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SPECIFIC REGULATION OF MALE RAT LIVER CYTOSOLIC ESTROGEN RECEPTOR BY THE MODULATOR OF THE GLUCOCORTICOID RECEPTOR*

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Modulator is a novel low-molecular-weight organic compound that regulates activities of glucocorticoid and mineralocorticoid receptors as well as protein kinase C. In this study we show that male rat liver cytosolic estrogen receptor activation is inhibited by modulator in a dose-dependent manner. Fifty percent inhibition is obtained with 1 unit/ml modulator purified from bovine liver which is within the physiological concentration for modulator. However, sheep uterine cytosolic estrogen and androgen receptors are insensitive to regulation by modulator. Exogenous sodium molybdate treatment inhibits activation of all of these receptors of liver or uterus origin in an identical manner, further differentiating the effects of modulator and the molybdate anion.

The unactivated steroid receptor complex is characterized as a heterooligomeric complex with low affinity to DNA and high affinity for steroid ligands (for a review, see 1). Upon hormone binding, the receptor undergoes compositional and conformational changes, referred to as activation or transformation, enabling binding to DNA and regulation of gene expression (1).

Low molecular weight regulators of steroid receptors have been the subject of several studies (see 14 for a review). One of these regulators, referred to as modulator, is a low-molecular weight (ca. 500 Da), heat stable, novel organic compound that inhibits the activation of glucocorticoid and mineralocorticoid receptors (2-4). Modulator also stabilizes the steroid binding

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Abbreviations used: TA, triamcinolone acetonide; DHT, dihydrotestosterone; DES, diethylstilbestrol; ER, estrogen receptor; GR, glucocorticoid receptor; AR, androgen receptor.

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ability of both liganded and unliganded unactivated glucocorticoid and mineralocorticoid receptors (2-4). Modulator was initially proposed to mimic the effects of exogenous sodium molybdate (2,3) which was reported to regulate other members of the steroid receptor superfamily as well (6, 7, 8, 9). Sodium molybdate is often employed during receptor purification because it stabilizes the unactivated receptor complex form. Sodium molybdate, in this respect, appears to be a nonselective inhibitor of steroid receptor activation. The actions of modulator are differentiated from molybdate on the basis that molybdate does not superactivate protein kinase C while modulator does (15). Here, we also demonstrate a difference in their effects on specific receptor systems.

In the present study, we evaluated the specificity of regulation of steroid receptors by modulator. This was accomplished by evaluating the effects of modulator on the glucocorticoid, estrogen, and androgen receptors from male rat liver and sheep uterine sources in comparison to the effects of sodium molybdate.

MATERIALS AND METHODS

Cytosol and Steroid Binding— Liver cytosol was prepared from adrenalectomized male Sprague-Dawley rats (Buckshire Farms) as described previously (3). Adult sheep uterus was obtained from a local slaughter house, dropped into liquid nitrogen and ground to a powder using a household-style coffee grinder. Uterine powder was then redissolved in 50 mM KH_2PO_4, 5 mM dithiothreitol, 10 mM monothioglycerol, and 20 mM sodium molybdate, pH 7.4 buffer and homogenized, centrifuged at 14,000 x g for 45 min and the supernatant was further centrifuged at 100,000 x g for 1 h. Supernatant(cytosol) was aliquotted and kept at -70°C until use. Steroid binding was performed by incubating aliquots of cytosol with 100 nM [^{3}H] triamcinolone acetonide (TA, for glucocorticoid receptor, GR), 10 nM [^{3}H] estradiol (for estrogen receptor, ER) or 10 nM [^{3}H] dihydrotestosterone (DHT, for androgen receptor, AR) for 3 h on ice in the presence or absence of 500-fold molar excess radioinert ligands to determine the nonspecific steroid binding, which was <5%, <12%, and <10% of total binding for TA, estradiol, and DHT respectively. Specific binding was determined by hydroxylapatite binding assay (2).
Assays—Activation Inhibition, DNA binding inhibition, hydroxylapatite binding and DNA-cellulose binding assays were performed as described previously (3).
Modulator Purification—Bovine livers were obtained from a local slaughter house. Overall modulator purification was similar to the small scale purification from rat liver described previously (2). Briefly, liver was first cut to small pieces and homogenized using a PolyTron-PT3000 (Brinkmann Inc.) homogenizer in 50 mM KH_2PO_4, 1 mM dithiothreitol, pH 7.0 buffer (1:1, w/v). Homogenate was centrifuged at 14,000 x g for 60 min and the supernatant was further centrifuged at 100,000 x g for 60 min. The 100,000 x g supernatant (cytosol) was stored at -30 °C until use.

One hundred ml aliquots of the cytosol were treated with trypsin (final concentration was 1 μM) at 37°C for 3 h followed by chilling on ice and centrifugation at 14,000 x g for 10 min. The resulting supernatant was chromatographed at 5 ml/min on a 1.8 L bed volume Sephadex G-15 gel filtration column (5 x 95 cm) equilibrated with 100 mM NH_4HCO_3, pH 7.8 buffer. The void volume was allowed to run through, and 9 ml fractions were collected. Three milliliter aliquots of every fifth fraction were lyophilized and relyophilized 4 times with water, resuspended in 550 μl of 50 mM HEPES, 100 mM KCl, pH 7.4 and 450 μl aliquots were subjected to activation inhibition assay.

Fractions with modulator activity were pooled and chromatographed at 1 ml/min over 250 ml bed volume Dowex-1 anion-exchange columns (2.5x50 cm) equilibrated with 100 mM NH_4HCO_3, pH 7.8. The column was washed with 2 L of the same buffer at 2 ml/min and eluted with a linear gradient of 100-500 mM NH_4HCO_3 buffer (250 ml each) at 2 ml/min, and 8 ml fractions were collected. Three milliliter aliquots of these fractions were lyophilized and relyophilized 4 times with water, resuspended in 550 μl of 50 mM HEPES, 100 mM KCl, pH 7.4

and 450 µl aliquots were subjected to activation inhibition assay. Fractions with modulator activity were pooled, extensively lyophilized and resuspended in water of 1/30 the original volume. Modulator purified by this method contains a mixture of modulator isoforms 1 and 2 (3). Analytical TLC demonstrates presence of one ninhydrin stained spot and a molybdic acid stained streak. Modulator preparations react with both of these chemicals.

Unit Definition—One unit of modulator is defined as the amount of modulator that inhibits glucocorticoid receptor activation by 50% in a reaction mixture containing 450 µl buffer (50 mM HEPES, 100 mM KCl, pH 7.4) and 50 µl rat liver cytosol labeled with [^{3}H] TA at 15°C for 1 h.

RESULTS AND DISCUSSION

Male Rat Liver vs. Sheep Uterus

To study the effects of modulator on a variety of steroid receptors, male rat liver and sheep uterus were selected as the sources for these receptors. Both male rat liver and sheep uterine cytosolic fractions contained glucocorticoid (GR) and estrogen receptors (ER) in significant amounts as determined by incubation with the respective radioligand in the presence or absence of 500-fold molar excess radioinert ligand followed by hydroxylapatite binding assay (data not shown). While androgen receptor was present in sheep uterine cytosolic fractions in significant amounts, male rat liver cytosol lacked androgen receptor as determined by binding to [^{3}H] DHT. All of these receptors bound to DNA-cellulose with low affinity when incubated with their respective radioactive ligands in low salt and low temperature conditions (i.e. 50 mM KH_2PO_4, 0°C) and maintained low DNA-cellulose affinity when incubated with 20 mM sodium molybdate at 15°C for 1 h (figure 1, open columns). When incubated at 15 °C in the absence of sodium molybdate, all of these receptors attained a higher affinity for DNA-cellulose (figure 1, filled columns).

Relative DNA-cellulose binding was calculated by the ratio of radioactivity associated with DNA-cellulose to radioactivity associated with hydroxylapatite (2). Male rat liver was reported to contain two distinct estradiol binding proteins, one of which is the putative estrogen receptor (ER) and the other is a male rat specific moderate-affinity high-capacity estradiol binding protein (11-13). Both the putative ER and the male-specific estradiol binding protein bound to hydroxylapatite but only the ER bound to DNA-cellulose resulting in a reduction of relative DNA-cellulose binding (data not shown) which could explain the apparent reduction in the activation of male rat liver cytosolic ER compared to sheep uterine ER (figure 1, lanes 2 and 6).

Since steroid binding stabilization assays are evaluated based on the binding to hydroxylapatite (2), the presence of male rat specific estradiol binding protein also interferred with the interpretation of the data regarding modulator's effects on steroid binding ability of estrogen receptor. Our attempts to separate the male-specific estradiol binding protein from the putative estrogen receptor by DEAE-cellulose, Sephadex G-100, or by eluting from sucrose gradient failed to provide us with estrogen receptors attaining significantly higher affinity for DNA-cellulose when heated to 15 °C (activation) (data not shown). This could be due to separation of a component(s) of the unactivated receptor complex involved in activation.

Male-specific estradiol binding protein is reported to be distinguishable from the estrogen receptor by inability of the estradiol binding protein to bind diethylstilbestrol, DES (11). When male rat liver cytosol was labeled with [^{3}H] DES, highly specific labeling of estrogen receptor was

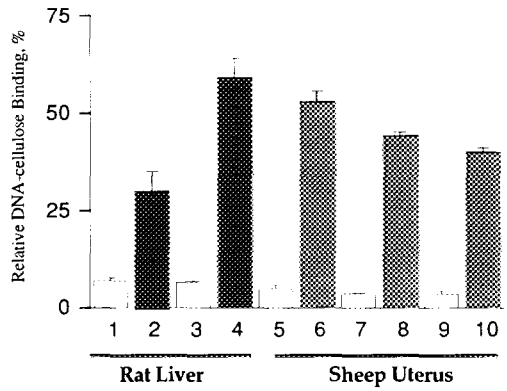

Figure 1. *DNA-cellulose binding characteristics of glucocorticoid, estrogen , and androgen receptors.* Male rat liver (lanes 1-4) or sheep uterine (lanes 5-10) cytosols were labeled with [^{3}H] estradiol (lanes 1,2,5, and 6) or [^{3}H] TA (lanes 3,4,7, and 8) or [^{3}H] DHT(lanes 9 and 10). 50 µl aliquots were incubated at 15°C with a buffer containing 50 mM HEPES, and 100 mM KCl, pH 7.4, in a final volume of 0.5 ml in the presence (odd numbered lanes) or absence (even numbered lanes) of 20 mM sodium molybdate. 100 µl aliquots from these reaction mixtures were subjected to DNA-cellulose or hydroxylapatite binding assays. Data presented are DNA-cellulose associated dpm as percent of hydroxylapatite associated dpm and represent one of three or more independent experiments. Mean hydroxylapatite associated radioactivity measurements were (dpm) : 1, 1830; 2, 1645; 3, 7250; 4, 6120; 5, 7565; 6, 5590; 7, 2740; 8, 2055; 9, 1050; 10, 950.

achieved, but DES-bound receptor did not respond to heating (i.e., activate) up to 37°C in low (100 mM KCl) or high (400 mM KCl) salt conditions (data not shown). Similar results were obtained when sheep uterine estrogen receptor was labeled with [^{3}H] DES suggesting that DES-bound estrogen receptor may be altered such that it cannot bind to DNA-cellulose under these assay conditions. This was further confirmed by size-exclusion chromatography when heat treated DES-bound estrogen receptor still eluted as higher molecular weight unactivated receptor complex form on Sephadex G-100 (data not shown).

Modulator Inhibits Activation of Liver GR and ER, and Uterine GR but not Uterine ER or AR

The effects of modulator on GR, ER and AR were evaluated in male rat liver and sheep uterine cytosolic fractions. Modulator inhibited activation of male rat liver GR and ER and sheep uterine GR in a dose-dependent manner (figure 2A, circles and squares and figure 2B, circles respectively). Fifty percent inhibition of these receptors was obtained at 1, 2, and 3 units/ml for liver ER, liver GR, and uterine GR respectively, which are within the physiological concentration of modulator purified from bovine liver (1-2 units/ml cytosol).

The inhibition of GR activation in different tissue systems such as rat liver (2,3), Sf9 insect cells overexpressing GR (unpublished data), CEM-C7 human T lymphocytes¶ have been

¶ N.M. Robertson, P.V. Bodine, M.Y. Çeliker, D. Saunders, E.S. Alnemri, G. Litwack; 75th Annual Endocrine Society Meeting (June 8-12, 1993) Las Vegas, NV; abstract 592.

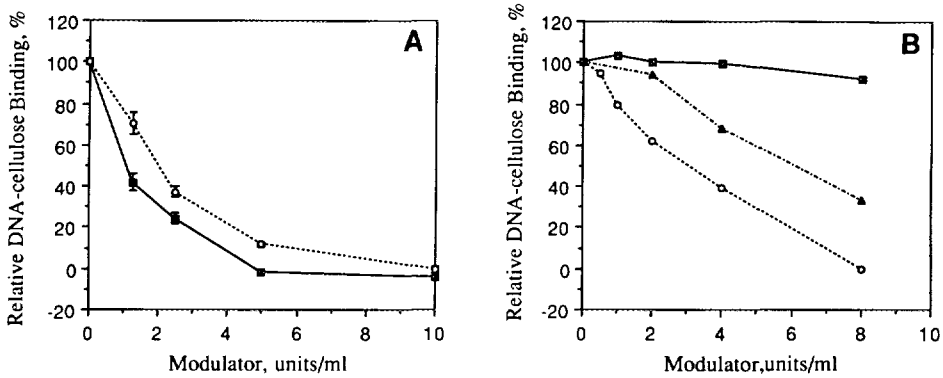

Figure 2. *Effects of modulator on estrogen, glucocorticoid, and androgen receptor activation.* Aliquots of male rat liver cytosol (A) or sheep uterine cytosol (B) labeled with [^{3}H] TA (circles), [^{3}H] estradiol (squares), or [^{3}H] DHT (triangles) were incubated with indicated amounts of modulator at 15°C for 1 hour. Aliquots from the reaction mixtures were subjected to DNA-cellulose or hydroxylapatite assays. Data are presented as percent of DNA-cellulose binding in the absence of modulator and represent averages of two separate experiments except where error bars are used indicating representation of three or more independent experiments.

demonstrated. We now report that sheep uterine cytosolic GR is also sensitive to regulation by modulator suggesting that modulator is a tissue- and species-independent inhibitor of glucocorticoid receptor.

In comparison, sheep uterine cytosolic estrogen receptor was insensitive to regulation by modulator at concentrations as high as 8 units/ml (figure 2B, squares). This could not be explained by tissue selectivity of modulator since glucocorticoid receptor of sheep uterine cytosol was inhibited by modulator. The unactivated estrogen receptor complex of sheep uterus could differ from that of rat liver in a way that is crucial for modulator's function. Further analysis of this selectivity may shed light onto the mechanism by which modulator inhibits receptor activation.

Activation of sheep uterine AR appeared to be inhibited by modulator (figure 2B, triangles). A similar inhibition pattern was observed when the activated AR was incubated with modulator (figure 3A, triangles) resulting in an overall 10 % inhibition with 8 units/ml modulator which is negligable. Therefore it was concluded that the apparent inhibition of AR by modulator was due to inhibition of the AR-DNA-cellulose interactions, not the activation process itself.

When modulator was added to the reaction mixture after cytosols were preheated at 25 °C for 30 min, it did not prevent activated ER and GR binding to DNA-cellulose at concentrations that inhibit receptor activation completely— concentrations smaller than 6 units/ml (Figure 3A). Since modulator did not inhibit activation of sheep uterine ER (figure 2B, squares) it can also be concluded that modulator did not interfere with DNA-cellulose binding ability of activated ER. However, binding of the activated AR to DNA-cellulose appeared to be inhibited by modulator in a dose responsive manner (figure 3A, triangles). Moreover, at high concentrations (6-10 units/ml) modulator somehow inhibited the receptor-DNA-cellulose interactions of all three receptors. Data presented are insufficient to explain this phenomenon and further investigation is required.

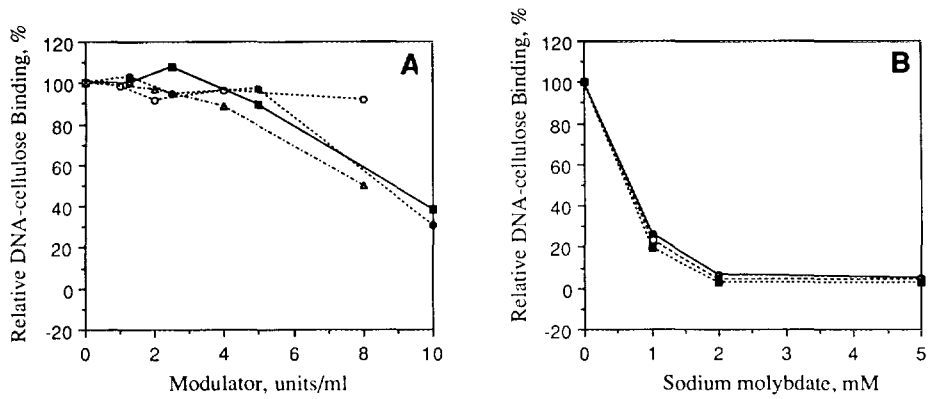

Figure 3. *A . Effects of modulator on activated estrogen, glucocorticoid, and androgen receptor.* Aliquots of male rat liver (solid symbols) or sheep uterine cytosol (open symbols) labeled with [^{3}H] TA (circles), [^{3}H] estradiol (squares) or [^{3}H] DHT (triangles) were incubated with indicated amounts of modulator at 15°C for 30 min after preheating the cytosol at 25°C for 30 minutes. Aliquots from the reaction mixtures were subjected to DNA-cellulose or hydroxylapatite assays. Data are presented as percent of DNA-cellulose binding in the absence of modulator or molybdate and represent averages of two independent experiments. *B. Effects of sodium molybdate on ER, GR and AR activation.* ER, GR, or AR of uterine or liver origin were subjected to activation inhibition assay in the presence of different amounts of sodium molybdate. Symbols as described above. Refer to legend of figure 1 for experimental design.

Sodium Molybdate Inhibits Activation of GR, ER, and AR of Liver or Uterus Origin

To determine whether sodium molybdate has a selective activity similar to that of modulator, radioliganded liver and uterine cytosolic fractions were incubated with different concentrations of sodium molybdate at 15°C for 1 h. Exogenous sodium molybdate inhibited the activation of all of the receptors studied in an identical manner at millimolar concentrations (figure 3B). Fifty percent inhibition was obtained with 0.5 mM sodium molybdate supporting our previous reports indicating that sodium molybdate was at least one hundred times less potent than modulator whose physiological concentration is now thought to be about 0.1 μM and 3 μM for modulators 1 and 2 respectively (unpublished data). Inhibition of sheep uterine ER and AR activation by sodium molybdate but not by modulator, suggests that modulator's mechanism of action is distinct from that of sodium molybdate.

In order to determine whether modulator acts by stabilizing the unactivated ER complex form as it does for rat liver glucocorticoid and rat colonic mineralocorticoid receptors (2-4), a series of sucrose density gradient centrifugations were performed. When [^{3}H] TA labeled rat liver cytosol was subjected to heat activation treatment followed by a sucrose gradient centrifugation of 5-30% in the absence of modulator, a dramatic shift from 8-10 S to 4-5 S was observed while in the presence of modulator a high 8-10 S peak was retained (data not shown). Since the 3-4 S peak of male-specific estradiol binding protein (12) and the 4-5 S peak of activated estrogen receptor

overlap, the changes in 4-5 S ER peak could not be observed. An increase of the 8 S peak was observed, however, when rat liver estrogen receptor was subjected to a similar treatment. This increase was not dramatic but it was reproducible (data not shown) suggesting that modulator stabilizes the unactivated complex form of ER.

In conclusion, modulator inhibits glucocorticoid receptor activation in a tissue- and species-independent manner and it appears to function differently on liver and uterine estrogen receptor. In addition, the higher potency and specificity of modulator compared to sodium molybdate suggests that modulator's mechanism of action is distinct from that of sodium molybdate. Therefore modulator is an endogenous, receptor-specific inhibitor of steroid receptor activation.

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