Glucocorticoids Stimulate Collagen and Noncollagen Protein Synthesis in Cultured Vascular Smooth Muscle Cells

DALE C. LEITMAN, STEPHEN C. BENSON,* and LORIN K. JOHNSON

Department of Pathology, Stanford University School of Medicine and Geriatric Research, Education, and Clinical Center, Palo Alto Veterans Administration Medical Center, Palo Alto, California 94304; *Department of Biological Sciences, California State University, Hayward, California 94542. Dr. Johnson's present address is California Biotechnology Inc., Mountain View, California, 94043.

ABSTRACT   The effect of glucocorticoids on collagen synthesis was examined in cultured bovine aortic smooth muscle (BASM) cells. BASM cells treated with 0.1 μM dexamethasone during their proliferative phase (11 d) were labeled with [³H]proline for 24 h, and the acid-precipitable material was incubated with bacterial collagenase. Dexamethasone produced an approximate twofold increase in the incorporation of proline into collagenase-digestible protein (CDP) and noncollagen protein (NCP) in the cell layer and medium. The stimulation was present in both primary mass cultures and cloned BASM. An increase in CDP and NCP was detected at 0.1 nM, while maximal stimulation occurred at 0.1 μM. Only cells exposed to dexamethasone during their log phase of growth (1–6 d after plating) showed the increase in CDP and NCP when labeled 11 d after plating. The stimulatory effect was observed in BASM cells treated with the natural bovine glucocorticoid, cortisol, dexamethasone, and testosterone, but was absent in cells treated with aldosterone, corticosterone, cholesterol, 17 β-estradiol, and progesterone. The increase in CDP and NCP was absent in cells treated with the inactive glucocorticoid, epicortisol, and totally abolished by the antagonist, 17α-hydroxyprogesterone, suggesting that the response was mediated by specific cytoplasmic glucocorticoid receptors. Dexamethasone-treated BASM cells showed a 4.5-fold increase in the specific activity of intracellular proline, which was the result of a twofold increase in the uptake of proline and depletion of the total proline pool. After normalizing for specific activity, dexamethasone produced a 2.4- and 2.8-fold increase in the rate of collagen and NCP synthesis, respectively. Cells treated with dexamethasone secreted 1.7-fold more collagen protein in 24 h compared to control cultures. The BASM cells secreted 70% Type I and 30% Type III collagen into the media as assessed by two-dimensional gel electrophoresis. The ratio of these two types was not altered by dexamethasone. The results of the present study deomonstrate that glucocorticoids can act directly on vascular smooth muscle cells to increase the synthesis and secretion of collagen and NCP.

Collagen is a major structural protein secreted into the extra-cellular space where it is involved in several diverse processes, including platelet aggregation, cell adhesion, differentiation, and cell proliferation (for a review, see reference 1). In addition to collagen's role in normal processes, alterations in collagen metabolism are key components in the pathogenesis of skin, and vascular diseases and pulmonary fibrosis (2). Although the underlying mechanisms that regulate collagen metabolism during normal and pathological states are poorly understood, many factors influence the rate and types of collagen synthesized (3). Prominent among these factors are several steroid hormones (4, 5), particularly glucocorticoids that exert diverse effects on collagen metabolism in connective tissue cells (for a review, see reference 6).

Most reports have indicated that glucocorticoids inhibit the rate of collagen synthesis in dermal fibroblasts (7), skin (8), granulation tissue (9), and hepatocytes (10). While the exact nature of this inhibition is unknown, collagen synthesis has

been shown to decline nonselectively (9) as a result of a generalized anti-anabolic effect which leads to a decrease in cell growth (11, 12), DNA synthesis (11, 12), and protein synthesis (8–11). Other investigators, however, have reported that glucocorticoid treatment selectively decreases collagen synthesis in rat skin (8) and fibroblasts from normal and keloid skin (12). More recently, it has been demonstrated that glucocorticoids produce a specific decrease in collagen messenger RNA in rat lung and skin (13) and chick tendon cells (14) when measured by an in vitro translation system.

In connective tissue-derived cells glucocorticoids are also known to inhibit the enzymes prolyl hydroxylase (15–17), lysyl hydroxylase (16), glucosyltransferase (16), galactosyltransferase (16), and lysyl oxidase (17), all of which are involved in the posttranslational modification and resultant maturation of collagen. The inhibition of these enzymes by glucocorticoids may result in a decline in net collagen by decreasing its stability, leading to enhanced degradation (1). Regardless of the mechanism of action of glucocorticoids at each of these loci, it is clear from the above studies that these steroids may be involved in regulating collagen levels by altering its synthesis, degradation, or cross-linking. While studies examining the glucocorticoid regulation of collagen synthesis in vitro have been limited primarily to connective tissue cells, the present report has examined their effects on vascular smooth muscle cells grown in culture.

Numerous laboratories have reported that vascular smooth muscle cells synthesize and secrete Types I and III collagens and, to a lesser extent, Type V collagen both in vivo and in vitro (18–20). Recently, attention has focused on the factors that influence the quantity and types of collagen synthesized by vascular smooth muscle cells because they are primarily responsible for elaborating collagen and other extracellular material that accumulates in the intima during atherosclerosis (21). In this regard, platelets (22), estradiol (23), insulin, and diabetic serum (24) have been shown to alter collagen synthesis in smooth muscle cells.

In view of the known effects of glucocorticoids on collagen metabolism in connective tissue, the increased rate of collagen synthesis and deposition during vascular diseases (25, 26), and our recent studies that indicate that glucocorticoids inhibit smooth muscle cell proliferation (27, 28), we investigated the effect of these steroids on collagen synthesis by both cloned and primary mass cultured bovine aortic smooth muscle cells. Our study demonstrates that glucocorticoid treatment of rapidly proliferating aortic smooth muscle cells results in a nonselective increase in the rate of collagen synthesis and secretion. In contrast, a progressive decrease in collagen synthesis was observed in cells exposed to glucocorticoids after reaching confluence.

MATERIALS AND METHODS

Dulbecco's modified Eagle's medium (DME),[1] Dulbecco's phosphate-buffered saline (PBS), trypsin, penicillin-streptomycin, fungizone, and glutamine were purchased from Grand Island Biological, Co. (Grand Island, NY). Bovine calf serum was obtained from Sterile Systems Inc., (Logan, UT). Tissue culture dishes were obtained from Costar Division, Bellco Glass Inc., (Vineland, NJ).

---

[1] *Abbreviations used in this paper:* DME, Dubecco's modified Eagle's medium; BASM, bovine aortic smooth muscle; CDP, collagenase-digestible protein; NCP, noncollagen protein; FGF, fibroblast growth factor; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; TEMED, N,N,N',N'-tetramethylethylenediame; SAS, solubilizer for aqueous samples; butyl PBD, 2-(4'-t-butylphenyl)-5-4"-biphenyl)-1,3,4-oxadiazole.

Acrylamide, bis, Coomassie Blue (R-250), TEMED, and ammonium persulfate were obtained from Bio-Rad Laboratories (Richmond, CA). Specially pure SDS was obtained from British Drug Houses (Poole, England). Ampholines were purchased from LKB Instruments (Rockville, MD). L-[5-³H]proline (10–20 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). Pepsin was obtained from Worthington Biochemicals (Freehold, NJ). Clostridial collagenase (Form III) was purchased from Advance Biofactures Corp. (Lynbrook, NY). SAS and butyl PBD were obtained from Research Products International Corp. (Mount Prospect, IL). Protosol and omnifluor were obtained from New England Nuclear (Boston, MA) and Sodium salicylate from Mallinckrodt Inc. (Paris, KY). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

Bovine aortic smooth muscle (BASM) cells were isolated from an adult aortic arch by collagenase/elastase digestion according to the method of Chamley-Campbell et al. (29). Following establishment of the primary culture, the cells were cloned in the presence of fibroblast growth factor (FGF) as previously described (27).

Stock BASM cells were maintained and subcultured in DME (H-16) containing 3.7 g/liter NaHCO₃, 50 U/ml penicillin, 50 μg/ml streptomycin, 2.5 μg/ml fungizone, and 0.6 mg/ml glutamine. For cloned cells, FGF (250 ng/ml) was added every other day to the stock cultures until the cells reached subconfluent densities. The cells were passaged weekly at a seeding density of 5 × 10⁴ cells/60-mm dish and incubated at 37°C in a 5% CO₂ humidified incubator. Smooth muscle extracellular matrix-coated dishes were prepared as described previously (28).

For experiments, stock cells were detached with 0.025% trypsin: 1 mM EDTA solution, and counted with a hemocytometer. Cells were seeded in 60-mm tissue culture dishes at a density of 50,000 cells/dish. Steroids were dissolved in 95% ethanol and added to the medium at a 1:1,000 dilution. Control cells received an equal volume of ethanol. 5 d after plating, the medium was changed and the steroid was replaced. When the cells reached confluence (day 10–11), they were washed with 3 ml of DME and preincubated for 1 h in 2 ml of DME containing penicillin, streptomycin, fungizone, 50 μg/ml sodium ascorbate, and 80 μg/ml β-aminopropionitrile. The medium was aspirated and replaced with 2 ml of fresh preincubation medium containing 10–20 μCi/ml L-5[³H]proline and the cells were incubated at 37°C for 24 h. Steroids were added during both the preincubation and incubation periods. Following incubation, the medium was harvested into plastic tubes containing phenylmethylsulfonyl fluoride, N-ethylmaleimide, EDTA, to yield final concentrations of 0.2, 10, and 2.5 mM, respectively. The cells were washed twice with PBS, and the first wash was combined with the appropriate medium. Cellular material was removed from the medium by centrifugation at 1,000 g for 5 min at 4°C. The media and tissue culture dishes were stored at −70°C until analysis.

Determination of the Amount of Incorporation of [³H]Proline into Collagen and Noncollagen Protein

MEDIUM: Proteins in the medium were precipitated with 10% TCA containing 1 mM proline in the presence of 100 μg/ml bovine serum albumin. After chilling on ice for 1 h, the samples were centrifuged at 35,000 g for 30 min at 4°C. Unincorporated [³H]proline was removed by resuspending the pellet twice in 10% trichloroacetic acid (TCA). The pellet was dissolved in 0.2 N NaOH, and the collagenase-digestable protein (CDP) and noncollagen protein (NCP) were quantitated as described by Peterkofsky and Diegelmann (30) using 100 U/ml purified Clostridial collagenase. The collagenase used in the following experiments was found to be free of proteolytic activity as assayed with [³H]tryptophan-labeled BASM medium proteins.

CELL LAYER: The cell layer was solubilized in 0.5 N NH₄OH and removed from the tissue culture dishes with the aid of a rubber policeman. TCA was added to the samples to a final concentration of 10%. The samples were then centrifuged, and the washed pellets were dissolved in 0.2 N NaOH as described above. An aliquot of the NaOH solution was either incubated with collagenase for CDP and NCP determination or added directly to SAS:butyl PBD toluene cocktail to determine total [³H]proline incorporation. The remaining portion was used to quantitate DNA by the method of Burton (31) using calf thymus DNA as a standard.

Determination of the Intracellular Proline Specific Activity and Rate of Protein Synthesis

Confluent control and dexamethasone-treated BASM cells were pulsed with 30 μCi/ml [³H]proline for 30 min at 37°C. After aspiration of the medium, the

cells were rapidly washed four times with 5 ml of ice-cold PBS and the tissue culture dishes were immediately stored at −70°C. The cell layer was solubilized in 0.5 N NH₄OH and precipitated with 10% TCA without proline. The acid-precipitable material was dissolved in 0.2 N NaOH and CDP, NCP, and DNA was determined. The first TCA-soluble fractions from three dishes were combined to quantitate the specific activity of the intracellular proline. To measure the amount of unincorporated [³H]proline, we counted an aliquot of the TCA-soluble fraction in SAS:butyl PBD toluene. The total intracellular proline content was determined by the colorimetric method of Troll and Lindsley (32) after the TCA was extracted five times with water-saturated ether. The rate of [³H]proline incorporation was normalized by dividing the total incorporation of [³H]proline into CDP and NCP by the specific activity of the free intracellular proline.

Preparation of Collagen Standards

Collagen Types I and III were purified from bovine placenta after digestion with pepsin for 24 h at 4°C (33). The pepsin-digested material was centrifuged at 10,000 g for 1 h at 4°C, and crystalline NaCl was added to the supernatant fraction to a final concentration of 0.7 M. The precipitate that developed after stirring overnight at 4°C was dissolved in 1 M NaCl, 50 mM Tris-HCl, pH 7.8. Types I and III collagens were purified by repeated salt fractionation at 2.6 and 1.6 M, respectively (34).

Determination of the Types and Specific Activity of Secreted Collagen

BASM cells were treated with dexamethasone and labeled with 10 μCi/ml [³H]proline for 24 h as described above. The media was harvested into protease inhibitors and dialyzed against 0.5 M acetic acid for 48 h at 4°C. Noncollagen protein was degraded by the addition of 2.5 μg/ml pepsin directly into the dialysis tubing. The samples were dialyzed for an additional 18 h, immediately frozen, and then lyophilized. To determine the specific activity of collagen, we dissolved the lyophilized samples in sample buffer (35) by boiling for 5 min. The collagen chains were separated by interrupted SDS PAGE according to the methods of Sykes (36) and Laemmli (35) using a 5% separating gel containing 0.5 M urea. Following SDS PAGE the gels were stained with 0.1% Coomassie Brilliant Blue (R-250) in 50% TCA for 15 min. The gels were destained overnight with 7% acetic acid:5% methanol solution, and the amount of collagen present was quantitated by measuring the area of absorbance of the Coomassie-Blue-stained bands at 595 nm with a Beckman spectrophometer (Beckman Instruments, Inc., Palo Alto, CA), equipped with a gel scanner. The absolute amount of collagen secreted by BASM cells was determined from a standard curve established using purified Type I collagen. To quantitate the amount of radioactivity in the α₁(I) and α₂(I) chains of Type I collagen, we excised the bands from the gel and solubilized in 10 ml of 3% protosol in omnifluor-toluene by shaking in a water bath for 18 h at 37°C. To determine which collagen types were secreted by the cells, we dissolved the lyophilized media proteins in 50 μl of lysis buffer, and performed nonequilibrium two-dimensional gel electrophoresis as described by O'Farrell et al. (37), except that the samples were electrophoresed for 4 h at 400 V in a pH 5–7 gradient. For the second dimension, we used a 5% separating gel containing 0.5 M urea to fractionate the collagen chains. Under these conditions, the α₁(III) chain of type III collagen could be separated from the α₁(I) chain without delayed reduction and interruption of electrophoresis. For fluorography, the gels were stained and destained as described above, incubated for 30 min in 1 M sodium salicylate (38), and dried on Whatman 3MM filter paper (Whatman Laboratory Products, Inc., Clifton, NJ) under vacuum with heating. The dried gels were exposed in Kodak K-OMAT film at −70°C.

Statistical Analysis

Each data point represents the mean of either duplicate or triplicate samples ± standard error of the mean. In figures where error bars are absent, the standard error was <10%.

RESULTS

This study was undertaken to examine the direct effect of glucocorticoids on collagen synthesis by vascular smooth muscle cells in vitro. In two recent studies (27, 28) we showed that primary cultures as well as a cloned strain of aortic smooth muscle cells were growth inhibited by glucocorticoids. To determine whether the biochemical response to glucocorticoids in the primary and cloned BASM cells was also similar,

cells from each culture were treated with 0.1 μM dexamethasone throughout their growth phase (11 d) and the incorporation of [³H]proline into medium CDP and NCP was quantitated. As shown in Table I, primary BASM cells treated with dexamethasone had a 2.2-fold increase in CDP and a 2.6-fold increase in NCP. The direction of the response was the same in the cloned BASM cells, although the magnitude of the increase was slightly less (1.7-fold increase in CDP and 2.2-fold increase in NCP). No major difference in the absolute amount of incorporation of [³H]proline into CDP and NCP was observed in the two BASM cultures. However, there was a greater percentage of collagen present in the medium of the cloned BASM. The results of this experiment demonstrate that dexamethasone produces a similar increase in the incorporation of [³H]proline into CDP and NCP in both the primary mass cultures and cloned BASM cells. Because of this observation, we used the cloned BASM cells in future experiments to further characterize the glucocorticoid effect since these cells provided the advantage of being a stable cell strain with an extended lifespan. Although the BASM were cloned and the stocks maintained in FGF, all cells in the following studies were grown and analyzed in the absence of FGF.

Influence of Glucocorticoids on [³H]Proline Incorporation into Cell Layer and Medium Protein

As shown in Fig. 1, when BASM cells were treated with dexamethasone during their proliferative phase (11 d) and then labeled at confluence with [³H]proline, a large increase in proline incorporation into acid-precipitable material was observed in both the cell layer and medium fractions during the 24-h labeling period. In the cell layer fraction (Fig. 1, upper panel) the glucocorticoid-induced increase was greatest in the earliest time periods examined (between 0.5 and 2 h). During this linear period of incorporation the rate of [³H]proline incorporation in the dexamethasone-treated cells was 3.0 × 10⁴ dpm/h/μg DNA compared to 0.7 × 10⁴ dpm/h/μg DNA in the control cells. After 6 h, the rate of proline incorporation began to plateau in both the treated and untreated cells and a steady state between synthesis, degradation, and secretion was apparently reached by 8 h, and was maintained for at least 24 h.

The data in Fig. 1, lower panel, show that [³H]proline

TABLE I

Effect of Dexamethasone on the Incorporation of [³H]Proline into CDP and NCP

	CDP	NCP	% Collagen
	(dpm/μg DNA)		
Primary			
Control	5,332 ± 130	3,183 ± 67	23.7 ± 0.04
DEX (0.1 μM)	11,979 ± 924	8,355 ± 801	21.5 ± 0.54
Cloned			
Control	8,123 ± 591	2,775 ± 153	35.1 ± 0.04
DEX	14,241 ± 193	6,077 ± 718	30.4 ± 1.6

Primary mass culture (second passage) and cloned smooth muscle cells were treated with 0.1 μM dexamethasone for 11 d. At confluence the cells were labeled with 10 μCi/ml [³H]proline for 24 h. The incorporation of radioactive proline into medium CDP and NCP was determined by collagenase digestion as described in Materials and Methods. The percent collagen was calculated according to Peterkofsky and Diegelmann (30), using the equation: % collagen = dpm(CDP)/dpm(CDP) + 5.4 dpm(NCP). Each determination represents the average of triplicate culture dishes ± SE.

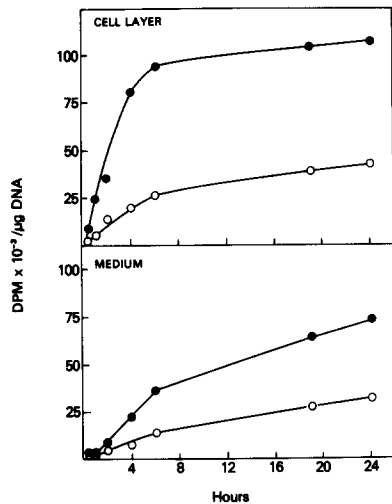FIGURE 1 Effect of dexamethasone on the rate of incorporation of [³H]proline into acid-precipitable material. Smooth muscle cells were grown in the absence (O) or presence (●) of 0.1 μM dexamethasone for 10 d. After the cells were labeled at confluence with 10 μCi/ml [³H]proline for various times, the total incorporation of proline into cell layer (*upper*) and medium (*lower*) protein was quantitated following precipitation with trichloroacetic acid. Each point represents the mean of duplicate culture dishes.

containing acid-precipitable material is first detected in the culture medium ~2 h after labeling. During the period of linear secretion (2-6 h), the dexamethasone-treated cultures secreted 9.4 × 10³ dpm/h/μg DNA while the control cells secreted 3.0 × 10³ dpm/μg DNA. Thus, during the initial linear period of labeling, dexamethasone increased [³H]proline incorporation by 4.3-fold in the cell layer and 2.9-fold in the medium. After 24 h these differences were 2.5-fold and 2.1-fold, respectively. In the following experiments, 24 h was chosen for a labeling time since the incorporation of proline into cell layer protein was at a steady state.

Glucocorticoid Dose-Response on Smooth Muscle Cell Collagen Synthesis

The effect of increasing concentrations of dexamethasone on the incorporation of [³H]proline into CDP and NCP was examined by digestion of acid-precipitable material in the cell layer and medium with bacterial collagenase. The data shown in Fig. 2 demonstrate that dexamethasone produced a dose-dependent increase in the incorporation of [³H]proline into both CDP and NCP. For both CDP and NCP in the cell layer and medium, the dose of dexamethasone required to elicit a half-maximal response was ~1 nM, and a maximal stimulation was observed at a steroid dose of 0.1 μM. At maximal steroid dose, dexamethasone increased the amount of CDP by 1.6-fold and NCP by 2.3-fold in the cell layer. This resulted in an ~50% decrease in the percentage of label appearing in the CDP fraction (0.85% collagen in control cultures vs. 0.52% collagen in cultures treated with 0.1 μM dexamethasone). No major change in percent collagen was observed in the medium fraction since dexamethasone produced a twofold increase in CDP and a 2.2-fold increase in NCP. When the incorporation of [³H]proline into the cell layer and medium CDP and NCP are considered together, dexamethasone-treated cultures had a 23% decrease in percent collagen. The results described above also show that 92% of all collagen present after 24-h labeling is found in the culture medium.
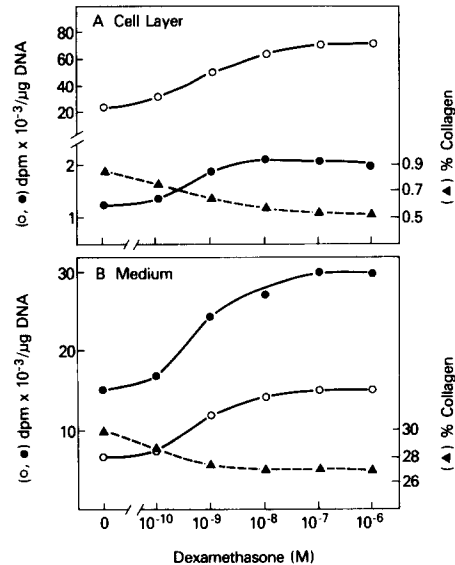

FIGURE 2 Effect of increasing concentrations of dexamethasone on the incorporation of [³H]proline into CDP and NCP. Smooth muscle cells were exposed to various concentrations of dexamethasone for 11 d and then were labeled with 20 μCi/ml [³H]proline for 24 h. The incorporation of proline into cell layer (A) and medium (B) CDP (●) and NCP (O) and percent collagen (▲) was determined as described in Materials and Methods. Each determination represents the mean of duplicate culture dishes.

Because of this observation the following studies examining the effect of glucocorticoids on collagen synthesis were limited to the medium fraction.

Influence of Proliferation Rate or Cell Density on the Cellular Sensitivity to Glucocorticoid Treatment

The influence of BASM proliferation rate on the glucocorticoid effect on CDP and NCP production was examined by exposing cultures to 0.1 μM dexamethasone for various periods of time during the growth phase. All cells were plated at 50,000 cells dish, and dexamethasone was added to duplicate cultures at the time of plating and every other day thereafter until the cells reached confluence. All cultures were then labeled with [³H]proline on the eleventh day after plating. Fig. 3A shows that the twofold increase in [³H]proline incorporation into secreted CDP and NCP occurred only in BASM cultures exposed to dexamethasone for 7-11 d before confluence. In contrast, cells exposed to dexamethasone for 0.5-5 d before reaching confluence showed a small decrease in CDP and no change in NCP. Growth rate analysis showed that the stimulatory effect was present in BASM cells exposed to dexamethasone only during their log phase of growth. We also examined the effect of dexamethasone on stationary BASM cells. In this case, cultures were grown in the absence of dexamethasone for 11 d and were then exposed to 0.1 μM dexamethasone for 1, 3, and 5 d after reaching confluence. Fig. 3B shows that dexamethasone produced a progressive decrease in the incorporation of [³H]proline into CDP without affecting proline incorporation into NCP. Thus, these results clearly demonstrate that the direction of the glucocorticoid response is dependent upon whether the BASM cells are proliferating or stationary when exposed to glucocorticoids.

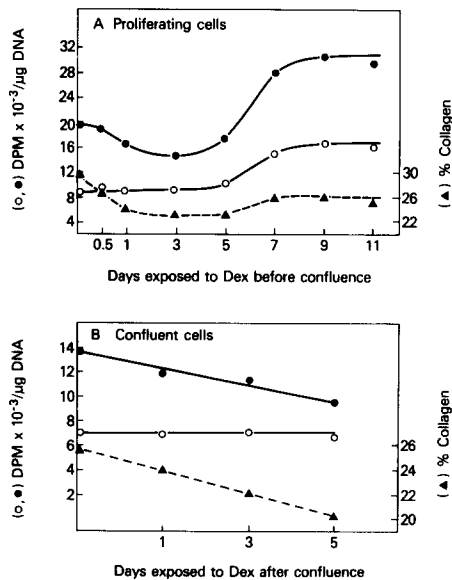We have shown previously that glucocorticoids inhibit the

FIGURE 3 Influence of proliferation rate or cell density on the response of smooth muscle cells to dexamethasone. (A) 0.1 µM dexamethasone was added to duplicate culture dishes beginning on the day of plating and every other day thereafter until the cells reached confluence. All cultures were labeled, on the eleventh day after plating, with 10 µCi/ml [³H]proline for 24 h. (B) Smooth muscle cells were grown in the absence of dexamethasone for 11 d. The cells were then treated with dexamethasone (0.1 µM) for various times, and all cultures were labeled for 24 h 5 d after reaching confluence. The medium fraction was analyzed for CDP (●), NCP (○), and % collagen (▲) as described in Materials and Methods. Each determination represents the mean of duplicate culture dishes.
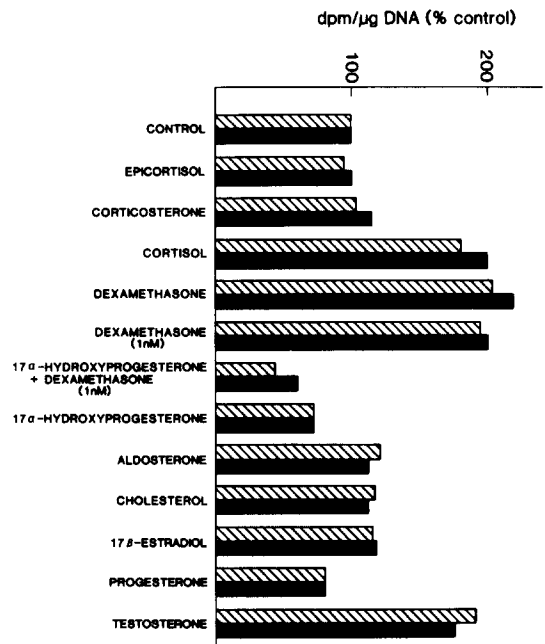

FIGURE 4 Effect of various steroids on the incorporation of [³H]-proline into CDP and NCP. All steroids were added to the medium on the day of plating and every other day thereafter until confluence to a final concentration of 0.1 µM unless otherwise indicated. All cultures were labeled on the eleventh day after plating with [³H]-proline for 24 h, and CDP (striped bar) and NCP (filled bar) was determined as described in Materials and Methods. Each determination represents the mean of duplicate culture dishes.

rate of proliferation and saturation density of this BASM clone by ~40% (27). These observations suggest the possibility that the increase in proline incorporation may have resulted from the lower saturation density attained in the glucocorticoid-treated cells rather than being a direct effect on protein synthesis. To examine this question, we took advantage of our previous observation that BASM extracellular matrix abolishes the glucocorticoid inhibition of cell proliferation (28). When BASM cells were grown on matrix in the presence of 0.1 µM dexamethasone for 11 d, CDP and NCP were increased to the same extent as seen in BASM cells grown on plastic (data not shown). Thus, it is apparent from these studies that the glucocorticoid stimulatory effect on CDP and NCP occurs independent of the inhibitory action of this steroid on BASM proliferation or saturation density.

Steroid Specificity of the Glucocorticoid Effect

The steroid specificity of the glucocorticoid effect on collagen and NCP was examined by treating smooth muscle cell cultures with various steroids throughout their proliferative phase (11 d). The data shown in Fig. 4 demonstrate that the stimulatory effect is glucocorticoid specific and is also manifested by certain natural glucocorticoids as well as dexamethasone. No increase in CDP and NCP was observed in BASM cells treated with corticosterone or the inactive glucocorticoid, epicortisol (17-α cortisol). Consistent with our previous results, 0.1 µM dexamethasone produced over a twofold increase in CDP and NCP, while hydrocortisone used at a concentration of 0.1 µM was as potent as 1 nM dexamethasone. The glucocorticoid antagonist, 17 α-hydroxyprogesterone, decreased CDP and NCP and completely abolished the

dexamethasone-mediated stimulation of CDP and NCP. A slight decrease in CDP and NCP was observed in BASM cells treated with progesterone, while minor increases occurred in cells treated with aldosterone, cholesterol, and 17-β estradiol. In addition to the glucocorticoids, testosterone was the only other steroid tested to produce a major increase in CDP and NCP. In view of the recent report by Horwitz and Korwitz (39) which identified testosterone receptors in the dog aorta, it seems likely that this effect is mediated through the androgen receptor system. Finally, none of the steroids tested produced a major alteration in the percent of collagen present in the medium.

Influence of Glucocorticoids on the Specific Activity of the Intracellular Proline Pool and Rate of Collagen and NCP Synthesis

To further examine the mechanism of the glucocorticoid stimulation of CDP, we treated smooth muscle cells for 11 d and then determined the synthetic rate of CDP and NCP during a 30-min pulse with [³H]proline to minimize any influence of degradation. To adequately estimate the initial rate of protein synthesis, we also determined the specific activity of the intracellular proline pool after this labeling period.

As shown in Fig. 5A, the dexamethasone-treated BASM cells had a 2.3-fold increase in the uptake of [³H]proline as determined by measuring the amount of radioactivity in the acid-soluble fraction of the cell layer. In addition, the dexamethasone-treated cells contained only one-half the quantity of total free proline inside the cells as assessed by colorimetric measurement (Fig. 5B). These two changes resulted in a 4.5-
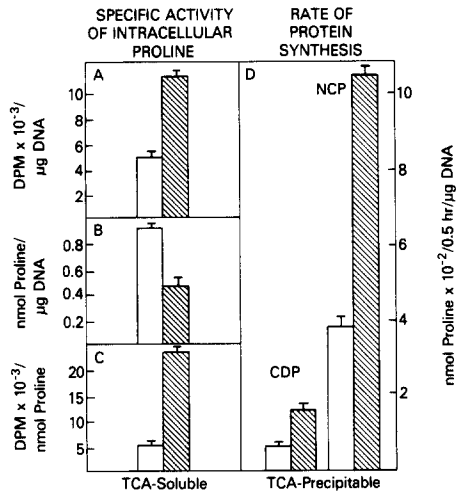
FIGURE 5 Effect of dexamethasone on the intracellular proline specific activity and rate of collagen and noncollagen protein synthesis. Smooth muscle cells were grown for 11 d in the absence (empty bar) or presence (striped bar) of 0.1 μM dexamethasone. At confluence the cells were labeled with 30 μCi/ml [^{3}H]proline for 30 min at 37°C. The uptake of radioactive proline (A), the total intracellular proline content (B), the intracellular proline specific activity (C), and the rate of collagen and non-collagen protein synthesis (D) was measured as described in Materials and Methods. Each determination represents the mean ± SE from three pooled culture dishes done in triplicate.
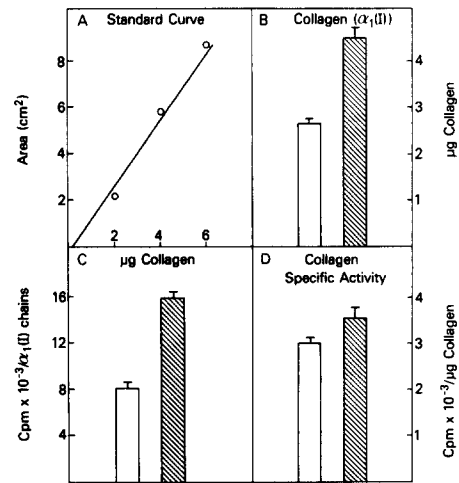

FIGURE 6 Effect of dexamethasone on the specific activity of secreted collagen. Control (empty bar) and dexamethasone-treated (striped bar) smooth muscle cells were labeled with 10 μCi/ml [^{3}H]proline for 24 h. The medium proteins were digested by pepsin, and the resultant collagen chains were separated by interrupted SDS PAGE. (A) Standard curve established by measuring the absorbance of Coomassie Blue of the α_1(I), band from purified bovine Type I collagen. (B) The absolute amount of the α_1(I) chains secreted by smooth muscle cells after 24 h. (C) The amount of radioactivity present in the α_1(I) band from smooth muscle cells. (D) The specific activity of secreted collagen. Each determination represents the mean + SE of medium samples from triplicate culture dishes.

fold increase in the specific activity of the proline pool (Fig. 5C). When the incorporation of [^{3}H]proline into CDP and NCP was normalized for the observed differences in the specific activity of the proline pool, dexamethasone produced an increase in the rate of collagen synthesis by 2.4-fold and NCP synthesis by 2.8-fold (Fig. 5D).

Influence of Glucocorticoids on the Specific Activity of Secreted Collagen

The previous studies have demonstrated that glucocorticoids increase the incorporation of [^{3}H]proline into CDP and NCP as a result of an increased rate of protein synthesis. However, these experiments did not provide direct evidence to show that glucocorticoids increase the absolute amount of collagen secreted by the cells. To address this question, we determined the specific activity of secreted collagen. In this case pepsin-digested [^{3}H]proline labeled medium proteins were separated by interrupted SDS PAGE, and the amount of collagen present in the gel was determined by measuring the absorbance of the Coomassie-Blue-stained bands. Fig. 6A shows that there is a linear relationship between the amount of purified Type I collagen standard loaded on the gel and the area of absorbance of the collagen band. When the media from control and treated cells were analyzed, it was found that the control cells secreted 2.7 μg of the α_1(I) chains of Type I collagen after 24 h compared to 4.5 μg secreted by the dexamethasone-treated cultures (Fig. 6B). To determine the amount of radioactivity present in collagen, the α_1(I) bands were excised from the gel and solubilized. Fig. 6C shows that the α_1(I) bands from the dexamethasone-treated cells contained twice the radioactivity of the α_1(I) band from the control cells. Using the above data, it was calculated that the specific activity of the α_1(I) chains was ~20% greater in the dexamethasone-treated cells (Fig. 6D). Similar results were

obtained when the α_2(I) chains of Type I collagen were analyzed for specific activity. These results demonstrate that dexamethasone increased the specific activity of secreted collagen by 20%, but cannot account for the twofold increase in the incorporation of [^{3}H]proline into collagen that we have consistently observed throughout this study. Furthermore, these data suggest that the glucocorticoid-treated BASM cultures secreted ~70% more collagen into the medium after 24 h.

Influence of Glucocorticoids on the Types of Collagen Secreted by BASM Cells

At the present time, five major tissue-specific and structurally distinct collagen types have been identified in mammalians (1). Although the role of each type is unknown, changes in the types of collagen produced in various tissues may be important in certain pathological conditions (2). Recently, it has been reported that several hormones, including 17 β-estradiol, alter the ratio of collagen types synthesized in cultured vascular smooth muscle cells (23). To investigate whether glucocorticoids change the proportion of collagen types secreted, [^{3}H]proline-labeled medium proteins were digested with pepsin, and the resultant collagen α-chains were separated by two-dimensional gel electrophoresis. As shown in Fig. 7 the α-chains of Type I and Type III collagens can be resolved by this technique using a pH 5–7 gradient in the first dimension. The α-chains present in BASM media from both the control (Fig. 7A) and the dexamethasone-treated cells (Fig. 7B) comigrated with the purified α_1(III) standard and the α_1(I) and α_2(I) standards (Fig. 7C) and demonstrate that these cells secreted Types I and III collagens. Densitometric scanning of the fluorograms revealed that both the untreated and dexamethasone-treated cells secreted ~70% Type I and
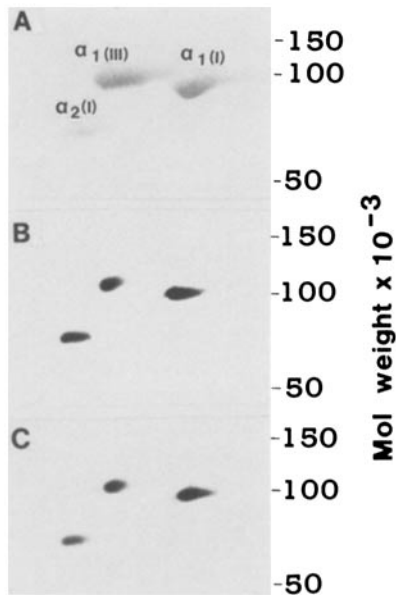
FIGURE 7 Effect of dexamethasone on the type of collagen secreted by smooth muscle cells. Smooth muscle cells were grown in the absence (B) or presence (C) of 0.1 μM dexamethasone for 11 d. The cells were labeled with 10 μCi/ml [^{3}H]proline for 24 h, and the medium was harvested and digested with pepsin. The resultant collagen chains were separated by two-dimensional electrophoresis, and the gels were fluorographed. (A) Coomassie-Blue-stained types I and III collagen standards purified from bovine placenta.

30% Type III collagens. These data indicate that while glucocorticoids increase the rate of collagen synthesis they do not specifically alter the types of collagen secreted by aortic smooth muscle cells.

DISCUSSION

Previous studies examining the effects of glucocorticoids on collagen synthesis have focused almost exclusively on connective tissue-derived cells. Although several reports have shown that glucocorticoids stimulate collagen synthesis (40, 41), most investigations have found that these steroids exert an inhibitory effect in connective tissue (6). The present study has demonstrated that glucocorticoids can act directly on vascular smooth muscle cells to increase the incorporation of proline into collagen and noncollagen protein. The stimulatory effect was not selective for collagen and was present in primary mass cultured as well as cloned cells. The finding that collagen synthesis is stimulated in smooth muscle cells and inhibited in connective tissue (6) by glucocorticoids shows that the direction of the biological response to these hormones is apparently dictated by the differentiation of the cell.

The increase in proline incorporation occurred with physiological concentrations of glucocorticoids. It was detectable at 0.1 nM dexamethasone and was maximal at 0.1 μM. These dose-response characteristics correlate well with the known binding affinities of the glucocorticoid receptor (42), the glucocorticoid induction of growth hormone (43), tyrosine aminotransferase (44), and other specific gene products (46), the inhibition of BASM proliferation (27), and fibroblast proliferation (11), and the inhibition of collagen synthesis in fibroblasts (9).

The stimulation of CDP and NCP was found in BASM cells treated with the natural bovine glucocorticoid, cortisol,

in addition to the synthetic glucocorticoid, dexamethasone. No major increase in CDP or NCP was observed in BASM cells treated with the inactive glucocorticoid, epicortisol, aldosterone, cholesterol, estradiol, and progesterone. Furthermore, the stimulatory effect was completely abolished by the glucocorticoid antagonist, 17 α-hydroxyprogesterone. These results suggest that the increased proline incorporation is mediated by specific cytoplasmic glucocorticoid receptors. More direct support for a receptor-mediated pathway is derived from recent studies that have identified glucocorticoid receptors in bovine (Lan, N., and L. K. Johnson, unpublished observations) and rat aortic BASM (46) and the aorta of dogs (39). In contrast to the report by Beldekas et al. (23) that showed that 17 β-estradiol produced a major decrease in CDP in bovine aortic BASM cells (23), we consistently observed a minor increase in CDP and NCP with this steroid. We also observed a major increase in CDP and NCP in BASM cells treated with testosterone. Although we did not examine vascular BASM cells for testosterone receptors, this effect was probably mediated by the androgen receptor system since testosterone receptors have been recently described in dog aorta (39).

The stimulation of CDP and NCP was found to be highly dependent on the time when the cells were exposed to dexamethasone. The effect was observed only in cultures treated with dexamethasone during their log phase of growth (1–6 d after plating). In contrast, when subconfluent or confluent cultures were exposed to dexamethasone, a small decrease in CDP and no change in NCP occurred. These results suggest that rapid proliferation is a necessary requirement for the stimulatory effect. In synchronous cell-lines it has been demonstrated that the cells are sensitive to glucocorticoids only during specific phases of the cell cycle. For example, the induction of alkaline phosphatase in HeLa cells (47) and tyrosine aminotransferase in hepatoma cells (48) is limited to the S and G_1 phases, while the glucocorticoid inhibition of proliferation of the human cell-line NHIK 3025 occurs only during the G_1 phase (49).

Recent studies in this laboratory have shown that glucocorticoids inhibit the proliferation and saturation density of both primary mass cultured and cloned BASM cells (27, 28). It has been reported that the rate of protein (50) and collagen (51) synthesis declines with increasing cell density in fibroblasts. These studies raise the possibility that the increase in CDP and NCP in the dexamethasone-treated cells was due to the lower saturation density reached by these cells rather than to a direct effect on protein synthesis. To address this question, we labeled cells with [^{3}H]proline during the late log phase of growth (6 d after plating) instead of at confluence (Leitman, D., and L. K. Johnson, unpublished observations). In addition, cells were grown in the presence and absence of dexamethasone on tissue culture dishes coated with a homologous extracellular matrix, which provided conditions whereby dexamethasone no longer inhibits the proliferation of this clone (28). In both of these cases, we observed an increase in CDP and NCP in the glucocorticoid-treated BASM cells. Thus, these studies exclude the possibility that the stimulatory effect resulted from differences in the time or density at which the treated cells attained confluence. It is also apparent that the glucocorticoid-induced inhibition of BASM proliferation can be dissociated from the increased proline incorporation, which suggests that the pathway of glucocorticoid action may diverge at some point to produce these separate effects.

The increased proline incorporation by the glucocorticoid-treated cells could have resulted from an increase in the rate of protein synthesis, decreased protein degradation, increased specific activity of intracellular proline, or by a combination of these processes. The site of glucocorticoid action was investigated by determining the rate of protein synthesis after correcting for differences in specific activity of proline. Although the steroid did apparently elevate the intracellular specific activity of the proline pool, the levels of proline incorporation when normalized for the proline specific activity still reflected a twofold increase in the rate of collagen and noncollagen synthesis after glucocorticoid treatment, assuming the extent of protein degradation to be negligible after a 30-min pulse.

If the fourfold increase in the intracellular specific activity of proline observed after a 30-min pulse was maintained throughout the labeling period, one would expect that the level of proline incorporation and protein specific activity would be elevated to the same extent had dexamethasone not produced an effect on the rate of protein synthesis. However, after a 24-h labeling period, we observed only a twofold increase in proline incorporation into protein and a 20% increase in the specific activity of secreted collagen. A possible explanation for this apparent disparity is that the specific activity of the proline pool may vary during the labeling period as a result of an increased rate of protein degradation in the steroid-treated cells. This would lead to a drop in the specific activity of the proline pool over time, due to the release of unlabeled proline into the cell. It is also possible that proline is being compartmentalized inside the cells such that the specific activity of the total intracellular pool may be significantly different from the proline pool accessible to prolyl-tRNA (52). Thus, although the total cellular proline specific activity may be much greater in the treated BASM cells, the specific activity of the pool that serves as the precursor for prolyl-tRNA may be similar to that in the control cells.

It is well established that vascular BASM cells synthesize predominantly Types I and III collagen (20). The exact proportion of these two types that are synthesized has been found to be highly variable, possibly due to differences in the age of the cells (19) and to the severity of pepsin treatment (18). We examined the possible glucocorticoid regulation of the collagen types secreted, since Type I collagen is increased relative to Type III collagen in atherosclerotic lesions (53) and with estradiol treatment of BASM cells (23). In the present study we modified the nonequilibrium two-dimensional gel electrophoresis technique of O'Farrell et al. (37) to separate the collagen chains of Types I and III. Using this method to separate pepsin-digested medium proteins, we found that both the control and steroid-treated cells secreted ~70% Type I and 30% Type III collagen, a ratio consistent with the findings of other studies using cultured vascular smooth muscle cells (54, 55). Thus, although glucocorticoid treatment increases total collagen production per cell, it does not alter the relative types of collagen chains expressed.

The most prominent feature in the pathology of atherosclerosis is the occurrence of smooth muscle cell proliferation and subsequent elaboration of collagen and other extracellular material that accumulates in the intima of the arterial wall (21). It has been reported that the rate of collagen synthesis is increased after experimental atherosclerosis in the rabbit aorta (25) and isolated rabbit atherosclerotic smooth muscle cells

(56). Furthermore, collagen is the most abundant protein present in the atherosclerotic lesions, comprising ~30% of the total dry weight (57, 58). However, the role of hormones in the regulation of collagen synthesis and deposition in normal and atherosclerotic blood vessels is unknown. Organ culture studies by Manthorpe et al. (59, 60) have demonstrated that collagen synthesis is decreased in the aorta from prednisone-treated rabbits. Our finding that glucocorticoids have a minor inhibitory effect on collagen synthesis in stationary BASM cells is consistent with their observations, since only a small number of smooth muscle cells are actively proliferating in the intact vessel (61). The use of an in vitro system has provided the opportunity to examine also the effect of glucocorticoids on collagen synthesis in proliferating BASM cells. Our results clearly demonstrate that glucocorticoids have the capacity to act directly on proliferating smooth muscle cells to stimulate collagen and noncollagen protein synthesis and secretion. Although caution must be employed when extrapolating the results of an in vitro study to the in vivo situation, our findings presented here and previously (27, 28) suggest that, if glucocorticoids have the same effects on proliferating smooth muscle cells during atherogenesis, they may promote the development of a less cellular but more fibrous lesion and thereby delay the regression of atherosclerotic lesions once formed.

REFERENCES

1. Bornstein, P., and H. Sage. 1980. Structurally distinct collagen types. *Annu. Rev. Biochem.* 49:957–1003.
2. Prockop, D. J., K. I. Kivirikko, L. Tuderman, and N. A. Guzman. 1979. The biosynthesis of collagen and its disorders. *New Engl. J. Med.* 301:77–85.
3. Muller, P. K., E. Kirsch, V. Gauss-Multer, and T. Krieg. 1981. Some aspects of the modulation and regulation of collagen synthesis in vitro. *Mol. Cell. Biochem.* 34:71–85.
4. Canalis, E., and L. G. Raisz. 1978. Effect of sex steroids on bone collagen synthesis in vitro. *Calcif. Tissue Res.* 25:105–110.
5. Fischer, G. M., and M. L. Swain. 1980. Influence of contraceptive and other sex steroids on aortic collagen and elastin. *Exp. Mol. Pathol.* 33:15–24.
6. Cutroneo, K. R., R. Rokowski, and D. F. Counts. 1981. Glucocorticoids and collagen synthesis: comparison of in vivo and cell cultures studies. *Collagen Rel. Res.* 1:557–568.
7. Russell, S. B., J. D. Russell, and K. M. Trupin. 1981. Collagen synthesis in human fibroblasts: effects of ascorbic acid and regulation by hydrocortisone. *J. Cell. Physiol.* 109:121–131.
8. McNelis, B., and K. R. Cutroneo. 1978. A selective decrease of collagen peptide synthesis by dermal polysomes isolated from glucocorticoid-treated newborn rats. *Mol. Pharmacol.* 14:1167–1175.
9. Kruse, N. J., D. W. Rose, W. Y. Fujimoto, and P. Bornstein. 1978. Inhibitory effects of glucocorticoids on collagen synthesis by mouse sponge granulomas and granuloma fibroblasts in culture. *Biochim. Biophys. Acta.* 540:101–116.
10. Diegelmann, R. F., W. J. Linkblad, and P. S. Guzelian. 1982. Effect of dexamethasone on collagen synthesis by primary cultures of rat hepatocytes. *Fed. Proc.* 41:618A. (Abstr.)
11. Verbruggen, L. A., and D. S. Salomon. 1980. Glucocorticoid receptors and inhibition of neonatal mouse dermal fibroblast growth in primary culture. *Arch. Dermatol. Res.*

269:111–126.

12. McCoy, B. J., R. F. Diegelmann, and I. K. Cohen. 1980. In vitro inhibition of cell growth, collagen synthesis, and prolyl hydroxylase activity by triamcinolone acetonide (40750). *Proc. Soc. Exp. Biol. Med.* 163:216–222.

13. Rokowski, R. J., J. Sheehy, and K. R. Cutroneo. 1981. Glucocorticoid-mediated selective reduction of functioning collagen messenger ribonucleic acid. *Arch. Biochem. Biophys.* 210:74–81.

14. Oikarinen, J., and L. Ryhänen. 1981. Cortisol decreases the concentration of translatable type-I procollagen mRNA species in the developing chick-embryo calvaria. *Biochem. J.* 198:519–524.

15. Counts, D. F., F. J. Rojas, and K. R. Cutroneo. 1979. Glucocorticoids decrease prolyl hydroxylase activity without the cellular accumulation of underhydroxylated collagen. *Mol. Pharmacol.* 15:99–107.

16. Oikarinen, A. 1977. Effect of cortisol acetate on collagen biosynthesis and on the activities of prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase and collagen glucosyltransferase in chick-embryo tendon cells. *Biochem. J.* 164:533–539.

17. Benson, S. C., and P. A. Luvalle. 1981. Inhibition of lysyl oxidase and prolyl hydroxylase activity in glucocorticoid treated rats. *Biochem. Biophys. Res. Commun.* 99:557–562.

18. Burke, J. M., G. Balian, R. Ross, and P. Bornstein. 1977. Synthesis of types I and III procollagen and collagen by monkey aortic smooth muscle cells in vitro. *Biochemistry.* 16:3243–3249.

19. Mayne, R., M. S. Vail, and E. J. Miller. 1978. Characterization of the collagen chains synthesized by cultured smooth muscle cells derived from rhesus monkey thoracic aorta. *Biochemistry.* 17:446–452.

20. Burke, J. M., and R. Ross. 1979. Synthesis of connective tissue macromolecules by smooth muscle. *Int. Rev. Connect. Tissue.* 8:119–157.

21. Ross, R., and J. A. Glomet. 1973. Atherosclerosis and the arterial smooth muscle cells. *Science (Wash. DC).* 180:1332–1339.

22. Burke, J. M., and R. Ross. 1977. Collagen synthesis by monkey arterial smooth muscle cells during proliferation and quiescence in culture. *Exp. Cell Res.* 107:387–395.

23. Beldekas, J. C., B. Smith, L. C. Gerstenfeld, G. E. Sonenshein, and C. Franzblau. 1981. Effects of 17 β-estradiol on the biosynthesis of collagen in cultured bovine aortic smooth muscle cells. *Biochemistry.* 20: 2162–2167.

24. Ledet, T., and J. Vuust. 1980. Arterial procollagen Type I, Type III and fibronectin: effects of diabetic serum, glucose, insulin, ketone, and growth hormone studied on rabbit aortic myomedial cell cultures. *Diabetes.* 29:964–970.

25. Fischer, G. M., M. L. Swain, and K. Cherian. 1980. Increased vascular collagen and elastin synthesis in experimental atherosclerosis in the rabbit. *Atherosclerosis.* 35:11–20.

26. Spector, S., A. Ooshima, K. Iwatsuki, G. Fuller, G. Cardinale, and S. Udenfriend. 1978. Increased vascular collagen biosynthesis by hypertension and reversal by antihypertensive drugs. *Blood Vessels.* 15:176–182.

27. Longenecker, J. P., L. A. Kilty, and L. K. Johnson. 1982. Glucocorticoid influence on growth of vascular wall cells in culture. *J. Cell. Physiol.* 113:197–202.

28. Longenecker, J. P., L. A. Kilty, and L. K. Johnson. 1983. Glucocorticoid inhibition of vascular smooth muscle cell proliferation: influence of homologous extracellular matrix and serum mitogens. *J. Cell. Biol.* 98:534–540.

29. Chamley-Campbell, J. H., G. R. Campbell, and R. Ross. 1981. Phenotype-dependent response of cultured aortic smooth muscle to serum mitogens. *J. Cell Biol.* 89:379–383.

30. Peterkofsky, B., and R. Diegelmann. 1971. Use of a mixture of proteinase-free collagenases for the specific assay of radioactive collagen in the presence of other proteins. *Biochemistry.* 10:988–994.

31. Burton, K. 1956. A study of the conditions and mechanism of the dipheylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* 62:315–323.

32. Troll, W., and J. Lindsley. 1955. A photometric method for determination of proline. *J. Biol. Chem.* 215:655–660.

33. Sage, H., R. G. Woodbury, and P. Bornstein. 1979. Structural studies on human Type IV collagen. *J. Biol. Chem.* 254:9893–9900.

34. Chung, E., and E. J. Miller. 1974. Collagen polymorphism: characterization of molecules with chain composition [α 1 (III)]₃ in human tissues. *Science (Wash. DC).* 283:1200–1201.

35. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature (Lond.).* 227:680–685.

36. Sykes, B., B. Puddle, M. Francis, and R. Smith. 1976. The estimation of two collagens from dermis by interrupted gel electrophoresis. *Biochem. Biophys. Res. Commun.* 72:1472–1480.

37. O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell. 1977. High resolution two dimensional electrophoresis of basic as well as acidic proteins. *Cell.* 12:1133–1142.

38. Chamberlain, J. P. 1979. Flurographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. *Anal. Biochem.* 98:132–135.

39. Horwitz, K. B., and L. D. Korwitz. 1982. Canine vascular tissues are targets for androgens, progestins, and glucocorticoids. *J. Clin. Invest.* 69:750–758.

40. Doherty, N. S., and H. Saarni. 1976. Stimulation by hydrocortisone of the rate of collagen synthesis in cultured fibroblasts. *J. Pharmacol. Pharmacol.* 28:656–657.

41. Saarni, H., and M. Tammi. 1978. Time and concentration dependence of the action of cortisol on fibroblasts in vitro. *Biochim. Biophys. Acta.* 540:117–126.

42. Baxter, J. D., and G. M. Tomkins. 1971. Specific cytoplasmic glucocorticoid hormone receptors in hepatoma tissue culture cells. *Proc. Natl. Acad. Sci. USA.* 68:932–937.

43. Yu, L.-Y., and F. C. Bancroft. 1977. Glucocorticoid induction of growth hormone synthesis in a strain of rat pituitary cells. *J. Biol. Chem.* 252:3870–3875.

44. Ernest, M. J., C.-L. Chen, and P. Feigelson. 1977. Induction of tyrosine aminotransferase synthesis in isolated live cell suspensions. *J. Biol. Chem.* 252:6783–6791.

45. Ringold, G. M., K. R. Yamamoto, G. M. Tomkins, J. M. Bishop, and H. E. Varmus. 1975. Dexamethasone-mediated induction of mouse mammary tumor virus RNA: a system for studying glucocorticoid action. *Cell.* 6:299–305.

46. Meyer, W. J., and N. R. Nichols. 1981. Mineralocorticoid binding in cultured smooth muscle cells and fibroblasts from rat aorta. *J. Steroid Biochem.* 14:1157–1168.

47. Cidlowski, J. A., and N. B. Cidlowski. 1982. Glucocorticoid receptors and the cell cycle: evidence that the accumulation of glucocorticoid receptors during the S phase of the cell cycle is dependent on ribonucleic acid and protein synthesis. *Endocrinology.* 110:1653–1662.

48. Sellers, L., and D. Granner. 1974. Regulation of tyrosine aminotransferase activity in two liver-derived permanent cells lines. *J. Cell Biol.* 60:337–345.

49. Bakke, O., and K. B. Eiknnes. 1981. Cell cycle-specific glucocorticoid growth regulation of a human cell line (NHIK 3025). *J. Cell. Physiol.* 109:489–496.

50. Steinberg, J. 1978. Collagen turnover and the growth state in in 3T6 fibroblast cultures. *Lab. Invest.* 39:491–496.

51. Aumailley, M., T. Krieg, G. Razaka, P. K. Müller, and H. Bricaud. 1982. Influence of cell density on collagen biosynthesis in fibroblasts cultures. *Biochem. J.* 206:505–510.

52. Hildebran, J. N., J. Airhart, W. S. Stirewalt, and R. B. Low. 1981. Prolyl-tRNA based rates of protein and collagen synthesis in human lung fibroblasts. *Biochem. J.* 206:505–510.

53. McCullagh, K. A., and G. Balian. 1975. Collagen characterisation and cell transformation in human atherosclerosis. *Nature (Lond.).* 258:73–75.

54. Layman, D. L., E. H. Epstein, Jr., R. F. Dodson, and J. L. Titus. 1977. Biosynthesis of type I and III collagens by cultured smooth muscle cells from human aorta. *Proc. Natl. Acad. Sci. USA.* 74:671–675.

55. Rauterberg, J., S. Allam, V. Brehmer, W. Wirth, and W. H. Hauss. 1977. Characterization of the collagen synthesis by cultured human smooth muscle cells from fetal and adult aorta. *Hoppe-Seylerâs Z. Physiol. Chem.* 358:401–407.

56. Pietilä, K., and T. Nikkari. 1980. Enhanced synthesis of collagen and total protein by smooth muscle cells from atherosclerotic rabbit aortas in culture. *Atherosclerosis.* 37:11–19.

57. Anastassiades, T., P. A. Anastassiadis, and O. F. Denstedt. 1972. Changes in the connective tissue of the atherosclerotic intima and media of the aorta. *Biochem. Biophys. Acta.* 261:418–427.

58. Levene, C. I., and J. C. F. Poole. 1962. The collagen content of the normal and atherosclerotic human aortic intima. *Br. J. Exp. Pathol.* 43:469–471.

59. Manthorpe, R., G. Helin, B. Kofod, and I. Lorenzen. 1974. Effects of glucocorticoid on connective tissue of aorta and skin in rabbits. Biochemical studies on collagen, glucosaminoglycans, DNA and RNA. *Acta Endocrinol.* 77:310–324.

60. Manthorpe, R., C. Garbarsch, and I. Lorenzen. 1975. Glucocorticoid effect on repair processes in vascular connective tissue. Morphological examination and biochemical studies on collagen, RNA and DNA in rabbit aorta. *Acta Endocrinol.* 30:380–397.

61. Chamley-Campbell, J. H., G. R. Campbell, and R. Ross. 1979. The smooth muscle cell in culture. *Physiol. Rev.* 59:1–61.