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Glucocorticoid receptor inhibits microtubule assembly in vitro

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Abstract

The effect of glucocorticoid hormones, purified glucocorticoid receptor (GR) and purified heat shock protein M_r 90 000 (hsp90) on microtubule (MT) assembly in vitro was tested by a spectrophotometric MT assembly assay and electron microscopy. GR significantly prolonged the nucleation phase, slowed down the assembly rate and reduced the maximal amplitude of MT assembly compared with control. The effects were partially reversed by the addition of glucocorticoid hormone. GR associated with MTs. These results indicate that GR affects MT assembly in vitro, which may be a functional correlate to the structural association of GR with MTs. This implies that factors affecting GR may affect MT assembly in vivo.

Keywords: Glucocorticoid receptor; Microtubules; Microtubule assembly; Co-localization

1. Introduction

Glucocorticoid hormones are known to inhibit growth of a number of different tissues and cell types. There is evidence that glucocorticoids block the cell cycle in G1 phase (Bakke 1986 and references therein) and these hormones have been considered to be mitotic spindle poisons (Dustin 1963). The molecular mechanism for this antiproliferative effect of glucocorticoids is unclear, but may involve a direct or receptor mediated interaction with cellular microtubules (MTs), such as the various mitotic spindle MTs or MTs in the centrosomes (Liang and Brinkley 1985).

There is both immunocytological and biochemical evidence for an interaction between the glucocorticoid receptor (GR) and the MT protein tubulin: (i) GR is co-localized with mammalian cell MTs in fixed cells, i.e. both cytoplasmic MTs during interphase and mitotic MTs during cell division (Akner et al., 1990, 1991, 1994); (ii) GR follows tubulin to newly formed plasma membrane protrusions upon artificially induced depolymerization of MTs using drugs such as colchicine, colcemid, vinblastine and nocodazole and GR also follows tubulin to vinblastine-induced paracrystals (Akner et al., 1990; Akner et al., 1991); (iii) tubulin immunoreactivity has been detected in purified preparations of activated GR (Akner et al., 1990); and (iv) activated GR has been found to associate with a cytosolic cytoskeletal protein complex containing tubulin (Pratt et al., 1989; Scherrer and Pratt 1992a,b). We have speculated that one mechanism by which glucocorticoids may act as a mitotic inhibitor and inhibit cell proliferation and cell division might be through GR localized in the mitotic spindle or in the centrioles in close proximity to spindle/centriolar MTs (Akner et al., 1991).

In this study we have used various glucocorticoid hormones and purified preparations of GR and analyzed their effects in an in vitro MT assembly assay (Gaskin et al., 1974). We also included heat shock protein M_r 90 000 (hsp90), since it is known to associate with the non-activated GR (Sanchez et al., 1985). The study was designed to analyze whether glucocorticoid hormones or purified preparations of GR and hsp90 had any effect on either of the following three parameters: (1) MT assembly kinetics; (2) morphology of newly assembled MTs; (3) association with newly assembled MTs. We present evidence that GR associates with MTs and also that it inhibits the assembly of MTs in vitro.

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2. Materials and methods

2.1. Purification of GR

Activated GR was purified from rat liver cytosol by sequential chromatography on phosphocellulose, DNA-cellulose and DEAE-Sepharose as described by Wrange et al. (1979). The final product held a protein concentration of 5–12 μg/ml (50–120 nM) in ETG buffer (1 mM EDTA, 20 mM Tris–HCl, pH 7.8, 10% (v/v) glycerol with 120 mM NaCl and 1 mM dithiothreitol, DTT).

2.2. Purification of hsp90

Hsp90 was purified from rat liver cytosol by a two-step procedure as previously described (Denis, 1988). The eluate was either lyophilized or frozen in EP buffer (1 mM EDTA, 20 mM Na-phosphate, pH 7.4 with 100 mM NaCl) with a protein concentration of 0.3 mg/ml (3.3 μM).

2.3. Preparation of tubulin

Bovine brains were obtained from a local slaughterhouse and the MT proteins were prepared by two cycles of temperature-dependent assembly-disassembly in MT polymerization (PM) buffer with 1 mM GTP (PM buffer: 100 mM PIPES, 0.5 mM $MgSO_4$, pH 6.8) as described (Wallin et al., 1985). The proteins were drop-frozen and stored in liquid nitrogen. These MT proteins are referred to as MTP and consist of approximately 80% tubulin and 20% MT associated proteins (MAPs) (Fridén et al., 1988).

2.4. In vitro MT assembly assay

Assembly of MT proteins at 2 mg/ml was started by placing the cuvette in a spectrophotometer at +37°C in a thermostated cuvette holder. The heat-induced increase in turbidity was monitored by the apparent increase in absorbance at 350 nm using an Ultrospec III® spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). The absorbance at 350 nm is proportional to the weight concentration of assembled long MTs and the change in absorbance at 350 nm over time can therefore be used as a measure of the amount of MT assembly (Gaskin et al., 1974; Wallin et al., 1988). The assembly of MTs can be divided into three different phases: nucleation, elongation and steady state. A typical control assembly curve is shown in Fig. 1. We defined three parts of the curve: (i) lag = nucleation phase = the time from placing the cuvette in the spectrophotometer at +37°C until onset of assembly; (ii) slope = the maximal slope of the assembly curve derived as y/x; (iii) amplitude = the maximal increase in MT assembly at the steady state phase. The effects of the test substances were analyzed by adding them before the onset of assembly at +4°C, either with or without pre-incubation.

The following test substances were added to the MT assembly buffer: (1) GR at a final assay concentration of 43–55 nM; (2) hsp90 at a final assay concentration of 12 μM (lyophilized material) or 0.18–1.1 μM (frozen material), respectively; (3) steroid hormones (dexamethasone, cortisol, β-estradiol) were diluted from stock solutions of 10^{-2} M in ethanol to final assay concentrations of 10^{-4}–10^{-6} M; (4) bovine serum albumin was diluted in PM buffer to a final assay concentration of 2 mg/ml (30 μM). The volumes were chosen such that the assembly mixture did not contain more than 2.8% (v/v) final ethanol concentration and that the total added volume of test substance did not exceed half of the total MT assembly buffer volume. Control buffers were: (a) PM buffer with or without 1% (v/v) ethanol; (b) ETG buffer; (c) EP buffer. After the reaction had reached a stable steady state phase, the lag, slope and amplitude were measured from each assembly curve, pooled and analyzed statistically. Since the GR and hsp90 buffers contained components possibly affecting the MT assembly assay, comparisons were made between on the one hand ETG or EP buffer and PM buffer and on the other between GR and ETG buffer as well as between hsp90 and EP buffer (see Discussion in Section 4).

2.5. Statistical analysis

The mean values were calculated for each effect parameter (lag, slope, amplitude) and the differences between controls and the corresponding test substances were analyzed by Student's t-test.

2.6. Electron microscopy

Light scattering analysis does not reveal the morphology of the newly assembled MTs; aberrant forms or aggregates can scatter light in the same range as normal MTs (Gaskin and Kress, 1977; Sharp and Perry, 1985; Chaudoreille et al., 1991). This might be incorrectly interpreted as a lack of effect or a stimulation of the assembly. The MT assembly studies were therefore supplemented by ultrastructural analysis. After MT assembly had reached a stable steady state phase, with or without added test substance, a drop of MT solution was applied to a Formvar-coated copper grid. After 20 s, the grid was dried with filter paper and stained with 1 % (v/v) uranyl acetate. Specimens were viewed in a Zeiss 902 A transmission electron microscope (Carl Zeiss, Oberkochen, Germany).

2.7. Gel electrophoresis

The SDS-PAGE electrophoresis was performed in 7.2% (w/v) separating gels as previously described (Akner et al., 1992). The samples were prepared as follows. After the newly assembled MTs, with or without added test substance, had reached a stable steady state phase, the mixture was immediately centrifuged at 180 000 × g for 5 min in an Airfuge® ultracentrifuge (Beckman Instruments, Palo Alto, CA, USA) allowing the newly formed MTs to be pelleted. The supernatant (175 μl) was carefully separated from the pellet and both samples were

promptly mixed with 175 and 150 μl of sample buffer, respectively, boiled for 3 min at 100°C and frozen at −20°C. On the day of analysis, the samples were thawed, sheared through a syringe and applied to the gel.

2.8. Western immunoblotting

Proteins were transferred from the SDS-PAGE gel to nitrocellulose (NC) filters as previously described (Akner et al., 1992). Filter strips were then incubated with monoclonal mouse anti-rat liver GR antibodies 'mab1' and 'mab7' produced in our laboratory (Okret et al., 1984), with final protein concentrations of 10–20 μg/ml followed by secondary horse radish peroxidase labeled antibodies as described (Akner et al., 1992). 'Mab1' was produced as conventional ascites, while 'mab7' was produced by a Diacult® system (Sjögren-Jansson and Jeansson, 1985). Both anti-GR antibodies were protein A purified.

2.9. Chemicals

Dexamethasone, cortisol, β-estradiol and bovine serum albumin were analytical grade products obtained from Sigma, St Louis, MO, USA.

3. Results

3.1. MT assembly kinetics

The results of addition of the test substances to MTP are shown in Table 1. Each experiment was repeated several times as indicated in the table. There were no differences whether the test substances were allowed to preincubate for 5–10 min or if MT assembly was allowed to start immediately after mixing the assembly components.

Buffer controls. Addition of 2.8% (v/v) ethanol or dilution of the assembly buffer with 25–50% (v/v) EP buffer did not cause any significant change compared with the control assembly curves. A similar dilution of the assembly mixture with ETG buffer did not affect the amplitude, but significantly prolonged the lag phase ($P < 0.05$) and reduced the slope ($P < 0.01$) compared with PM buffer with or without ethanol.

GR. The effects of GR are illustrated in Table 1 and Fig. 1. Purified, activated GR at 43–55 nM significantly reduced the amplitude compared with both ETG and PM buffer controls. Moreover, GR caused a further significant prolongation of the lag phase and reduction of the slope compared with ETG buffer. When adding GR and dexamethasone together before onset of assembly, the amplitude was still significantly reduced compared with control, but the lag and slope were not different from control. Without addition of GTP to the PM buffer GR by itself did not induce MT assembly (not shown).

There were no significant effects of either dexamethasone, cortisol (not shown), β-estradiol, hsp90 or bovine serum albumin (not shown) on the MT assembly curve.

3.2. Morphology of newly assembled MTs

Electron microscopic evaluation revealed that the newly polymerized MTPs exhibited a normal MT morphology (not shown). Both ETG buffer as well as GR in ETG buffer induced a similar small degree of aggregation. None of the test substances, i.e. GR, hsp90 or steroid hormones, at the final assembly concentrations mentioned

Table 1

Effects of different test substances on MTP assembly

Test substance	Lag			Slope			Amplitude		
	Mean	SD	*P*-value	Mean	SD	*P*-value	Mean	SD	*P*-value
A. *Steroid hormones*									
PM buffer/ethanol control ($n = 7$)	1.1	0.11		6.3	0.43		0.42	0.06	
Dex 10^{-4} M ($n = 3$)	1.0	0.11		6.0	0.10		0.42	0.07	
Dex 10^{-5} M ($n = 7$)	1.2	0.17		5.9	0.97		0.39	0.06	
Dex 10^{-6} M ($n = 3$)	1.3	0.06		5 7	0.92		0.38	0.08	
β-Estradiol 10^{-5} M ($n = 2$)	1.1	0.21		7.0	0.92		0.47	0.02	
B. *GR*									
ETG buffer ($n = 3$)	1.4	0.06		3.6	0.26		0.38	0.01	
GR 43–55 nM ($n = 5$)	1.7	0.12	<0.01	1.6	0.17	<0.001	0.35	0.01	<0.01
GR 31–62 nM + Dex 10^{-5} M ($n = 3$)	1.2	0.06		3.8	1.07		0.34	0.01	<0.01
C. *Hsp90*									
EP buffer ($n = 1$)	1.2			6.4			0.42		
Hsp90 12 μM, lyophilized ($n = 2$)	1.2	0.14		7.3	0.42		0.46	0.02	
Hsp90 0.18–1.1 μM, EP buffer ($n = 2$)	1.2	0.14		7.1	1.06		0.46	0.06	

Mean effects of test substances on the MTP assembly curve in terms of lag (minutes), slope and amplitude (Δ amplitude units) compared to the respective buffer controls within each group, A–C. Statistically significant differences within each group in relation to control with the respective buffer only are indicated by the *P*-value. Dex = dexamethasone. For other abbreviations, see text.

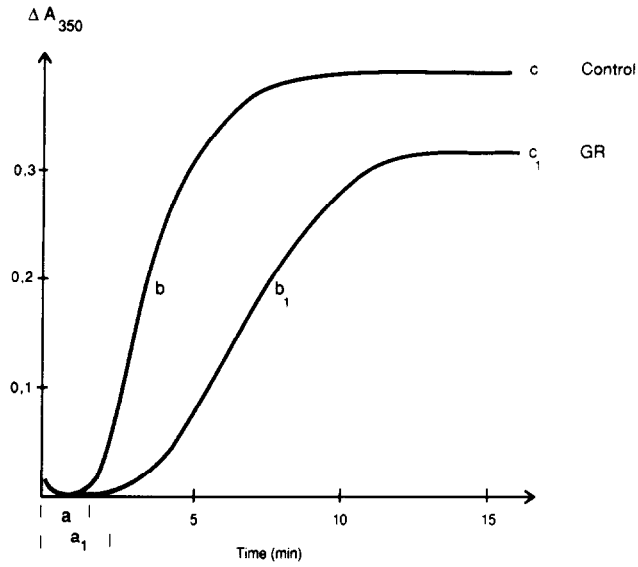

Fig. 1. Effect of glucocorticoid receptor (GR) on microtubule (MT) assembly. The figure shows two representative experiments where purified bovine brain MT proteins were assembled in the absence (control) or presence (GR) of purified rat liver GR. The control curve corresponds to ETG buffer without GR. a, b and c refer to the control curve, a_1, b_1 and c_1 illustrate the effect of GR. GR causes a prolongation of the lag phase (a_1) and a reduction of both the slope (b_1) and the amplitude (c_1) of MT assembly.

above, induced any detectable structural MT aberrations (not shown). Purified GR added directly to the EM grid did not produce any aggregates (not shown).

3.3. Association of GR with newly assembled MTs

Western immunoblots showed that activated GR, when added at the onset of MT assembly, was present both in the supernatant and the pellet fraction of the polymerization mixture at steady state (Fig. 2). This was observed with both of the used anti-GR antibodies. GR alone without MTP could not be pelleted (not shown). This indicates that activated GR associates with MTs during assembly.

4. Discussion

We have previously shown that GR exhibits both a nuclear and an extranuclear distribution in mammalian cells, where it co-stains (Akner et al., 1990, 1991) and co-purifies (Akner et al., 1990) with the MT protein tubulin. The present study provides evidence that GR also associates with and affects the polymerization pattern of assembled MTs. This is in line with the results of Pratt et al. (1989) who showed that GR in L-cell cytosol is converted from soluble to particulate form under conditions that favor tubulin polymerization. Furthermore, the activated GR has been shown to associate with a cytoskeletal protein complex containing tubulin; DNA binding was not required for this association (Scherrer and Pratt, 1992a,b). Thus, there is now evidence that GR associates with MTs using both purified and cytosolic proteins.

The observed association between GR and MTs may be due to protein–protein interaction between GR and tubulin or between GR and one of the MAPs in the MTP assembly mixture. One may speculate that GR itself is a MAP and interacts with the $\alpha\beta$-tubulin polymer in a similar manner as other MAPs. Several MAPs contain a highly conserved MT binding motif (Lewis et al., 1988). By computer-assisted homology screening, we have compared the primary amino acid sequences of, on the one hand, human GR (Hollenberg et al., 1985) and on the other, published sequences of MAP-1B (Noble et al., 1989), MAP-2 (Lewis et al., 1988) and tau (Lee et al., 1988) without finding that GR contains any significant homology with the reported MT binding motifs.

GR at 43–55 nM concentration induced a significant inhibition of MT assembly, whereas glucocorticoid hormones, β-estradiol or hsp90 did not show any effects compared to controls. The GR buffer (ETG) alone slowed down the assembly process, while the addition of GR further significantly reduced the assembly speed and also reduced the amplitude. We observed a similar degree of aggregation with ETG buffer alone and with GR in ETG, respectively, indicating that this buffer causes some degree of aggregation of MTP during assembly. EP buffer also gave rise to some smaller aggregates. It would have

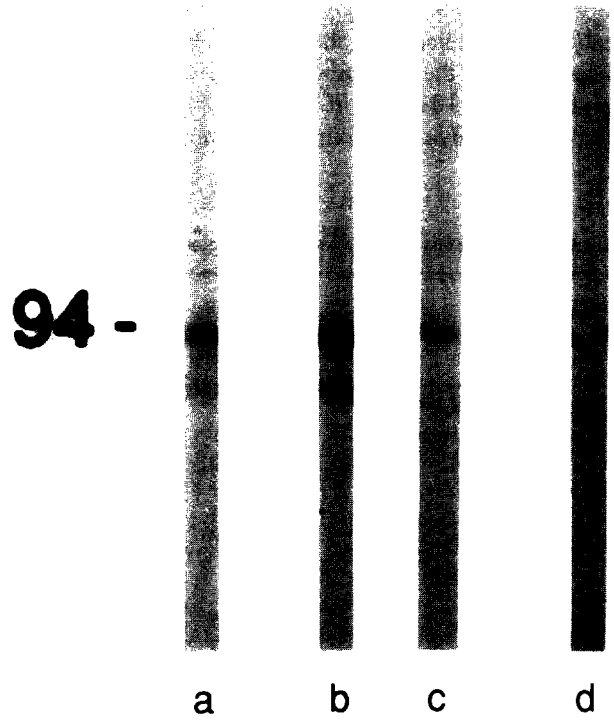

Fig. 2. Association of GR and tubulin. GR was added to the MT protein mixture, MTs were allowed to polymerize for 15 min and the supernatant was separated from the pellet. The figure shows Western immunoblots of the supernatant (a,b) and pellet (c,d) fractions at a stable steady state phase, detected by monoclonal antibodies against GR (Okret et al., 1984). a,c, 'mab7'; b,d, 'mab1' Molecular weight × 10^{-3} is indicated to the left.

been preferable to dilute GR in PM buffer alone without introducing the problem of buffer mixing. Since GR is an unstable molecule, we decided to maintain the buffer conditions previously shown to keep the protein intact (Wrange et al., 1979). The GR protein was not affected by the limited dilution with PM buffer as judged from the intact M_r 94 kDa species detected by immunoblotting (Fig. 2). The effect of combined addition of activated GR and glucocorticoid hormone indicates that glucocorticoids may somewhat reduce the effect of activated GR on MT assembly. We added 'activated' GR, i.e. GR exhibiting specific DNA binding activity after being dissociated from hsp90, induced by the combined effect of glucocorticoids and heat during GR purification. The degree of dissociation of glucocorticoid from GR during sample preparation and during the MT assembly assay is unknown, however, the observed effect of further addition of hormone to GR indicates some degree of hormone dissociation. The observation that the conversion of GR from a soluble to a particulate form is inhibited by molybdate (Pratt et al., 1989) may be indicative of a different effect of non-activated and activated GR, respectively, on MT assembly. It is thus possible that non-activated GR transduces a larger modulatory effect by glucocorticoids on MTs; we were, however, not able to test this hypothesis owing to the lack of purified, non-activated GR.

There were no detectable effects of hsp90 alone. Preliminary experiments where GR and hsp90 have been added together have not yielded any consistent results.

4.1. Interaction between glucocorticoid hormones and MTs

There are previous reports in the literature that glucocorticoid hormones may affect MTs; control rat C_6 glioma cells (clone ST1) seldom display a defined MT network, but rather a 'dispersed' tubulin pattern (Armelin and Armelin, 1983). Incubation of these cells with 700 nM cortisol for 48 h induced the formation of a typical, colchicine sensitive MT network in most of the cells (Armelin and Armelin, 1983). Treatment of a human cervix cancer cell line, NHIK 3025, with 100 nM dexamethasone for 4 days induced morphological changes of MTs (Bakke, 1986). The effect was counteracted by simultaneous treatment with the glucocorticoid antagonist RU 38486, indicating involvement of GR (Bakke, 1986). It is unclear how these findings relate to the results presented here.

On simultaneous application of cortisone and colchicine, cortisone was found to decrease the effect of colchicine on the mitotic ratio, both in the rat epidermis and the outer orbital gland (Isotalo and Teir, 1953). This is in line with the previously described apparent antagonistic effects between cortisone and colchicine (Dustin, 1963).

According to a recent report, glucocorticoid-induced gene expression is maintained in the absence of intact microtubules (Szapary et al., 1994). This does not rule out the possibility that extranuclear and/or extragenomic actions by glucocorticoids may be of importance in the regulation of, e.g. MT homeostasis.

4.2. Interaction between other steroid hormones and MTs

Both natural and synthetic estrogens have been reported to inhibit tubulin assembly and induce structural MT aberrations, which may produce, e.g. mitotic arrest and aneuploidy (Rao and Engelberg, 1967; Tucker and Barrett, 1986; Sato et al., 1987; Wheeler et al., 1987; Chaudoreille et al., 1991). The synthetic non-steroidal estrogen and carcinogen, diethylstilbestrol (DES), has been shown to bind to the colchicine site on tubulin and to exert a dose-dependent effect on tubulin assembly (Hartley-Asp et al., 1985; Sharp and Parry, 1985; Chaudoreille et al., 1991) and cell growth (Tucker and Barrett, 1986) in vitro. 17β-Estradiol significantly and reversibly decreases MT number and length in rat endometrial epithelial cells after only 35 s incubation (Szego et al., 1988). 17β-Estradiol has been shown to reverse formation of vinblastine-induced MT-containing paracrystals (Wilson et al., 1982) and DES protects rats from the toxicity of vinblastine in vivo (Cutts, 1968). Colchicine inhibits the 17β-estradiol-induced uterine water retention (Wilson et al., 1982; Alexandre et al., 1988). Intracerebroventricular injection of colchicine to guinea pigs induces the appearance of both estrogen receptor (ER) and progestine receptor (PR) immunoreactivity in atypical brain areas, indicating intracellular interaction between ER and MTs as well as PR and MTs in some neurons (Blaustein and Olster, 1993).

There is also evidence in support of a transient interaction between the calcitriol (vitamin D3) receptor and MTs during the initial phase (0–3 min) of calcitriol interaction with the cell (Barsony and McKoy, 1992). Finally, there is immunocytological evidence that some steroid hormones such as 17β-estradiol, progesterone and testosterone localize at the pericentriolar material (Nenci and Marchetti, 1978), which is known to contain MTs (Gould and Borisy, 1977). Several MAPs are able to induce MT assembly in vitro and it has been speculated that they may exert similar functions in vivo (Weingarten et al., 1975; Hiller and Weber, 1978). It is thus conceivable that also other proteins, e.g. GR, may affect the MT assembly process. Taken together, the results in this study indicate that GR inhibits MT assembly and that this inhibition may be modulated by glucocorticoid hormones.

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