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Cytoplasmic Steroid Effects on Nuclear RNA Polymerase Activity in Canine Mammary Carcinomas

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Abstract—*DNA-dependent RNA polymerase activity has been studied in isolated nuclei from canine mammary tumours. Initial experiments showed high levels of RNase activity in this tissue; accordingly, routine assays were terminated before loss of acid-precipitable radioactivity was evident. RNA polymerase A and B activity in isolated nuclei were shown to be increased by addition of receptor-containing cytosol previously incubated with oestradiol-17β, dihydrotestosterone or R5020. Where no receptor was present, as measured by saturation binding assays and sucrose density gradient analysis, there was no corresponding increase in polymerase activity. The steroid antagonists tamoxifen and cyproterone did not elicit any response even when their corresponding steroids produced a 1- to 2-fold stimulation of polymerase activity. Steroid-induced effects proved to be dose-dependent, with half maximal responses for oestradiol-17β* 8×10^{-8}M, *R5020* 2×10^{-6}M *and dihydrotestosterone* 9×10^{-6}M.

INTRODUCTION

THE NORMAL development and function of the secondary sex organs are dependent on the presence of gonadal hormones. Such a dependence often persists with the advent of neoplasia in these organs. The canine mammary gland is especially prone to neoplastic change [1–3] and it has been considered that such lesions could be hormonally influenced.

Current views consider that the steroid hormones achieve their effects via specific cytoplasmic receptor proteins [4, 5] to which they bind, and the resulting complex is then translocated to the nucleus. Close association of the complex with chromatin results in gene activation and an enhancement of RNA polymerase activity with the subsequent synthesis of new mRNA [6, 7].

Receptor proteins have previously been demonstrated in the cytosol fraction from canine mammary tumours that bind oestrogens, androgens and progestagens [8–10]. It was the intention of this study to examine the effect of these steroids in association with cytosols from canine mammary carcinomas on the activity of DNA-dependent RNA polymerases in the nuclei from these same tumours, and to relate the results to the presence or absence of specific cytoplasmic receptors.

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MATERIALS AND METHODS

[5-^{3}H]-UTP, [1,2,4,5,6,7-^{3}H]-5α-dihydrotestosterone and [2,4,6,7-^{3}H]-oestradiol-17β were obtained from Amersham International Ltd. (Amersham, Bucks, U.K.), and [17-methyl-^{3}H]-17α,21-dimethyl-19-nor-pregna-4,9-diene-3,20-dione ([^{3}H]-R5020) and R5020 from New England Nuclear (Dreieich, W. Germany). Nucleotides and α-amanitin were bought from Boehringer Corp. (London, U.K.). Diethyl pyrocarbonate (DEP), bovine serum albumin (BSA), calf thymus DNA, dithiothreitol, trisma, charcoal, oestradiol-17β and 5α-dihydrotestosterone (DHT) were obtained from Sigma (London) Chemical Co. Ltd. (Poole, Dorset, U.K.). 1-2α-Methylene-6-chloro-pregnen-4,6-diene-17α-hydroxy-3,20-dione (cyproterone) was kindly donated by Schering ag Berlin (W. Germany). Tamoxifen was a gift from I.C.I. Pharmaceuticals Ltd. (Cheshire, U.K.). Scintillant 299 was obtained from Packard Instrument Co. Ltd. (Illinois, U.S.A.). All other reagents were obtained from B.D.H. Chemicals (Poole, Dorset, U.K.) and were of Analar grade.

Canine mammary tumours were obtained from local veterinary surgeons and transported to the laboratory on ice. Histological evaluations were made on frozen sections and those diagnosed as carcinomas were selected for investigation.

Isolation and purification of nuclei

All procedures were carried out at 0–4°C unless otherwise indicated.

Tissue was weighed, finely minced and homogenised in 3 vol. of 0.25 M sucrose, 1 mM $MgCl_2$, filtered through nylon mesh and centrifuged at 800 *g* for 10 min. The crude nuclear pellet was resuspended in 2.2 M sucrose, 1 mM $MgCl_2$ and gently homogenised in a glass–Teflon homogeniser. The resulting nuclear suspension was layered on 2.2 M sucrose, 1 mM $MgCl_2$, and centrifuged at 50,000 *g* for 1 hr at 4°C. The pellet was resuspended in 0.25 M sucrose, 1 mM $MgCl_2$ and used in polymerase assays.

Determination of RNA polymerase activity

The assay mixture (325 μl) contained 120 mM Tris–HCl, pH 7.9, 30 mM KCl, 400 μM dithiothreitol, 600 μM NaF or 825 mM DEP, 10% glycerol, 600 μM ATP, CTP and GTP, 40 μM UTP and 123 nM [^{3}H]-UTP (25 Ci/mmol), 10 mM $MgCl_2$ or 5 mM $MnCl_2$, 100 mM NH_4SO_4, 25 ng α-amanitin and 50 μl of nuclei (100–200 μg/ml DNA). The incubations were maintained at 37°C for 17 min and the reaction terminated by cooling on ice. Samples were precipitated with 10% trichloroacetic acid (TCA), 1 mM $Na_4P_2O_7$ and yeast RNA (50 μg/50 μl) followed by centrifugation at 800 *g* for 10 min. Pellets were washed three times with 5% TCA (500 μl) and once in ethanol (500 μl), and solubilised in 20% PCA (250 μl) for 1 hr at 60°C. Radioactivity was determined in a 200-μl sample in 5 ml of scintillant 299 in an Isocap liquid scintillation spectrophotometer. Counting efficiency was 25–30% and correction was made for quenching by the external standard method.

Labelling of cytosol with steroids

The supernatant from the 800 *g* centrifugation in the isolation of nuclei was further spun at 114,000 *g* for 1 hr. The resulting cytosol was incubated alone or in the presence of steroids or steroid antagonists at 1×10^{-5}M for 1 hr at 25°C. Cytosol was further incubated with isolated nuclei for 30 min at 37°C and centrifuged at 800 *g* for 10 min. Nuclei were resuspended in 0.25 M sucrose, 1 mM $MgCl_2$, and used in RNA polymerase B assays to determine the effect of steroid-labelled cytosol on transcription, their specificity for steroids and, by incubating cytosol containing the appropriate receptor with increasing concentrations of steroid from 1×10^{-4}M to 1×10^{-10}M, the dose-dependence of RNA polymerase B activity.

Estimation of cytoplasmic receptor sites

Cytoplasmic receptor sites were measured using a modified version of the method of d'Arville and Pierrepoint [8].

Tissue was homogenised using a glass–Teflon homogeniser in 3 vol. buffer consisting of 10 mM Tris–HCl, pH 7.4, 1 mM EDTA and 0.5 mM dithiothreitol (Buffer A), filtered through nylon mesh and centrifuged at 114,000 *g* for 1 hr at 4°C. The resulting cytosol was then vortexed with an equal volume of pelleted dextran-coated charcoal (0.5% Norit A, 0.05% dextran and 0.1% gelatin) and incubated for 10 min at 0°C followed by centrifugation at 800 *g* for 10 min. An aliquot of the charcoal-treated cytosol was used for protein determination.

Cytosol (250 μl) was vortexed with an equal volume of buffer containing 5 nM [^{3}H]-steroid in the presence and absence of 500 nM unlabelled steroid and incubated at 0°C for 4 hr. Dextran-coated charcoal (500 μl) was added to each tube, vortexed and allowed to stand for 10 min at 0°C prior to centrifugation at 800 *g* for 10 min. An aliquot (500 μl) was removed from the supernatant for determination of radioactivity.

Sucrose density gradient analysis

Cytosol was incubated for 10 min with an equal volume of pelleted dextran-coated charcoal and centrifuged at 800 *g* for 10 min. Aliquots (1 ml) were incubated with 5 nM tritiated steroids in the presence and absence of 500 nM unlabelled steroid at 0°C for 4 hr. Unbound steroid was removed with dextran-coated charcoal and a 400-μl aliquot layered onto linear sucrose density gradients (5 ml; 5–20% w/v) in buffer A as described by Martin and Ames [11]. An equal aliquot of bovine serum albumin (1 mg/ml) was layered onto a similar gradient to provide a standard with a coefficient of sedimentation of 4.6 S. Gradients were centrifuged at 114,000 *g* for 16 hr at 4°C. Fractions (200 μl) were obtained by upward displacement into scintillation vials, and radioactivity was determined in 10 ml scintillant 299. BSA fractions were prepared by the addition of 1 ml of water and their optical density measured at 280 nm.

Chemical analyses

DNA was determined by the method of Burton [12] using calf thymus DNA as a standard. Protein estimations were performed using the method of Lowry *et al.* [13] using crystalline BSA as a standard.

RESULTS

Optimal conditions have been established for the determination of DNA-dependent RNA polymerase activities in isolated nuclei from canine mammary carcinomas. Initial experiments showed that steroid effects on enzyme activity were similar for both type A and B enzymes and consequently further experiments concentrated on investigations of RNA polymerase B, with the associated benefits of a simpler and more reproducible assay system.

The results presented for the incorporation of [^{3}H]-UMP by RNA polymerase B in canine mammary carcinomas (Fig. 1a) are atypical of those obtained in different tissues by other workers [14, 15] and by this laboratory using rat liver nuclei in the same assay system (Fig. 1b). The difference in the results from those of the saturation kinetics usually observed in this type of assay suggests the presence of considerable ribonuclease activity. By replacing sodium fluoride with the more potent ribonuclease inhibitor diethylpyrocarbonate at concentrations of 0–1.38 M, normal reaction kinetics were produced at a concentration of 825 mM (Fig. 1a), though at the expense of loss of incorporation. However, the high concentration of DEP necessary and the reduction in incorporation were unacceptable and routine assays were therefore terminated after 17 min of incubation, before loss of incorporation was evident.

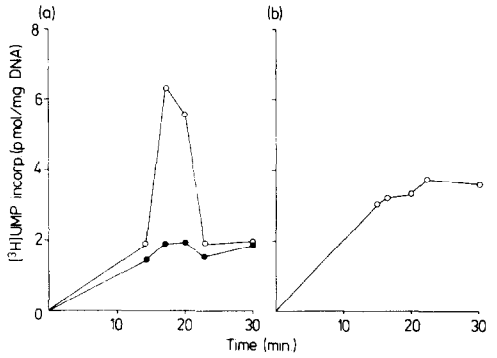

Fig. 1. Effect of diethylpyrocarbonate on RNA polymerase B activity of canine mammary carcinoma nuclei. (a) (○) *Nuclei incubated under normal assay conditions at* 37°C; (•) *replacement of NAF by diethylpyrocarbonate* (825 mM). *In (b)* (○) *rat liver nuclei were used to show normal reaction kinetics. At indicated times incubations were terminated and the incorporation of* [^{3}H]*-UMP determined. Each value represents the mean of* 4 *determinations.*

The effect of steroid receptor complexes on transcription was examined for a number of steroids. Stimulation of RNA polymerase B activity above the levels seen in controls in the absence of steroid was dependent on the presence in the cytosol of the specific receptor for the steroid used, as measured by a saturation binding assay (Table 1) and sucrose density gradient analysis (Fig. 2). Stimulation was steroid-specific for the receptor used (Fig. 3); incubation of cytosol containing oestradiol, and DHT receptors with tamoxifen and cyproterone respectively did not produce stimulation of polymerase activity when added to nuclei.

Steroid effects on transcription were dose-dependent (Fig. 4); however, the concentration of steroid required to elicit a half maximal response was always greater than that expected from the range of dissociation constants of the respective steroids for their receptors in canine mammary tumours [8]. It is likely that this is due to steroid receptor complexes formed with endogenous steroid in control cytosols stimulating transcription above basal levels, and therefore elevated doses of steroid are required to show a stimulation of polymerase activity above this level.

DISCUSSION

Previous work in this laboratory has shown the presence of specific receptors in canine mammary neoplasms [8–10]. Among these tumours are a

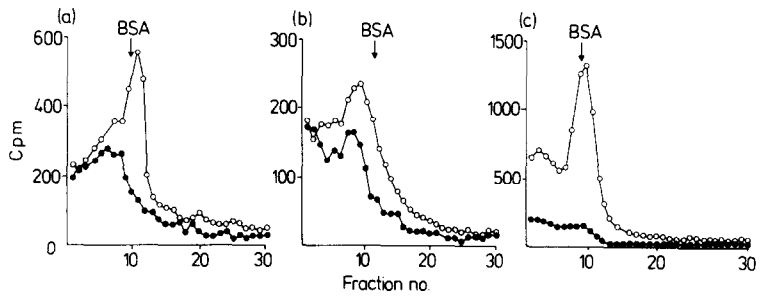

Fig. 2. Radioactive profiles of cytosol preparations from canine mammary carcinomas. (a) (○) *Profile from cytosol incubated with* [^{3}H]*-oestradiol-17β;* (•) *cytosol incubated with* [^{3}H]*-oestradiol-17β in the presence of* 100*-fold excess of unlabelled oestradiol-17β. (b)* (○) *Profile from cytosol incubated with* [^{3}H]*-R5020 alone;* (•) *cytosol incubated with* [^{3}H]*-R5020 in the presence of* 100*-fold excess of unlabelled R5020. (c)* (○) *Profile from cytosol incubated with* [^{3}H]*-5α-dihydrotestosterone alone;* (•) *cytosol incubated with* [^{3}H]*-5α-dihydrotestosterone in the presence of* 100*-fold excess of unlabelled 5α-dihydrotestosterone.*

Table 1. Effect of steroid receptor complexes on transcription

No.	Oestradiol-17β receptor site concentration (fmol/mg protein)	RNA polymerase B activity as a % stimulation of control	R5020 receptor site concentration (fmol/mg protein)	RNA polymerase B activity as a % stimulation of control	DHT receptor site concentration (fmol/mg protein)	RNA polymerase B activity as a % stimulation of control
1501	-ve	N.D.	-ve	N.D.	-ve	N.D.
510	-ve	N.D.	4.8	—	-ve	—
239	16.5	163 ± 15	-ve	N.D.	-ve	—
521	32.1	153 ± 7	46.5	120 ± 8	56.7	132 ± 10
3006	127.2	159 ± 15	67.8	117 ± 12	38.51	—
207	-ve	—	52.13	188 ± 11	4.0	119 ± 11
6002	21.7	—	10.4	130 ± 2	36.3	—
8000	-ve	—	-ve	—	102	151 ± 9
6006	6.2	—	-ve	—	10	183 ± 4
6008	4.5	123 ± 5	7.0	135 ± 2	10	126 ± 5
529	8.0	140 ± 15	-ve	N.D.	6.4	132 ± 17
2027	8.0	128 ± 6	16.3	148 ± 3	3.6	N.D.
532	4.1	N.D.	7.6	113 ± 6	5.1	N.D.
528	-ve	N.D.	-ve	N.D.	-ve	N.D.
522	-ve	N.D.	-ve	N.D.	10.6	—
2018	-ve	N.D.	-ve	N.D.	-ve	N.D.
3008	-ve	N.D.	-ve	N.D.	-ve	N.D.
5006	-ve		-ve	N.D.	-ve	N.D.

Cytoplasmic steroid receptor concentration and corresponding stimulation of RNA polymerase B activity produced by addition of steroid labelled cytosol to nuclei. Percentage stimulation and standard deviations compared to controls (non-steroid treated cytosol). (-ve) Receptor undetectable; (N.D.) no difference to controls; (—) RNA polymerase B assay not performed. Results are means of 4–6 determinations.

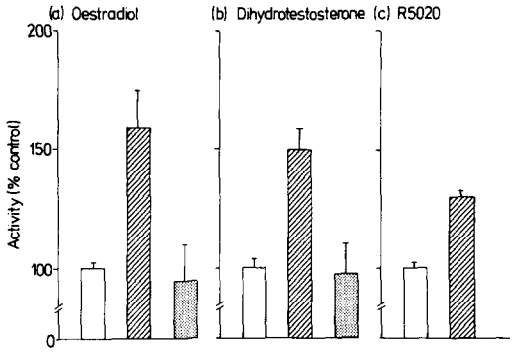

Fig. 3. Specificity of assay system for steroids. Cytosol from mammary carcinomas containing the appropriate receptor were incubated with (a) oestradiol-17β ▨ and tamoxifen ▩ at 1×10^{-5}M; (b) 5α-dihydrotestosterone ▨ and cyproterone ▩ at 1×10^{-5}M; (c) R5020 ▨ 1×10^{-5}M for 1 hr at 25°C, and incubated with nuclei at 37°C for 30 min. RNA polymerase B activities were determined and results expressed relative to controls incubated in the absence of added steroid (□).

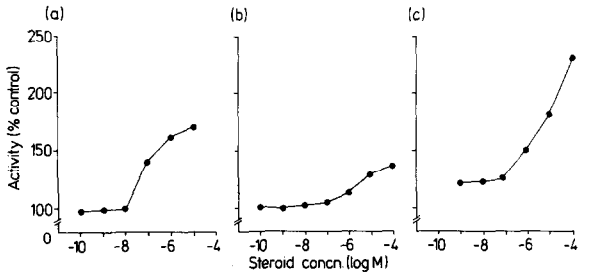

Fig. 4. Dose-dependence of steroidal stimulation of RNA polymerase B activity. Cytosol was incubated with increasing concentrations of (a) oestradiol-17β; (b) R5020; and (c) dihydrotestosterone for 1 hr at 25°C, and incubated with isolated nuclei for 1 hr at 37°C. RNA polymerase B activities were determined. Results are the mean of 4–6 determinations and are expressed relative to controls incubated in the absence of added steroid.

subset which may be classified histologically as malignant and defined as carcinomas [16]. The present work has concentrated on determining the function of these steroid receptor systems in promoting increases in RNA production associated with growth in these tumours with the aim of developing steroid antagonist therapy.

These studies show a distinct loss in acid-precipitable incorporated radioactivity in RNA polymerase assays with time. This effect can be abolished by using diethylpyrocarbonate and therefore it seems likely that RNase levels are elevated. Work using rat mammary gland during lactation and R3230AC mammary adenocarcinoma showed increased RNase activities [17]. We suggest that canine carcinomas are constantly in a state which allows RNase levels to be permanently elevated.

Canine mammary carcinomas are steroid-sensitive only when they contain specific cytoplasmic receptors. This has been similarly shown using rat mammary tumours [18]. If the cytoplasm contains more than one steroid receptor a stimulation of polymerase activity is found when the respective steroid is incorporated

into the assay. RNA polymerase B and to a lesser extent RNA polymerase A were both stimulated by cytoplasm–steroid combinations, which complies with studies using other tissues such as rat ventral prostate [19] and calf uterine endometrium [20].

The failure by tamoxifen and cyproterone to elicit an increase in [^{3}H]-UMP incorporation in contrast to the 0.7- to 1.3-fold stimulation found with oestradiol-17β and DHT suggests that receptor complexes with these compounds cannot bind to chromatin in a manner that will promote transcription.

The presence of specific steroid receptors in the cytoplasm enabled us to show that the stimulatory effect was dose-dependent for all three steroids tested. However, their half maximal responses were somewhat increased compared to the KDs from Scatchard analysis of canine mammary neoplasms [9]. This increase could be due to the presence of endogenous steroid, which would require a greater amount of steroid to elicit the corresponding effect. Sucrose density gradient analysis of these tissues showed a receptor with a sedimentation coefficient of 5 S for oestradiol-17β of 4 S for R5020 and of 4 S for DHT.

These results emphasise previous suspicions of the hormonal responsiveness of certain mammary carcinomas in the bitch. It is most pertinent that out of the 43 receptor site concentrations and RNA polymerase tests undertaken in this study, in no instance was a stimulation of polymerase activity achieved by the steroids examined in the absence of the respective receptor. These studies also emphasise the multiplicity of hormone-dependence in these tumours.

The clinical implications from this work are that rational forms of anti-hormone therapy may be studied in those animals from which receptor-positive carcinomas have been removed.

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