37°. At the end of the incubation the media were poured away and 400 μl aliquots of them were analyzed. The proteolytic digestion of the sample was carried out by papain (500 μg/sample, 60000 U/g, Merck, Germany) in the presence of 0.005 M Na-EDTA and 0.005 M cysteine-HCl at 60° for 6 hr [17]. After cooling the samples to room temperature, GAGs were precipitated for 3 hr with 1% cetylpyridinium chloride (CPC, Merck) in 0.02 N NaCl. The precipitate was then collected onto a MF-Millipore^R-membrane (pore size 0.45 μm, Millipore Corp., MA, U.S.A.). The nonprecipitable glycoproteins and free radioactivity were washed through with 0.05% CPC in 0.02 N NaCl until no radioactivity was detected in the washings. The Millipore^R membrane with the CPC-precipitable GAGs was dissolved in 10 ml of "Cello-POP" in a counting vial (Cello-POP, PPO 15 g, POPOP 50 mg, ethylenglycolmonomethylaether 600 ml and toluene 1000 ml) and counted in a Packard Tri-Carb Scin-

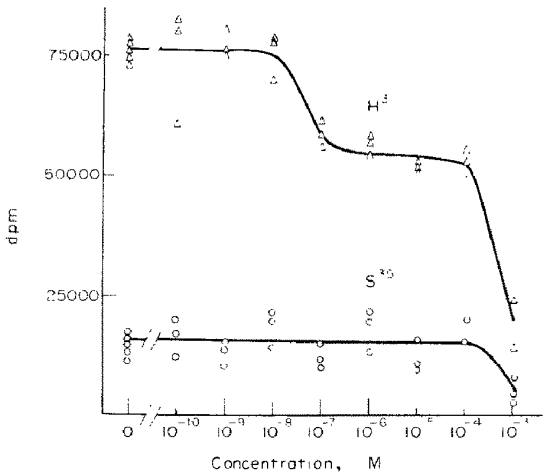

Fig. 1. Effect of cortisol on the amounts of [³H]glucosamine and [³⁵S]sulphate labeled GAGs in the medium. Values are given in dpm per tube.

tillation Spectrometer (model 3375, Packard Instrument Corp., Inc., IL, U.S.A.) with a counting efficiency of 13.2 per cent for tritium and about 90 per cent for [³⁵S] sulphur.

For further fractionation of medium GAGs, cells were grown in 25 cm² tissue culture flasks containing 4 ml medium. Fresh medium, containing 3 μCi/ml of [³H]glucosamine and cortisol ($1 \times 10^{-9} - 1 \times 10^{-5}$ M, in controls no drug) was added to the flasks and incubated for 30 hr. After incubation 2 ml of the media were dialyzed for 5 days against 0.02 M phosphate buffer (pH 7.4) and the remaining 2 ml of the media at the same time against 0.05 M Tris–HCl-buffer (pH 7.4) at + 4° by changing the dialysis fluid twice a day. The samples dialyzed against the Tris–HCl-buffer were fractionated by DEAE-cellulose ion-exchange chromatography [18]. The column (2.5 × 13 cm, DE 52 microgranular, Whatman Ltd., Maidstone, U.K.) was eluted at + 4° with a linear gradient of 0–0.6 M NaCl in 0.05 M Tris–HCl and 2 M urea, pH 7.4, over a total volume of 600 ml. Fractions of approximately 7 ml were collected and 2 ml of each were counted with 15 ml of "Tergitol-POP" in a scintillation spectrometer (Tergitol-POP: PPO 15 g, POPOP 50 mg, tergitol 600 ml and toluene 400 ml) with a counting efficiency of 12.0 per cent.

Aliquots (0.5 ml) of the media dialyzed against 0.02 M phosphate buffer were fractionated by Sepharose 2B gel filtration [18]. The column (0.9 × 14 cm, Sepharose 2B, Pharmacia Fine Chemicals, Uppsala, Sweden) was eluted with 0.02 M phosphate buffer, pH 7.4, with a flow 1 ml/13 min, 30 fractions of 0.4 ml were collected and each of them was analyzed by CPC-precipitation by collecting the labeled GAGs on Millipore® membrane as above.

RESULTS

Effect of cortisol on the synthesis of GAGs. The effect of different concentrations of cortisol on the incorporation of [³H]glucosamine into medium GAGs is shown in Fig. 1. Cortisol concentrations 1×10^{-8} M or less had no effect, while concentration 1×10^{-7} M lowered [³H]glucosamine incorporation to a level of about 35 per cent (30–45 per cent depending on the fibroblast culture used) below that of the controls. This new level was maintained up to cortisol concentration 1×10^{-4} M. Concentration above this lowered [³H]glucosamine incorporation into GAGs steeply, and it was about 20 per cent of controls at 1×10^{-3} of cortisol.

Low concentrations of cortisol did not affect the synthesis of sulphated GAGs. Decreased synthesis was observed only at a cortisol concentration of 1×10^{-3} M when the amount of [³⁵S]sulphate-labeled GAGs decreased to 25 per cent of the controls in the medium.

The above result obtained from [³H]glucosamine and [³⁵S]sulphate incorporations suggested that low levels of cortisol inhibited only the synthesis of non-sulphated GAGs. This was confirmed by DEAE-chromatography, where a similar fall in the synthesis of hyaluronic acid was observed between 1×10^{-7} M–1×10^{-5} M of cortisol (Fig. 2). At cortisol concentrations 1×10^{-9} M–1×10^{-5} M no changes were observed in the amounts of labeled sulphated GAGs or glycoproteins.

Effect of cortisol on the mol. wt distribution of newly-synthesized GAGs. The mol. wt distribution of [³H]glucosamine labeled GAGs, purified from contaminating labeled glycoproteins, is shown in Fig. 3. The GAG radioactivity was found to be distributed in the first sixteen of the twenty fractions containing incorporated [³H]glucosamine, while the

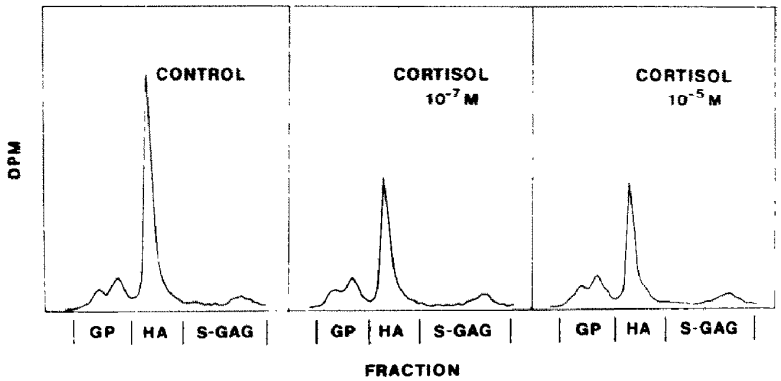

Fig. 2. Effect of cortisol on the [³H]glucosamine labeled fractions of cell culture medium in DEAE-chromatography. The graphs are means of duplicate.

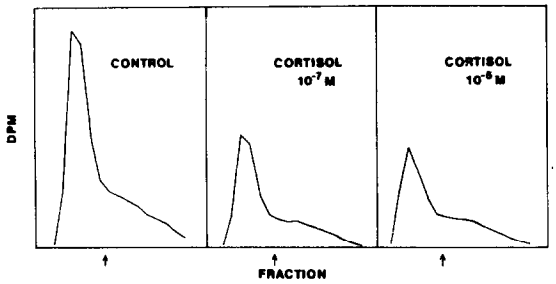

Fig. 3. Effect of cortisol on Sepharose 2B elution profiles of the [^{3}H]glucosamine labeled GAGs from the cell culture medium. The GAGs were purified from contaminating glycoproteins by CPC-precipitation. The lines are means of duplicates. The arrows show the end of fraction five.

remaining four fractions contained only labeled glycoproteins. The profiles of the Sepharose 2B chromatography curves were identical although the total synthesis of GAGs was decreased by cortisol. When the first five GAG-containing fractions were pooled to calculate the relative amount of GAGs in the excluded volume it was noticed that the relative amount of the high mol. wt fractions had not changed (Table 1).

DISCUSSION

The amount of [^{3}H]glucosamine labeled GAGs decreased by about 35 per cent with 1×10^{-7} M cortisol and a further decrease was observed with cortisol concentrations higher than 1×10^{-4} M. This nonlinear decrease in GAG-synthesis by cortisol was observed also by Castor and Dorstewitz [19] who found that the degree of inhibition of mucopolysaccharide synthesis in a synovial cell culture was about the same between cortisol concentrations 2×10^{-7} M–2×10^{-5} M. About 80 per cent or even more of incorporated [^{3}H]glucosamine in the medium GAGs was found in hyaluronic acid. Therefore the changes in the [^{3}H]glucosamine labeled GAGs mainly reflect variations in the synthesis of hyaluronic acid.

The decrease in the amount of labeled hyaluronic acid at low cortisol concentrations seemed to be independent of the synthesis of glycoproteins or sulphated GAGs in the short term culture used. A decrease in the synthesis of hyaluronic acid by cultured skin fibroblasts at low concentrations of different anti-inflammatory steroids has been also found in short time incubations, while the synthesis of sulphated GAGs remainded constant [20]. The changes in the amounts of labeled GAGs in cell layers followed those obtained in the media.

The second decrease in the amount of [^{3}H]glucosamine labeled GAGs in the medium observed at 1×10^{-3} M of cortisol was connected to a similar inhibition in the synthesis of both sulphated and non-sulphated GAGs. The inhibition in the synthesis of sulphated GAGs *in vitro* has been observed earlier at high cortisol concentrations, i.e. 1×10^{-4} M or more [8, 21]. There may be a question of a general inhibition of synthetic processes as suggested also by the inhibition of collagen synthesis at these high cortisol concentrations [21, 22].

The inhibition of cortisol seemed to be limited to the hyaluronate synthesis at low concentrations. Since UDP-*N*-acetylglucosamine is a common intermediate both in the synthesis of hyaluronic acid and sulphated GAGs, it is suggested that the regulation step in hyaluronic acid synthesis by cortisol occurs after UDP-*N*-acetylglucosamine formation.

One specific regulatory site to hyaluronic acid synthesis may be hyaluronic acid synthetase, which catalyzes the synthesis of hyaluronic acid from UDP-*N*-acetylglucosamine and UDP-glucuronic acid [23]. This enzyme has a short half life (2–3.5 hr), making rapid changes in the synthesis of hyaluronic acid possible. The activity of this enzyme correlates to the synthesis rate of hyaluronic acid and may be the site of cortisol action.

No changes in the mol. wt distribution of GAGs were observed, even though their total radioactivity fell to about 60 per cent of the controls. However, cortisol increases the mol. wt and relative viscosity of the abnormal hyaluronic acid in the synovial fluid during treatment of rheumatoid arthritis [14, 24]. Rheumatoid synovial fibroblasts in a culture synthesize hyaluronic acid that is not excluded from Sepharose 2B [18]. In our preliminary experiments *in vitro*, cortisol did not affect this altered hyaluronic acid distribution, either, even though it decreased the total synthesis of hyaluronic acid about 60 per cent also by these cells.

The normal early morning values of human plasma cortisol are known to be 6×10^{-8} M–6×10^{-7} M, and the cortisol level falls to nearly zero at night [25, 26]. The inhibition in the synthesis of hyaluronic acid was observed at physiologic plasma concentrations, indicating that this phenomenon might have some relevance in connective tissue regulation and diseases *in vivo* as well in their treatment. In acute inflammation the synthesis of hyal-

Table 1. Effect of cortisol on the amount of labeled GAGs in the cell culture medium and its distribution in Sepharose 2B chromatography. The amount of labeled GAGs in the excluded volume was calculated by pooling the radioactivities in the first five fractions. The values are means of duplicates

Amount of labeled GAGs (DPM)	Cortisol concentration (M)					
	0	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}
Total	100110	103785	86219	52891	42783	49116
Excluded volume	66680	67823	48560	35596	27509	30894
Excluded volume (% of total)	66.0	65.4	63.8	67.3	64.3	62.9

uronic acid is increased and its inhibition by steroids may have a useful therapeutic influence. In topical corticosteroid therapy as well as in the experimental treatment of normal skin, the early degenerative changes are found in the connective tissue ground substance, while changes in cells and collagen are less prominent [27]. Thus the inhibition of the hyaluronate synthesis seems to be an early event, which is followed by changes in the cell number and the production of fibrous proteins in prolonged local steroid treatment resulting in skin atrophy.

Acknowledgements—The author would like to thank Professor V. K. Hopsu-Havu and Dr. M. Tammi for their helpful discussion and criticism during the preparation of this manuscript. He is grateful to Professor E. Kulonen, in whose laboratory this work was carried out. This investigation was supported by a grant from the Emil Aaltonen Foundation.

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