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PROGESTERONE-INDUCED INACTIVATION OF NUCLEAR ESTROGEN RECEPTOR IN THE HAMSTER UTERUS IS MEDIATED BY ACID PHOSPHATASE

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SUMMARY: Inactivation of hamster uterine estrogen receptor was assayed in nuclear KCl extracts (30 min, 37°C, pH 7.5) after progesterone treatment *in vivo* for 2h. At very low concentrations (≥0.05 mM), molybdate and vanadate blocked the progesterone-induced increase in receptor inactivation. In contrast, only high concentrations (≥10 mM) of the inhibitors blocked receptor inactivation in extracts from untreated hamsters. Gel electrophoresis and inhibition curves for phosphatases in nuclear extract demonstrated that acid, rather than alkaline, phosphatase activity is most likely responsible for these effects. These data suggest that progesterone antagonizes estrogen action in the hamster uterus by promoting estrogen receptor dephosphorylation leading to inactivation.

INTRODUCTION: Steroid hormone action in the target cell is believed to depend on retention of the steroid-receptor complex on nuclear "acceptor" sites (1,2). In the uterus of the hamster (3) and rat (4), progesterone antagonizes estrogen action by diminishing retention of the nuclear estrogen receptor (Re)[1]. This inactivation mechanism is selective for the occupied estrogen-Re complex (5). Inhibitors of protein and RNA synthesis can block progesterone-dependent loss of nuclear Re, suggesting that progesterone induces formation of a factor, termed estrogen receptor-regulatory factor (ReRF), which promotes inactivation of Re in the uterine nucleus (6). We have shown recently that ReRF can be extracted from uterine nuclei by 0.5 M KCl and that ReRF can inactivate uterine nuclear Re *in vitro* at 37°C (7). Sodium molybdate and sodium vanadate prevented the progesterone-dependent increase in Re inactivation. However, at the high inhibitor concentrations (10 mM) used, it was unclear whether these agents stabilized Re indirectly through inhibition of phosphatases or by

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1. ABBREVIATIONS: Re, estrogen receptor; ReRF, estrogen receptor-regulatory factor.

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direct interaction with the receptor (7-10). We now report that ReRF activity is inhibited by very low concentrations of molybdate and vanadate, and we provide evidence that ReRF action on Re is mediated by acid rather than alkaline phosphatase activity.

METHODS: Adult female golden hamsters (Engle Labs, Farmersburg, IN) were treated between 0730h and 0900h on day 4 of the estrous cycle (proestrus) with 5 mg progesterone in 0.3 ml corn oil subcutaneously. The animals were killed 2h later along with untreated controls. Uteri were removed and homogenized as described before (3) in four volumes (v:w) of buffer A_{30} (50 mM Tris-HCl, 1 mM EDTA, 12 mM thioglycerol, 30% (v:v) glycerol, pH 7.5). For measurement of phosphatase activities, buffer TMZ_{30} was used (50 mM Tris-HCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 30% glycerol, pH 7.5). Nuclear extract was prepared from the low-speed nuclear pellet as described (3,11) using the corresponding buffer containing 0.5 M KCl.

Measurement of Re Inactivation at 37°C In Vitro. Re inactivation was assayed as described before (7). Briefly, duplicate aliquots (0.4 ml) of nuclear extract were diluted (1:1) with buffer A_{30} plus 0.5 M KCl containing different concentrations of inhibitors and were pre-incubated at 0°C for 30 min. One set of samples was then incubated at 37°C for 30 min while the second set was kept at 0°C. Total Re was determined in 0.3 ml portions of each sample following incubation at 30°C for 1h with a saturating (3 nM) concentration of [1,2,6,7-$^3H_4$]estradiol (97 Ci/mmol, New England Nuclear). Nonspecific binding was measured in a parallel incubation with 1 μM diethylstilbestrol, and bound steroid was assayed in each sample after treatment with dextran-coated charcoal (3,11). Re inactivation (or Re loss) at 37°C is expressed as a percentage of the total Re measured in the corresponding extract kept in the cold.

Measurement of Phosphatases. Phosphatase activities in nuclear extract were assayed by colorimetric determination of p-nitrophenol released from the substrate p-nitrophenylphosphate during a 30 min incubation at 37°C (12). Each reaction mixture contained (in a final 0.55 ml volume): 0.05 ml sample extract, 7 mM p-nitrophenylphosphate, 0.5 mM $MgCl_2$ and either 45 mM glycine, pH 10.4 (alkaline phosphatase) or 45 mM sodium acetate, pH 5.5 (acid). One unit of enzyme activity is defined as the amount of enzyme catalyzing production of 1 μmole product per minute at 37°C.

Gel Electrophoresis of Nuclear Phosphatases. Nuclear extracts were dialyzed for 2-3h at 3°C against 200 volumes of buffer TMZ_{30} to remove salt. Aliquots (0.1-0.3 ml) were applied to 5 X 100 mm cylindrical gels consisting of 0.5% agarose and 3.0% acrylamide (13). Electrophoresis was run for 2h at 3°C (1 mA/gel) in the buffer system of Laemmli (14) in the absence of detergent. Each gel was stained for histochemical localization of either alkaline (15) or acid (16) phosphatase. Gels were scanned on a Quick Scan Jr. densitometer (Helena Labs, Beaumont, TX). Electrophoresis reagents were obtained from Bio-Rad. All phosphatase reagents were purchased from Sigma.

RESULTS: Figure 1 (Panels A and B) shows the concentration-dependence of vanadate and molybdate inhibition of Re inactivation *in vitro*. Progesterone treatment increased the amount of Re inactivated at 37°C relative to the control in the absence of inhibitors (34% vs 25% Re loss, respectively). Both inhibitors blocked this increased Re inactivation at very low concentra-

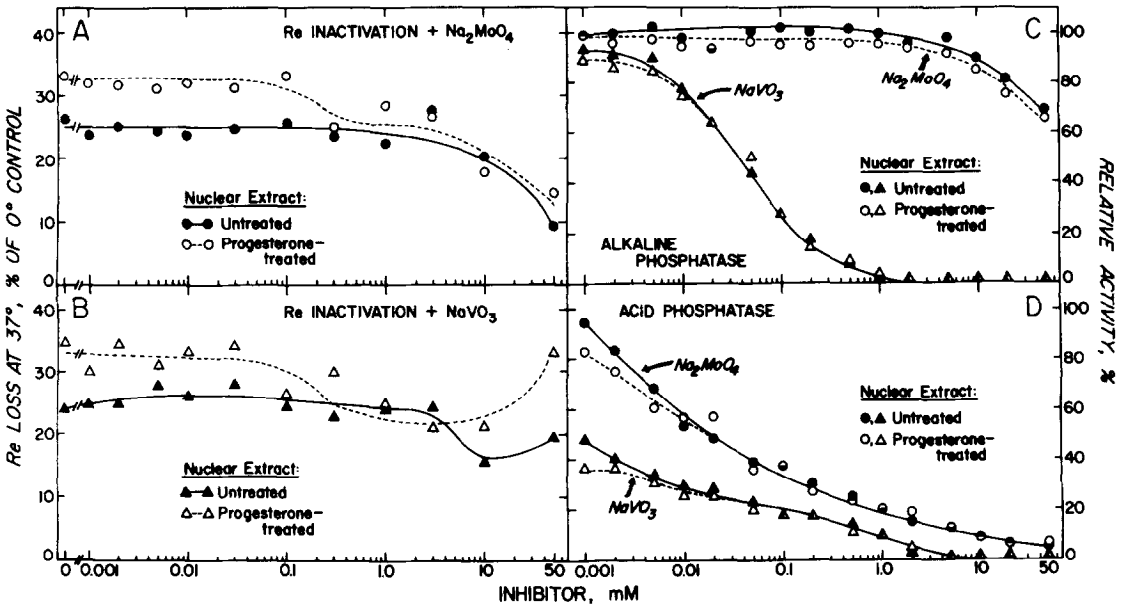

Figure 1: Inhibition of Estrogen Receptor Inactivation and Phosphatase Activities in Uterine Nuclear Extract by Molybdate and Vanadate. Uterine nuclear extracts were prepared from untreated hamsters (closed symbols) and from animals treated for 2h with progesterone (open symbols). Panels A and B: Re loss at 37°C relative to 0°C controls was measured in portions of nuclear extract (buffer A_{30}) in the presence of increasing concentrations of sodium molybdate (A) or sodium vanadate (B). Each value is the mean of single determinations made in two experiments. Mean values for total Re (cpm/ml) at 0°C (inhibitor-free controls) were: Untreated: 12300 (A) and 12500 (B); progesterone-treated: 11300 (A) and 10900 (B). Panels C and D: Aliquots (50 µl) of nuclear extract (TMZ_{30} buffer) were assayed for alkaline (C) or acid (D) phosphatase activity in solution as described in Methods. Each point is the mean of duplicate measurements made in the presence of different concentrations of molybdate (circles) or vanadate (triangles). Enzyme activity is expressed as a percent of that in the corresponding extract measured in the absence of inhibitors. Under these conditions, phosphatase activities were: Acid: 53.8 mUnits/ml in control (untreated) and 52.2 mUnits/ml in progesterone-treated extract; alkaline: 39.1 mUnits/ml in control and 38.5 mUnits/ml in progesterone-treated.

tions, between 0.03 mM and 0.3 mM. In a more detailed study, half-maximal inhibition by both inhibitors occurred at approximately 0.05 mM (data not shown). At higher inhibitor concentrations, Re loss curves for uterine nuclear extract from progesterone-treated and untreated animals were identical. This "endogenous" Re inactivation was inhibited by molybdate (half-maximum at approx. 20 mM). This activity was inhibited slightly by 10 mM vanadate, but 50 mM vanadate produced a stimulation of Re inactivation. These results suggest the presence of a factor in the extract from progesterone-treated animals which promotes Re inactivation *in vitro* above the level observed in

control extract. That molybdate and vanadate inhibit this activity suggests that the action of this factor is phosphatase-mediated.

The data in Figure 1C and 1D illustrate the effects of molybdate and vanadate on acid and alkaline phosphatase activities in uterine nuclear extract in vitro. Alkaline phosphatase activity (Fig. 1C) in uterine nuclear extract from both control and progesterone-treated animals was very sensitive to sodium vanadate (half-maximal inhibition at 0.05 mM) and relatively insensitive to molybdate (half-maximal inhibition at >50 mM). Inhibition curves for acid phosphatase activity in uterine nuclear extract were nonsigmoidal, indicating the presence of many enzymes with different sensitivities to these inhibitors (Fig. 1D). Both inhibitors block acid phosphatase activity substantially at concentrations less than 0.1 mM. Interestingly, there is a greater inhibition of acid phosphatase activity in the progesterone-treated extract than in the control extract at low inhibitor concentrations. The significance of this finding is presently unclear. Collectively, the data in Figure 1 show that progesterone-dependent Re inactivation is probably not mediated by alkaline phosphatase but may depend on acid phosphatase activity.

The possibility that progesterone modulates the activity of uterine nuclear acid phosphatase was investigated by electrophoresis of uterine nuclear extract followed by direct histochemical assay of enzyme activity in agarose-acrylamide gels (Fig. 2). As suggested by the data in Figure 1D, acid phosphatases in uterine nuclear extract are heterogeneous (Fig. 2A). At least five electrophoretically distinct acid phosphatases are detected in control nuclear extract. Several of these activities having high electrophoretic mobility appear to be stimulated two hours after progesterone injection. In contrast, only one major species of soluble alkaline phosphatase was detected in uterine nuclear extract following electrophoresis (Fig. 2B). This activity was not altered by progesterone treatment.

DISCUSSION: These studies demonstrate that progesterone-dependent enhancement of Re inactivation in uterine nuclear extract is phosphatase-mediated. The possibility that molybdate and vanadate block progesterone-induced Re loss by

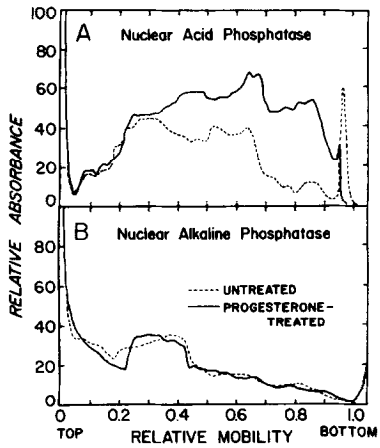

Figure 2: Effect of Progesterone Treatment on Acid and Alkaline Phosphatases in Uterine Nuclear Extract. Aliquots of nuclear extract (0.3 ml) prepared in TMZ_{30} buffer (8:1, v:w) were dialyzed and then electrophoresed on agarose-acrylamide gels (Methods). The gels were stained for acid phosphatase activity (Panel A) and scanned at 445 nm or were stained for alkaline phosphatase activity (Panel B) and scanned at 570 nm. Dashed lines: control (untreated) nuclear extract; solid lines: extract from progesterone-treated hamsters. This experiment is representative of three replicates.

direct action on Re is untenable, because these inhibitors are effective at much lower concentrations than are required for direct stabilization of receptors (8-10). The enzyme responsible for progesterone-dependent Re inactivation is not an alkaline phosphatase, since the Re inactivation mechanism is very sensitive to molybdate inhibition whereas uterine nuclear alkaline phosphatase is not. Moreover, the alkaline phosphatase present in nuclear extract is rapidly inactivated at 37°C in buffer A_{30}, and it is inhibited by EDTA, which is present in buffer A_{30} (data not shown). Hence, under conditions which permit measurement of progesterone-dependent Re inactivation, alkaline phosphatase activity is unstable. The data presented herein support the conclusion that acid phosphatase(s) mediate(s) the progesterone-induced Re inactivation. Inhibition curves for progesterone-induced Re loss (Fig. 1A and 1B) and acid phosphatase activity (Fig. 1D) are in reasonable agreement. Furthermore, progesterone treatment increases activity of one or more uterine nuclear acid phosphatases (Fig. 2A). Whether progesterone stimulates synthesis of this (these) enzyme(s) or enhances activity indirectly through an enzyme modulator is not known (6,7). The phosphatase which mediates progesterone-induced Re inactivation may have optimal activity in the acid pH range, but it

is exceptional in that this enzyme is active at pH 7.5. Although it is tempting to speculate that ReRF could be a modulator or cofactor which alters the properties of a pre-existing acid phosphatase to increase catalytic activity at neutral pH, our efforts to detect a progesterone-regulated "neutral" phosphatase in uterine nuclear extract have not been successful.

In agreement with others (17,18), we have found that Re inactivation in nuclear extract from uteri of untreated hamsters is inhibited by molybdate. If, as those authors have suggested, this mechanism is phosphatase-mediated, then our studies indicate that acid phosphatase is responsible for this activity. This conclusion is supported by the finding that vanadate is a powerful inhibitor of alkaline phosphatase but is a poor inhibitor of endogenous Re inactivation. That this inactivation mechanism is not mediated by alkaline phosphatase is further supported by the finding that Re in nuclear extract from untreated hamster uterus is stable at 37°C at pH 8.4 (data to be published elsewhere).

In conclusion, these studies provide further support for our hypothesis that progesterone controls estrogen action in the uterine nucleus by promoting dephosphorylation of the estrogen-Re complex, resulting in receptor inactivation. The finding that this mechanism is mediated by acid phosphatase(s) is a major step toward the understanding of ReRF action in the target cell and may represent a novel mechanism for hormonal modulation of the action of other hormones.

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