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MOLECULAR ASPECTS ON THE MECHANISM OF ACTION OF TESTOSTERONE IN RAT BONE MARROW CELLS

J. MINGUELL and L. VALLADARES*

Departamento de Ciencias Químicas y Fisológicas, Sede Santiago Sur, Universidad de Chile, Casilla 3931, Santiago, Chile

SUMMARY

The fate of [^{3}H]-testosterone in female rat bone marrow cells has been studied. The hormone is taken up by the cytosol and nuclear fractions to a different extent, the nucleus taking up the larger amount of the hormone. Receptor proteins have not been detected in the cytosol and the free hormone is transported from cytoplasm to nucleus, where it becomes bound to a soluble nuclear receptor. The hormone–nuclear soluble receptor complex is then bound to chromatin components in a process that seems to be hormone and tissue specific.

INTRODUCTION

Steroid sensitive tissues contain hormone-binding proteins called receptors which exhibit high affinity for the hormone and account for the concentration of the hormone in target tissues. In most of the tissues studied, the hormone in association with the cytosol receptor migrates from the cytoplasm to the nucleus, where the complex associates with nuclear components [1].

Steroid hormones have been shown to bind *in vitro* and *in vivo* to several nuclear components such as DNA, histones and non-histone proteins, histone-DNA complexes and enzymes [2]. It has been postulated that as a consequence of these interactions some transcriptional properties of chromatin are changed. These changes could be related to the molecular mechanism of action of steroid hormones on the so-called target tissues.

These general considerations for the mode of action of steroids apply to androgen sensitive tissues, such as prostate where receptor proteins have been identified in the cytosol, showing specificity for testosterone or 5-α-dihydrotestosterone (5α-DHT) [3]. The receptor–hormone complex is then transported into the nucleus, producing there some of the typical responses elicited by the androgens in the target tissues [3, 4 5]. Although bone marrow is not considered as a typical androgen-dependent tissue, we have demonstrated that testosterone produces changes in both total and nuclear RNA metabolism in normal or polycythemic rat bone marrow [6, 7]. Also, the hormone stimulates the activity of a highly specific ribonuclease mainly localized in the nucleus of bone marrow cells [7, 8]. The fact that testosterone stimulates bone marrow metabolism by itself and is not mediated by other polypeptide hormones [6, 9], allows one to postulate that once the hormone reaches the marrow from the blood, it associates with cytoplasmic components and is then transported into the nucleus, where the main molecular effects of the hormone have been detected.

Due to the above facts, it was of interest to study the fate of the hormone inside the bone marrow cells, both at cytoplasmic and nuclear sites. Some of the results obtained are described in this communication.

MATERIAL AND METHODS

Female Wistar rats (200–250 g) were used. When *in vivo* experiments were performed, each rat was given intraperitoneally 6 μCi [^{3}H]-testosterone in 0·2 ml saline. Thirty minutes later they were killed and bone marrow removed as described [8]. Cells were gently homogenized in 50 mM Tris–HCl buffer, pH 7·4 containing 5×10^{-3} M $MgCl_2$ and 5×10^{-3} M NaCl and the suspension centrifuged at 600 *g* for 15 min. The pellet obtained was washed twice with 0·25 M sucrose solution containing 5×10^{-3} M $MgCl_2$. The 600 *g* supernatant, after centrifugation at 105,000 *g* for 1 h, yielded a clear supernatant that was used as the cytosol fraction. Alternatively, for experiments reported in Fig. 1, cytosol was prepared by gently homogenizing cells in 50 mM Tris–HCl buffer, pH 7·4 containing 2 mM mercaptoethanol (TmE buffer), followed by a centrifugation at 105,000 *g* for 1 h. Excessive frothing and shearing were carefully avoided.

* Departamento de Ciencias Médico Biológicas y Básicas, Facultad de Medicina, Sede Santiago Sur. Universidad de Chile.

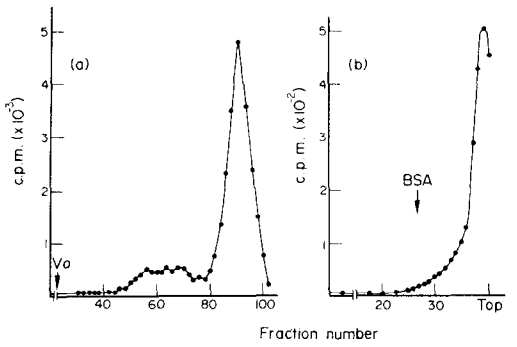

Fig. 1. The search of a cytosol receptor for testosterone in bone marrow.
(a) Cytosol (13 mg protein) was incubated with [^{3}H]-testosterone (0·15 μCi) in TmE buffer for 4 h at 25°C at a final volume of 2·4 ml. At the end of the incubation, 2 ml of the incubate was applied to a Sephadex G-25 column and processed as described in Material and Methods. (b) Cytosol (5·7 mg of protein) prepared in TmE buffer containing 10% glycerol was incubated for 4 h at 4°C at a final volume of 1 ml. At the end of the incubation, 0·18 ml of the incubate was applied to the top of a sucrose–glycerol gradient and processed as described under Material and Methods. Bovine Serum Albumin (BSA) (Sigma) in TmE buffer was used as a sedimentation marker.

The purified nuclear fraction was prepared as previously described [8]. All the above mentioned steps were performed at 0–4°C.

Linear sucrose gradients (5–15% w/w containing 10% glycerol) were prepared from ribonuclease-free sucrose (Serva, W. Germany) in TmE buffer in nitrocellulose tubes using a Buchler density gradient system. After overnight equilibration, samples were layered on top of the gradient and the charged tubes were centrifuged for 16 h at 4°C in a SW 39 rotor in a Beckman L2 65B ultracentrifuge. At the end of the run, the tubes were pierced and fractions of 9 drops each were collected and measured for radioactivity.

Columns of Sephadex G-25 (2·5 × 23 cm.) were packed and equilibrated with TmE buffer. Dextran blue was used for the determination of the void volume (Vo) of each column. Samples were separated on the columns, using TmE buffer as the eluting medium, at a constant elution rate of 0·33 ml/min. Each fraction was monitored by extinction analysis at 260 and 280 nm and by scintillation spectrometry. The whole procedure was conducted at 4°C.

For the preparation of the 0·15 M or 0·30 M KCl extracts, one ml of TmE buffer containing 1×10^{-3} M $MgCl_2$ and 0·15 M or 0·30 M KCl was added to a pellet of purified nuclei (630 μg DNA). The suspension was stirred in a Vortex mixer, left at 4°C for 45 min, and then centrifuged at 5000 *g* for 10 min. The supernatants thus obtained were used as the nuclear KCl extracts.

For the analysis of the components in the nuclear KCl extracts that bind testosterone, the above nuclear KCl extracts (850 μg protein) were incubated with 0·1 μCi of [^{3}H]testosterone in TmE buffer containing 0·15 M or 0·30 M KCl at 4°C for 30 min at a final volume of 1 ml. At the end of the incubation, free [^{3}H]-testosterone was removed by the addition of a 2 ml suspension (previously pelleted) of charcoal–Dextran [10]. The mixture was left at 4°C for 5 min and then centrifuged at 400 *g* for 5 min. The clear supernatant thus obtained, containing the nuclear components that bind testosterone, was subjected to sedimentation analysis or used for studies of interaction with chromatin. Chromatin was prepared from bone marrow, liver and spleen nuclei following the method described by Paul *et al.*[11].

For the experiments involving binding of testosterone-labelled KCl extract of nuclei with chromatin, various amounts of [^{3}H]-testosterone- 0·15 M KCl nuclear extract were incubated with 50 μg DNA of bone marrow chromatin suspended in TmE buffer containing 0·15 M KCl. Samples were incubated at 4°C for 30 min and chromatin was separated, washed and analysed as described by Schrader *et al.*[12].

Radioactivity was extracted from subcellular fractions after three extractions of each sample with ethylacetate. Polar and non-polar metabolites of [^{3}H]-testosterone were analysed by the method described by Bruchowsky *et al.*[13]. Extracts were evaporated to dryness and radioactivity measured after the addition of scintillator (4 g PPO, 400 ml ethanol and 600 ml toluene), in a Nuclear Chicago Scintillation Spectrometer model Mark 1, at a counting efficiency for tritium of 30%.

DNA and protein were determined according to Burton[14] and Lowry *et al.*[15], respectively. [1,2-^{3}H]-testosterone (specific radioactivity 30 mCi/mmol) and 5α-dihydro [1,2-^{3}H]-testosterone (specific radioactivity 40 mCi/mmol) were purchased from New England Nuclear Corp.

All chemicals used were analytical grade.

RESULTS

Distribution of radioactivity in cell fractions

After giving a 30 min pulse of [^{3}H]-testosterone to female rats, radioactivity in bone marrow cell fractions was measured. As shown in Table 1, approximately 85% of the radioactivity present in the homogenate was recovered in both nuclear and cytosol fractions, and more than 50% of the total radioactivity was found in the nuclear fraction.

Table 1. Distribution of radioactivity in bone marrow 30 min following intraperitoneal administration of [^{3}H]-testosterone to female rats

Fraction	(d.p.m. *g*) bone marrow	(%)
Homogenate	460	100
600 *g* pellet	269	59
Cytosol	113	25
Cytosol pellet	60	13

The results are from a typical experiment of a series of three. Bone marrow from 3 rats (approximately 1·2 g) was pooled, cells were fractionated and radioactivity extracted and measured as described under material and methods.

The fact that the whole radioactivity in the homogenate was extracted with chloroform–methanol indicates that the radioactivity present in bone marrow cells is made up only of non-polar testosterone derivatives [13]. Under *in vitro* conditions, a similar pattern of radioactivity distribution in cell fractions was obtained. As shown in Table 2, more than 60% of the recovered radioactivity was present in the nuclear fraction. The nature of the radioactivity was not determined in these experiments, but the non-polar character of the extracted compounds and the previous findings showing the failure of bone marrow cell suspensions to transform testosterone into 5 α-dihydro-testosterone or androstenedione [16] suggest that in this tissue testosterone is the main component.

It is worth mentioning that under *in vitro* conditions, the entry of the hormone into the cell is temperature dependent; however, the distribution of radioactivity within the cell is not affected by temperature. In fact, at 4°C or at 30°C, almost the same percentage of radioactivity is present in the nuclear fraction. This is in contrast to what happens in other steroid-sensitive tissues, where the entry of the hormone or the receptor–hormone complex into the nucleus is temperature dependent [1].

The search for a cytosol receptor in bone marrow

In the androgen-sensitive tissues a cytosol receptor has been described that specifically binds testosterone or 5 α-dihydrotestosterone and accounts for the transfer of the hormone to the cell nucleus [3, 17]. Since there is a considerable transfer of testosterone from cytosol to nucleus in bone marrow, a search for receptors was undertaken. After several attempts we have not been able to detect any association of the hormone with high molecular weight components of the cytosol. Some of the typical results obtained are shown in Fig. 1.

When the incubation mixture of cytosol with [^{3}H]-testosterone was applied to a Sephadex G-25 column (Fig. 1a), no radioactivity was eluted in the void volume of the column. More than 95% of the radioactivity was eluted as free [^{3}H]-testosterone (peak 1) and the remaining radioactivity was recovered as a possible random association complex of the hormone with the Tris–base of the buffer (peak 2).

Cytosol prepared and incubated with [^{3}H]-testosterone in buffer containing 10% glycerol [18], and further analysed by sedimentation in sucrose gradients (Fig. 1b), shows that radioactivity was present only in the gradient regions where free [^{3}H]-testosterone sediments.

Attempts to isolate the reaction products, after incubating different amounts of cytosol and [^{3}H]-testosterone, by using ammonium sulphate precipitation [19] or by treatment with Dextran–coated charcoal [10] have not been successful. These results prompted us to conclude that bone marrow cytosol either lacks a typical receptor for testosterone or that its concentration is too low to be detected by the methods used.

Table 2. *In vitro* retention of radioactivity in bone marrow subcellular fractions after one hour incubation of bone marrow suspension with [^{3}H]-testosterone

Temperature	(c.p.m.) in 600 *g* pellet	(c.p.m.) in 600 *g* supernatant	% (c.p.m.) in 600 *g* pellet
4°C	276	146	67
	233	130	64
30°C	387	162	71
	364	189	66

Bone marrow from 8 rats were removed, collected and pooled in Krebs–Ringer bicarbonate buffer. A cell suspension in the same buffer, containing 2·36 mg DNA in each case, was incubated at the temperature indicated with [^{3}H]-testosterone (0·020 μCi) in a final volume of 2 ml.

At the end of the incubation, cells were pelleted and rinsed twice with the incubation buffer. Cells were handled as described in Material and Methods and radioactivity in the 600 *g* pellet and supernatant was extracted and measured.

Figures given correspond to 2 separate experiments and were corrected for DNA and radioactivity lost during the procedure.

The transfer of testosterone from cytoplasm to nucleus

The absence of a cytosol receptor that could account for the transfer of testosterone from cytoplasm to nucleus demanded experiments to show how the hormone is transported. The results of such experiments are presented in Table 3.

The incubation of a purified nuclear fraction with [^{3}H]-testosterone shows that the hormone is taken up by the nucleus. When Triton X-100 was omitted from the purification step and nuclei obtained were incubated with [^{3}H]-testosterone, the hormone was retained by the nuclei to the same extent as with the Triton X-100 purified nuclei. If cytosol were added to the incubation medium, no increase in the amount of [^{3}H]testosterone retained by the nuclei was observed.

From these results, we might conclude that bone marrow nuclei have mechanisms for the uptake and retention of testosterone by processes not mediated by cytosol components or by the outer nuclear membrane which is removed by Triton.

The interaction of testosterone with nuclear components

In vitro experiments using purified nuclear fractions were performed to study the fate of the hormone within the nuclei. After incubating the nuclear fraction with [^{3}H]testosterone, nuclei were extracted with KCl at two different concentrations. When the nuclei were treated with 0·3 M KCl, approximately 70% of the total radioactivity was extracted and the remaining 30% was strongly associated with the nuclear pellet.

Since 0·3 M KCl extracts soluble nuclear proteins and some of the chromatin-associated proteins [20], it is assumed that in the 0·3 M KCl extract of bone marrow nuclei, various components that can bind testosterone could be present. This assumption proved to be correct when the 0·3 M KCl extract was sedimented in a sucrose gradient. Results in Fig. 2a, show that two components that bind testosterone are present with sedimentation coefficient lower than 4 *S*.

Table 3. The uptake of [^{3}H]-testosterone by bone marrow nuclei

Nuclei prepared	Radioactivity (c.p.m./mg DNA)
With Triton (3)	3·420 ± 185†
Without Triton (3)	3·680 ± 420
With Triton + cytosol* (3)	3·200 ± 356

A suspension of purified nuclei (628 μg DNA) in Tris–HCl buffer, pH 7·4, containing 1×10^{-3} M $MgCl_2$ was incubated with [^{3}H]-testosterone (0·15 μCi) for 45 min at 4°C. At the end of incubation, nuclei were washed twice in the buffer and radioactivity counted.

Figure in parentheses represent number of determinations.

* Cytosol (230 μg protein) was added at the beginning of the incubation.

† Standard deviation.

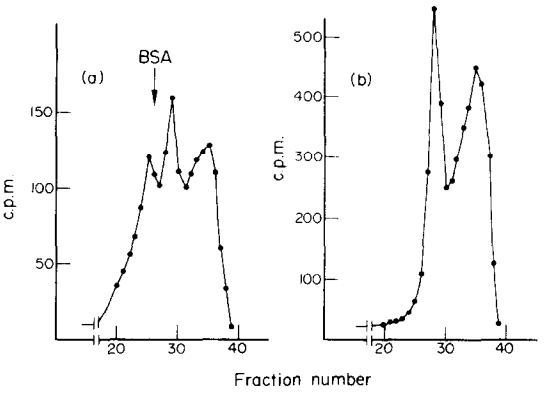

Fig. 2. Sucrose gradient analysis of [^{3}H]-testosterone-nuclear KCl extracts.
(a) A sample of [^{3}H]-testosterone-nuclear 0·30 M KCl extract (3500 c.p.m.) was applied to a sucrose gradient. (b) A sample of [^{3}H]-testosterone-nuclear 0·15 M KCl extract (7600 c.p.m.) was applied to a sucrose gradient. Details on the preparation of the [^{3}H]-testosterone nuclear KCl extracts, sucrose gradients and analysis of gradient fractions are described under material and methods. Bovine serum albumin (BSA) was used as sedimentation marker.

The partial isolation of one of these two components was achieved by lowering the concentration of KCl to 0·15 M. At this concentration chromatin protein is not extracted [12]. The treatment of nuclei with 0·15 M KCl produces an extract that binds testosterone well when compared with that derived after extracting nuclei with 0·30 M KCl. If the radioactivity associated with the 0·30 M extract is considered as 100%, that extracted with 0·15 M KCl represents approximately 65%.

The 0·15 M KCl extract, when applied to a sucrose gradient and subjected to ultracentrifugation, shows that only one component that binds the hormone is present (Fig. 2b). These results suggest that in the nuclei of rat bone marrow cells, there is a soluble component that strongly binds the hormone once it reaches the nucleus, and another component extractable with 0·30 M KCl that could be a chromatin component.

To check the above suggestions, experiments described in Fig. 3 were performed. It can be seen that chromatin prepared from bone marrow nuclei and allowed to react with either free [^{3}H]-testosterone or with the 0·15 M KCl nuclear extract previously labelled with [^{3}H]-testosterone, binds the hormone to a different extent. The binding of the free hormone to chromatin is very poor under a wide range of [^{3}H]-testosterone concentrations. However, when the hormone

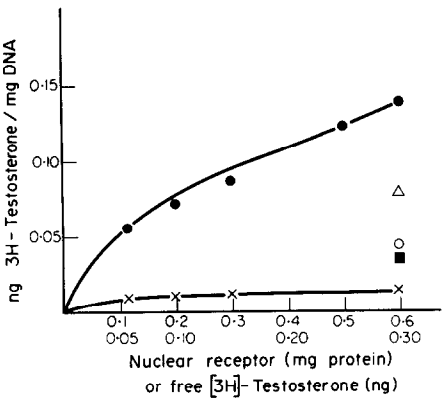

Fig. 3. Binding of [^{3}H]-testosterone-labeled nuclear soluble receptor to chromatin.
The preparation of the [^{3}H]-testosterone-labeled nuclear receptor (0·15 M KCl extract), chromatin and details on incubations, chromatin washes and collection on Millipore filters are described under Material and Methods. (●) bone marrow chromatin, (■) liver chromatin, (○) spleen chromatin (×) binding of free [^{3}H]-testosterone to bone marrow chromatin (△) [^{3}H]-dihydrotestosterone (0·2 μCi) was used instead of [^{3}H]-testosterone to load the bone marrow nuclear receptor.

is first bound to the 0·15 M KCl nuclear extract and then allowed to react with chromatin, a significant increase on the amount of bound testosterone can be observed.

The binding of the bone marrow nuclear soluble receptor–hormone complex to chromatin is better when the hormone associated with the receptor is testosterone rather than 5 α-dihydrotestosterone and also when the acceptor is chromatin from bone marrow cells rather than from other tissues. As shown in Fig. 3, when 5 α-dihydrotestosterone was used instead of testosterone to load the nuclear soluble receptor, the binding to chromatin was poor. Also, it can be seen that liver or spleen chromatin binds the bone marrow nuclear soluble testosterone complex to a different extent from bone marrow chromatin. Whether this represents tissue specificity is not known, but saturation and competition experiments show that the nuclear receptor exhibits high affinity and low capacity of binding to testosterone (unpublished results.)

DISCUSSION

Several workers have previously attempted to study by different approaches the way androgens might affect rat bone marrow metabolism. Current evidence indicates that androgens stimulate bone marrow metabolism, directly inducing a selective response. This is the induction and processing of some RNA species and the synthesis of the specific protein, hemoglobin [6, 7, 21]. This allows us to consider bone marrow as a target tissue for testosterone, and thus to presume the existence in bone marrow cells of mechanisms for the uptake and retention of testosterone from plasma, for the transfer of the hormone from cytoplasm to nucleus, and for the molecular expression of the hormone action.

However, the results presented in this communication show that there are several differences between bone marrow and many of the other target tissues for androgens described in the literature. Briefly, they are:

(a) bone marrow cells lack the capability to transform testosterone into 5 α-DHT or androstenedione [16]. Therefore, it seems that in this tissue the active androgen is testosterone.

(b) attempts to detect high molecular weight components in the cytosol that bind testosterone have failed. This could mean that marrow cells either lack or possess in only very low amounts cytosol receptors for testosterone.

(c) in bone marrow the free hormone diffuses from cytoplasm to the nucleus in a process neither mediated by cytosol components nor by the outer nuclear membranes.

Some of these characteristics presented by bone marrow cells are also exhibited by other tissues under steroid control. Thus in the rat kidney cytosol, high affinity testosterone binding proteins are either absent or in a very low concentration [22]. It is also known that in the foetal kidney, unbound aldosterone crosses the nuclear membrane and forms complexes with nuclear components without the participation of a cytosol intermediate [23].

Receptor and acceptor sites have been described in androgen-sensitive tissues [24]. According to results shown in Fig. 2 and 3, bone marrow cell nucleus has both a receptor and an acceptor site for testosterone. The receptor site, extractable at 0·15 M KCl and present in the nuclear soluble fraction, is a protein with a sedimentation coefficient similar to that of bovine serum albumin and showing high affinity for testosterone and very poor affinity for 5 α-DHT. We assume that once the free hormone penetrates the nucleus, it becomes bound to this nuclear receptor, the whole complex or the hormone moiety being then transferred to the acceptor site(s) represented by components of chromatin not yet characterized.

These results and the fact that in nuclei previously loaded with [^{3}H]-testosterone and later extracted with increasing concentrations of KCl (0·3 M–1·2 M), approximately 30% of the radioactivity is always strongly associated with the remaining nuclear pellet suggest the presence in bone marrow nuclei of two acceptor

sites for the nuclear receptor [^{3}H]-testosterone complex. One could be a chromatin protein (non-histone proteins?) and the other could be DNA. Similar acceptor sites for receptor–hormone complexes have also been described in other steroid-sensitive tissues. [2, 12, 24].

The results presented here show that bone marrow cells, in spite of the absence of a cytosol receptor for testosterone, possess mechanisms to assure a specific response for the hormone within the nucleus [7].

If the nuclear hormone–receptor complex is indeed the inducer unit for testosterone modulation of nuclear transcription of DNA, then the binding of the receptor–hormone complex to the genome may prove to be of major importance to our understanding of testosterone action in bone marrow cells. Studies with the purified receptor and acceptors will be directed toward exploiting the binding effects with the goal of altering chromatin *in vitro*.

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