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Microtubules are not Required for Glucocorticoid Receptor Mediated Gene Induction

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Steroid-free glucocorticoid receptors are generally considered to reside in the cytoplasm of cells. After the binding of steroids, the receptors translocate into the nucleus in a manner that has been proposed to involve microtubules. However, some results with inhibitors of microtubule assembly argue to the contrary. In all of these studies, only the whole cell localization of receptors has been examined; the biological activity of these receptors has not been determined. We now report that steroid-induced gene expression is maintained in the absence of intact microtubules. This argues that microtubules are not required for either the nuclear translocation or biological activity of glucocorticoid receptors.

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INTRODUCTION

The mechanism responsible for the relocalization of whole cell glucocorticoid receptors from the cytoplasm to the nucleus, after steroid binding and activation to a form with increased affinity for DNA and nuclei, remains obscure after many years of investigation [1–4]. Several lines of evidence suggest that tubulin-containing fibers, or microtubules, may be intimately involved. An almost identical whole cell immunocytochemical staining of steroid-free human fibroblasts by anti-receptor and anti-tubulin antibodies has been seen both in interphase and mitotic cells and in cells treated with colchicine, which is a well-known inhibitor of microtubule assembly due to β-tubulin unfolding [5–7]. Thus, it was proposed that steroid-free glucocorticoid receptors interact with cytoplasmic microtubules in intact cells [8–10]. Unactivated receptors are associated with heat shock protein 90 (hsp90) and whole cell immunofluorescence with anti-hsp90 and anti-tubulin antibodies suggest an identical cellular organization [11]. Cell-free activated, but not unactivated, receptor–steroid complexes from L cells become associated with particular complexes containing tubulin in a manner that does not require DNA binding by the receptors. However, the formation of this particulate complex was not inhibited by colchicine [11, 12]. From these observations, it has been speculated that the nuclear binding of activated glucocorticoid receptor–steroid complexes may involve transport along microtubules [13, 14].

Studies with other steroid receptors have been of little help in determining whether microtubules are involved in the whole cellular localization of receptors. Added steroid caused a rapid (30–45 s) reorganization of vitamin D receptors to immunochemically detected, microtubule-like structures that appeared identical to those detected by anti-tubulin antibodies. Furthermore, tubulin disrupting agents such as colchicine were found to prevent both the alignment of receptors along microtubules and the subsequent steroid-induced nuclear binding [15]. However, it has been reported that colchicine does [16] and does not [17] block the nuclear binding of estrogen receptor–steroid complexes in rat uteri. Finally, elegant work with a mutant progesterone receptor, which lacks the nuclear translocation sequence at amino acids 638–642 and is cytoplasmic in the absence of added steroid, demonstrated that the nuclear binding of progesterone receptors required neither an intact cytoskeletal network nor microtubules [18].

In all of the previous reports, only the presence or absence of receptors in the nucleus was studied. There

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was no indication whether those complexes that bound to nuclei in the absence of microtubules were still biologically active. Therefore, we have now examined whether glucocorticoid receptors retain biological activity and can induce gene transcription under conditions where all microtubules are disaggregated.

EXPERIMENTAL

Materials

Chemicals were commercially available from the following suppliers: [^{14}C]chloramphenicol (57 mCi/mmol; New England Nuclear Research Products-Dupont, Boston, MA); $CaCl_2$, (Fisher, Fair Lawn, NJ); dexamethasone (Dex), *p*-phenylenediamine, and fibronectin (Sigma, St Louis, MO); phosphate-buffered saline (PBS; without Mg^{++} and Ca^{++}; Quality Biological, Rockville, MD); Hydrofluor (National Diagnostics, Manville, NJ); colchicine (Fluka Chemie AG, Buchs, Switzerland); paraformaldehyde, E.M. grade (Polyscience, Inc., Warrington, PA); methanol, HPLC grade (J. T. Baker Inc, Phillipsburg, NJ); monoclonal mouse β tubulin (Amersham, Arlington Height, IL); affinity purified goat anti-mouse immunoglobulin (IGg) labeled with lissamine and chrompure goat IGg (Jackson ImmunoResearch Inc., Westgrove, PA).

Cell culture

Monolayer cultures of HeLa cell (epithelial adenocarcinoma from human cervix; gift from Dr G. Hagger, NIH) were grown in Dulbecco's minimum essential medium (D-MEM; Gibco-Bethesda Research Labs, Gaithersburg, MD) supplemented with 10% heat inactivated fetal calf serum. All cells were maintained in a 5% CO_2 atmosphere at 37°C.

Transient transfections

GREtkCAT, which was originally named PRE-PBL7 and contains two repeats of 23 bp glucocorticoid/progesterone responsive elements (GRE/PRE) of the rat tyrosine aminotransferase (TAT) gene, was obtained from Dr B. O'Malley through Dr J. Ashwell (NIH). The DNA was transfected into HeLa cells using the calcium phosphate precipitate method as described previously [19]. Transfected cells were pretreated for varying lengths of time with different concentrations of colchicine before adjusting to 1% EtOH $\pm$ 10^{-6} M dex. The cells were incubated for an additional 22 h before being harvested. Chloramphenicol acetyltransferase (CAT) activity was determined as described previously [19] and expressed as absolute CAT activity (pmol/[min][mg protein]).

Confocal microscopy

The cells were plated on fibronectin-treated glass "Chamber slides" (Nunc Inc., Naperville, IL) and grown at 37°C in 5% CO_2; each well (18 × 18 mm) received 4×10^4 cells in a total volume of 1.5 ml of medium. Cells were incubated with 1% ethanol $\pm$ colchicine for 4 or 24 h, washed twice with PBS, treated with 3% formaldehyde for 10 min at room temperature (r.t.), fixed with 100% methanol (−10°C) for 6 min, and rinsed twice with PBS. Fixed cells were preincubated in blocker solution (1.9 mg/ml of goat IGg in PBS with 0.3 mg of glycine) at r.t. for 30 min, treated with mouse anti-β-tubulin diluted 1:500 in blocker solution for 60 min at r.t. on a rocker shaker and rinsed three times for 10 min with blocker solution. The cells were then incubated for 60 min with lissamine-labeled goat anti-mouse IGg secondary antibody (diluted 1:100 in blocker) on a shaker while protected from light, washed twice with the blocker for 10 min, and once with PBS for 10 min, and then mounted on a slide in *p*-phenylenediamine. Controls were prepared by replacing the primary antibody with a non-immune, serum mouse IGg (10% solution in PBS). Confocal microscopy was performed with a Nikon Optiphot microscope equipped with a Bio-Rad MRC-600 (Cambridge, MA) scanning confocal imaging system with a krypton/argon laser. Lissamine fluorescence was detected with a 568 DF10 excitation filter, 560 DFLP dichroic reflector, and 585 EFLP emission filter.

RESULTS

HeLa cells were selected for an assessment of the involvement of microtubules in nuclear translocation of biologically active glucocorticoid receptors for three reasons. First, HeLa cells contain reasonable levels of functional glucocorticoid receptors (about 20,000 per cell) ([20], P. K. Chakraborti and S. S. Simons Jr., unpublished results). Second, the receptors in HeLa cells have been found to undergo a cytoplasmic to nuclear translocation upon the addition of steroid [3]. Third, this system mimics a previous study with human fibroblasts in which receptors were found to associate with microtubules [10]. The biological response chosen was the glucocorticoid receptor mediated induction of the transiently transfected GREtkCAT reporter gene. This reporter contains just a synthetic tandem repeat of the second GRE of the liver specific rat TAT gene upstream of the thymidine kinase (tk) promoter driving the CAT gene. The simplicity of this reporter gene minimizes any indirect effects of the microtubule disrupting agent colchicine on glucocorticoid-induced expression. For example, we have recently shown that there are no tissue specific factors that modify the induction of the GREtkCAT construct in HeLa vs rat liver cells [19].

A dose–response curve for the effect of various concentrations of colchicine on the induction of the GREtkCAT reporter gene by saturating concentrations of the synthetic glucocorticoid Dex was determined in transiently transfected HeLa cells. As shown in Fig. 1, there was no appreciable effect of up to 10 μM

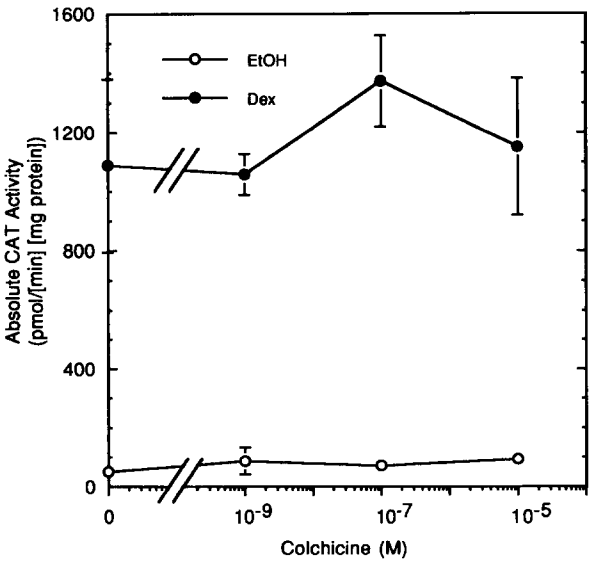

Fig. 1. Effect of colchicine preincubation time on Dex induction of CAT activity. Duplicate plates of HeLa cells, transiently transfected with the GREtkCAT reporter gene, were preincubated for 4 h with medium containing the indicated concentrations of colchicine followed by ethanol ± 1 μM Dex for 22 h before being assayed for CAT activity as described in Experimental. Error bars indicate the range of duplicate values. Similar results were obtained in at least one additional experiment.

colchicine on either the basal level of CAT expression or the ability of Dex to induce the CAT enzyme. In a preliminary experiment, even 1 mM colchicine did not prevent Dex induction of CAT gene expression (data not shown). Varying the time of preincubation with 10 μM colchicine had little effect on either the basal level, or the Dex inducible level, of CAT activity during the subsequent 22 h incubation (Fig. 2). Thus, the time of preincubation was not critical for whether or not an effect of colchicine could be observed.

Most of the cells displayed a more rounded shape after 2 h in 10 μM colchicine, suggesting that the microtubules had been rapidly depolymerized. The cells remained rounded up until they were harvested. These results indicated that colchicine is sufficiently stable to maintain microtubule depolymerization throughout the entire steroid induction period. This conclusion was confirmed by a confocal microscopic examination of the whole cell immunofluorescence of cells treated with the same mouse anti-β-tubulin antibody as used by others [10]. The filamentous structures of control cell, characteristic of microtubules, were absent in cells pretreated for 4 or 24 h with 10 μM colchicine (Fig. 3).Thus, the normal microtubule architecture of HeLa cells was prevented by colchicine treatment during the entire time period in which glucocorticoids were capable of inducing the expression from a responsive gene.

DISCUSSION

Steroid-free glucocorticoid receptors are generally thought to reside in the cytoplasm of cells and must migrate into the nucleus in order to induce the transcription of responsive genes [1–4]. Thus, any agent or process that blocks the nuclear binding of receptor–steroid complexes would concomitantly prevent glucocorticoid receptor mediated gene induction [21]. We have disrupted the assembly of microtubules with 10 μM colchicine (Fig. 3) without altering the capacity of a responsive gene to be induced by glucocorticoids (Figs 1 and 2). This argues that microtubules are not

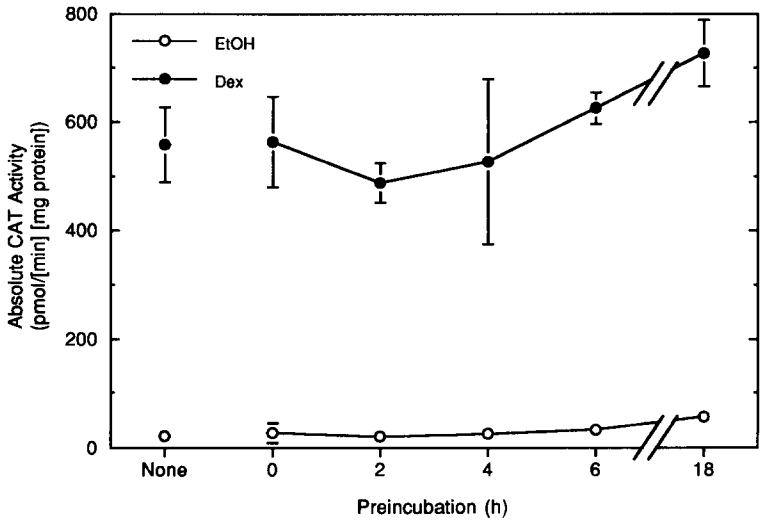

Fig. 2. Effect of varying preincubation time with 10 μM colchicine on Dex induction of CAT activity. Duplicate plates of HeLa cells, transiently transfected with the GREtkCAT reporter gene, were preincubated for the indicated times with 10 μM colchicine, at which point ethanol ± 1 μM Dex was added. Further incubation and analysis of CAT activity was conducted as for Fig. 1. Error bars indicate the range of duplicate values. Similar results were obtained in at least one additional experiment.

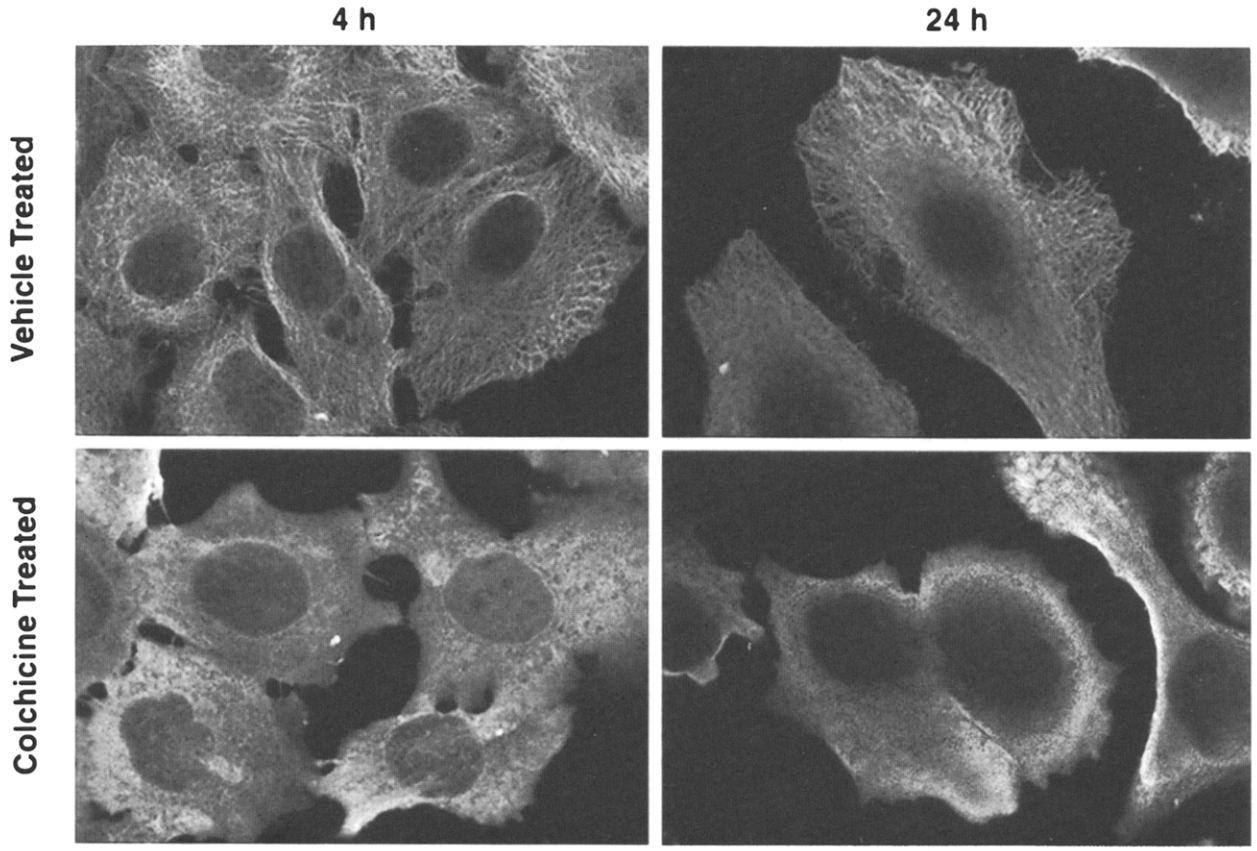

Fig. 3. Effect of colchicine on integrity of microtubules in intact cells. HeLa cells were treated as indicated with vehicle or 10 μM colchicine for 4 or 24 h before being processed as described in Experimental for the visualization of microtubules by anti-tubulin antibody and confocal microscopy ($\times$ 400 for 4 h, $\times$ 600 for 24 h). Not shown are the non-immune controls which displayed no fluorescence.

required for either the nuclear binding or the expression of transcriptional activity by glucocorticoid receptor–steroid complexes that, initially, are predominantly cytoplasmic.

These results are not necessarily in conflict with reports of a microtubular-like intracellular arrangement of receptors since this behavior has been observed almost exclusively with steroid-free receptors [8–10, 13, 14]. It was originally suggested that the nuclear translocation of receptor–steroid complexes after activation occurred along microtubules because of the prior attachment of the receptors to such a convenient tertiary structure. More recently, a component of the unactivated complexes (heat shock protein 56, or hsp56) has been found to coimmunoadsorb with the heavy and intermediate chains of dynein, which is involved in the movement of particles along microtubules [14]. Our results indicate that microtubules are not required for the nuclear migration of activated glucocorticoid receptor–steroid complexes even though the unactivated receptors appear to be tightly bound to microtubules. What additional processes are necessary for the disassociation of receptors from microtubules concomitant with activation are not known but they may involve the deoligomerization or loosening of those proteins associated with unactivated receptors [13, 14].

Colchicine does block the rapid intracellular reorganization and nuclear translocation of vitamin D receptors [15]. However, it may be significant that the vitamin D receptor is a member of a sub-class of steroid receptors that, unlike glucocorticoid receptors, are not associated with hsp90 [22, 23] and thus could be subject to different mechanisms for intracellular movement. Consistent with this is the observation that cGMP stabilizes vitamin D receptors in intact cells [15] while we could find no effect of cGMP on glucocorticoid receptors in a cell-free system [24a].

The present study extends the conclusion that microtubules are not required for the nuclear binding of macromolecules such as progesterone [18] and estrogen [17] receptors, or of hsp90 [24b], an induced 72 kDa heat shock protein and microinjected adenovirus E1A protein [25]. These earlier studies used antibodies to detect the bulk of the antigenic proteins. Since the number of genes regulated by steroid hormones is usually much less than the total number of receptor molecules [26], it is conceivable that a small sub-population of biologically active receptors could have been missed by these immunocytochemical techniques. By following the induced biological response, we have now established that microtubules are not required for transcriptional activation even by

a potentially undetected sub-population of activated receptor–steroid complexes.

Because we have monitored the biological consequences of nuclear bound receptors as opposed to the whole cell localization of receptors, we cannot say that the quantity of nuclear bound receptors is the same both with and without 10 μM colchicine. However, we [27] and others [28] have found that the level of receptor-induced gene expression is directly proportional to the number of available receptors. The fact that similar levels of gene induction were observed in the presence or absence of colchicine and depolymerized microtubules suggests that both the total number of biologically active receptors, and the capacity of the HeLa cells to respond to activated glucocorticoid receptor–steroid complexes, did not change dramatically.

What, then, is directing the nuclear translocation of biologically active glucocorticoid receptors if microtubules are not required? It is possible that all of the biologically active receptors already reside in the nucleus but this seems unlikely in view of the extensive evidence that most of the steroid-free glucocorticoid receptors are cytoplasmic [1–4]. Cytoskeletal networks not disrupted by the various agents used so far may be other candidates. Alternatively, the cellular distribution may be controlled by a simple partitioning, or shuttling [18, 29], of the free and bound receptors. In fact, such a partitioning between two different aqueous phases has been observed for both glucocorticoid [30] and estrogen [31] receptors. Further studies should help to resolve these issues.

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