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

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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, 17β-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 demonstrate 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 extracellular 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

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been shown to decline nonselectively (9) as a result of a general 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), glycosyltransferase (16), galactosyl-transferase (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), 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, Belco Glass Inc. (Vineland, NJ).

1 Abbreviations used in this paper: DME, Dulbecco'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, trichloracetic acid; TEMED, N,N,N',N'-tetramethylethylenediamine; SAS, solubilizer for aqueous samples; butyl PBD, 2-(4'-t-butylphenyl)-5,4'-biphenyl)-1,4-oxadiazoole.

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-[3-H]proline (10-20 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). Pepsinogen was obtained from Worthington Biochemicals (Freehold, NJ). Clistriodial collagenase (Form III) was purchased from Advanced 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 Chapman-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 2 × 10⁶ 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-amino- propionitrile. The medium was aspirated and replaced with 2 ml of fresh preincubation medium containing 10-20 μCi/ml L-[3-H]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 phenylmethysulfonyl 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 [3-H]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 [3-H]proline was removed by resuspending the pellet twice in 10% trichloracetic acid (TCA). The pellet was dissolved in 0.2 N NaOH, and the collagen-digestable protein (CDP) and noncollagen protein (NCP) were quantitated as described by Peterkosky 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 NaOH 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 tolueone 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 NaOH and precipitated with 10% TCA without proline. The acid-precipitable material was dissolved in 0.2 N NaOH and CDP, NCP, and DNA were estimated.

The data in Fig. 1, lower panel, show that [3H]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 incorporation into medium CDP and NCP was greater 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 \times 10^6 dpm/h/μg DNA compared to 0.7 \times 10^6 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.

Table I

<table>
<thead>
<tr>
<th></th>
<th>CDP (dpm/μg DNA)</th>
<th>NCP (%)</th>
<th>% Collagen</th>
</tr>
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<tbody>
<tr>
<td>Primary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5,332 ± 130</td>
<td>3,183 ± 67</td>
<td>23.7 ± 0.04</td>
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<td>DEX (0.1 μM)</td>
<td>11,979 ± 924</td>
<td>8,355 ± 801</td>
<td>21.5 ± 0.54</td>
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<tr>
<td>Cloned</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8,123 ± 591</td>
<td>2,775 ± 153</td>
<td>35.1 ± 0.04</td>
</tr>
<tr>
<td>DEX</td>
<td>14,241 ± 193</td>
<td>6,077 ± 718</td>
<td>30.4 ± 0.16</td>
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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 + dpm NCP)) × 100. Each determination represents the average of triplicate culture dishes ± SE.
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 × 10^3 dpm/h/µg DNA while the control cells secreted 3.0 × 10^3 dpm/µg DNA. Thus, during the initial linear period of labeling, dexamethasone increased [\(^{3}\)H]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 [\(^{3}\)H]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 [\(^{3}\)H]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 [\(^{3}\)H]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.

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/plate, 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 [\(^{3}\)H]proline on the eleventh day after plating. Fig. 3A shows that the twofold increase in [\(^{3}\)H]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 [\(^{3}\)H]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...
concentration of 0.1 AM was as potent as 1 nM dexamethasone, decreased CDP and NCP and completely abolished the glucocorticoid inhibition of cell proliferation. The glucocorticoid antagonist, 17α-hydroxyprogesterone, increased CDP and NCP, while hydrocortisone used at a lower concentration of 0.1 AM dexamethasone produced over a twofold increase in CDP and NCP for 11 d, CDP and NCP were increased to the same extent as seen in BASM 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, epocortisol (17α-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 with increasing amounts of dexamethasone. The data shown in Fig. 5A 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, epocortisol (17α-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.

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, epocortisol (17α-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.

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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 with 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-
fold increase in the specific activity of the proline pool (Fig. 5C). When the incorporation of \(^{[\text{H}]}\)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 \(^{[\text{H}]}\)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 \(^{[\text{H}]}\)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 \(\mu\)g of the \(\alpha_1(I)\) chains of Type I collagen after 24 h compared to 4.5 \(\mu\)g secreted by the dexamethasone-treated cultures (Fig. 6B). To determine the amount of radioactivity present in collagen, the \(\alpha_1(I)\) bands were excised from the gel and solubilized. Fig. 6C shows that the \(\alpha_1(I)\) bands from the dexamethasone-treated cells contained twice the radioactivity of the \(\alpha_1(I)\) band from the control cells. Using the above data, it was calculated that the specific activity of the \(\alpha_1(I)\) chains was \(20\%\) greater in the dexamethasone-treated cells (Fig. 6D). Similar results were obtained when the \(\alpha_1(I)\) 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 \(^{[\text{H}]}\)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 mammals (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 \(17\beta\)-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, \(^{[\text{H}]}\)proline-labeled medium proteins were digested with pepsin, and the resultant collagen \(\alpha\)-chains were separated by two-dimensional gel electrophoresis. As shown in Fig. 7 the \(\alpha\)-chains of Type I and Type III collagens can be resolved by this technique using a \(\text{pH} 5-7\) gradient in the first dimension. The \(\alpha\)-chains present in BASM media from both the control (Fig. 7A) and the dexamethasone-treated cells (Fig. 7B) comigrated with the purified \(\alpha_1(III)\) standard and the \(\alpha_1(II)\) and \(\alpha_2(II)\) 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...
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 G2 phases, while the glucocorticoid inhibition of proliferation of the human cell-line NHIK 3025 occurs only during the G1 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 [3H]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.

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