Requirement for a reduced sulphydryl entity in the protection of molybdate-stabilized estrogen receptor

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The separate and combined effects of molybdate and dithiothreitol on the stability of human uterine 9 S estrogen receptor were studied. Maximal, short-term, protection of the 9 S estrogen receptor was achieved by the joint inclusion of both stabilizing agents in cytosol buffers. This molybdate–dithiothreitol-mediated stability was dependent on reducing agent concentration inferring sulphydryl involvement in 9 S receptor protection by molybdate. The study also showed that molybdate–dithiothreitol could not prevent the gradual decay of the 9 S estrogen receptor to the 4 S form in cytosols stored at 4°C over prolonged periods.

Estrogen *Receptor Stability* *Molybdate* *Leupeptin* *Dithiothreitol*

1. INTRODUCTION

The native 9–10 S form of the estrogen receptor is preserved by the inclusion of molybdate in cytosolic extracts from human breast carcinoma and myometrium and in low ionic strength gradients during sedimentation studies [1,2]. Evidence suggests that molybdate exerts its stabilizing effect by inhibiting the the activity of receptor modifying enzymes such as phosphatases and calcium dependent proteases [3–5]. Here we illustrate the influence of molybdate on receptor structure in the absence and presence of dithiothreitol and present data which supports the involvement of a reduced sulphydryl moiety in the protection of the 9 S estrogen receptor.

2. MATERIALS AND METHODS

Human myometrial cytosol was prepared as in [5] in phosphate (P) or phosphate–molybdate (PM) buffers containing 10 mM potassium dihydrogen orthophosphate (Ajax Chemicals, Sydney NSW) at pH 7.4, without or with 20 mM sodium molybdate (Mallinckrodt, St Louis MO), respectively. From the phosphate-buffered cytosol a series of cytosols containing 1 mM dithiothreitol (D, Calbiochem, Carlingford VIC) and/or 20 mM molybdate were prepared. The resultant cytosols, with buffer compositions of P, PD, PM and PMD, were incubated for at least 16 h at 4°C with 5 nM [^{3}H]estradiol-17β (54 Ci/mmol, Radiochemical Centre, Amersham). Free and loosely bound hormone was removed by charcoal adsorption prior to the cytosols being examined by sedimentation analysis and chromatography on columns (1.6 × 30 cm, flow rate 30 ml/h) of Sephacryl S-300 (Pharmacia, Lane Cove NSW). Buffers corresponding to those of the cytosols were used in gradient preparation and column elution. Unless otherwise indicated all gradients were centrifuged in an MSE-75 ultracentrifuge at 145000 × g for 16 h at 4°C using a titanium swing-out rotor. Standard proteins were obtained from Serva (Heidelberg) and Blue dextran from Pharmacia (Uppsala).

3. RESULTS

The individual and combined effects of molybdate and dithiothreitol on the sedimentation properties of the estrogen receptor are shown in fig. 1. Because ultracentrifugal analysis was initially restricted to 3 samples/run cytosols in P, PD and PMD buffers were examined in the first centrifugation. The last 2 extracts were analyzed again, together with PM buffered cytosol, 24 h later. The

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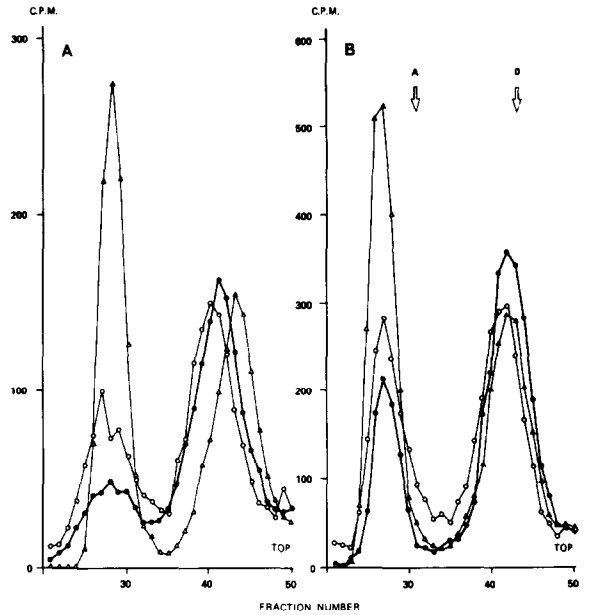

Fig. 1. The individual and combined effects of molybdate and dithiothreitol on the sedimentation rate of the estrogen receptor. The stabilizing agents were added to a phosphate-buffered myometrial cytosol to give cytosols equilibrated in P, PD, PM and PMD buffers. (A) After labelling with [^{3}H]estradiol the cytosols in P (●), PD (○) and PMD (■) buffers were subjected to glycerol gradient centrifugation in the corresponding buffers. (B) The cytosols in PM (●), PD (○) and PMD (■) buffers were left standing at 4°C and were treated in a similar manner 24 h later. Sedimentation markers: (A) aldolase (7.9 S) and (O) ovalbumin (3.6 S).

profiles in fig. 1 indicate that although some enhancement in the concentration of the more rapidly sedimenting 9 S estrogen receptor is observed on separate inclusion of molybdate and dithiothreitol a much more pronounced increase is obtained by the combined action of the stabilizing agents in the extracts.

After deriving a 9 S:4 S ratio for each profile from the respective receptor concentrations the results of fig. 1 were expressed in semiquantitative form by calculating the relative increase of this parameter in the modified cytosols compared to that in the unstabilized extract (table 1, myometrium 1). On average dithiothreitol effected a 2.4-fold increase in the 9 S:4 S ratio while molybdate, by itself, was less effective in promoting 9 S receptor stability and produced only a 1.4-fold rise

Table 1

Effect of molybdate and dithiothreitol, together or alone, on the 9 S:4 S ratio of estrogen receptors (increase (-fold) in 9 S:4 S ratio with respect to that observed in unprotected cytosol)

Agent(s) added	Myometrium no.				
	1	2	3	4	5
Dithiothreitol	2.4	1.2	2.7	1.2	1.0
Molybdate	1.4	2.1	2.0	2.2	1.8
Molybdate + dithiothreitol	3.8	3.1	11.1	4.8	2.7

All experimental details were as in fig. 1. The 9 S:4 S ratios were derived from sedimentation analysis profiles and the increase in this parameter compared to that in unprotected cytosol was calculated. The results for cytosols with dithiothreitol and molybdate–dithiothreitol are the average of two consecutive sedimentation analyses on the same extracts run 24 h apart

(table 1). In the presence of reducing agent however, molybdate elevated the proportion of 9 S:4 S estrogen receptor by an average of 3.8-fold (table 1). Although the degree of response to the stabilizing compounds was variable between extracts from different human myometria table 1 shows that in each case the response was maximal if molybdate was added with dithiothreitol. Gel filtration of the modified cytosols on Sephacryl S-300 gave results (not shown) which were consistent with the findings derived from sedimentation analysis.

Stabilization of the 9 S receptor by molybdate–dithiothreitol, as measured by the 9 S:4 S ratio, appears also to be dependent on reducing agent concentration. Fig. 2 shows that molybdate had little effect on 9 S receptor stability until a threshold concentration of 0.1–0.2 mM had been reached for dithiothreitol. The 9 S:4 S ratio was further enhanced by 0.5 mM dithiothreitol, but with additional reducing agent (1 mM) only a slightly higher degree of protection was observed (fig. 2). The apparent requirement for a minimum dithiothreitol concentration for increased 9 S receptor stability with molybdate is consistent with results which indicate that molybdate restricts the reducing capacity of dithiothreitol (unpublished). The effective oxidizable concentration of 1 mM dithiothreitol in the presence of 20 mM molybdate, as determined

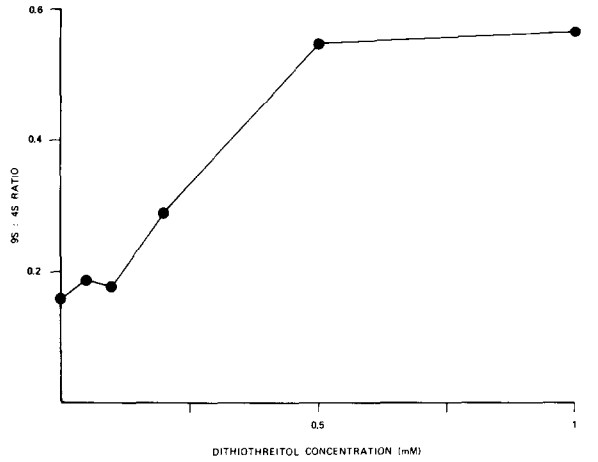

Fig. 2. The influence of dithiothreitol concentration on the ratio of 9 S:4 S estrogen receptors in molybdate stabilized cytosols. Dithiothreitol was added to PM buffered myometrial cytosol to give extracts with reducing agent at 0, 0.05, 0.1, 0.2, 0.5 and 1 mM. The receptors were labelled with 5 nM [^{3}H]estradiol at 4°C for 16 h and the 9 S:4 S ratios determined after centrifugation at 237000 × *g* for 17 h through glycerol gradients prepared in the corresponding buffers. Centrifugation was carried out in a Beckman L-8 ultracentrifuge using an SW 55Ti rotor.

by ferricyanide titration, is 0.86 mM.

The repeated ultracentrifugal analysis of cytosols containing dithiothreitol, with and without molybdate, revealed that the 9 S:4 S ratio was maintained, if not slightly increased over the 24 h period between runs (fig. 1). The longer term influence of molybdate on the stability of the 9 S estrogen receptor in the presence and absence of reducing agent was monitored by Sephacryl S-300 chromatography. An almost complete conversion of the larger receptor form to the smaller 4 S unit was observed after 9 days storage at 4°C. The time dependent reduction in the peak height-based 9 S:4 S ratio for the molybdate-stabilized cytosols is shown in fig. 3. The rate of this decrease was not influenced by reducing agent, even though the initial concentration of the 9 S receptor was much higher in the extract containing dithiothreitol (fig. 2).

4. DISCUSSION

The stability of the hormone-binding site in estrogen receptors is dependent on the presence of

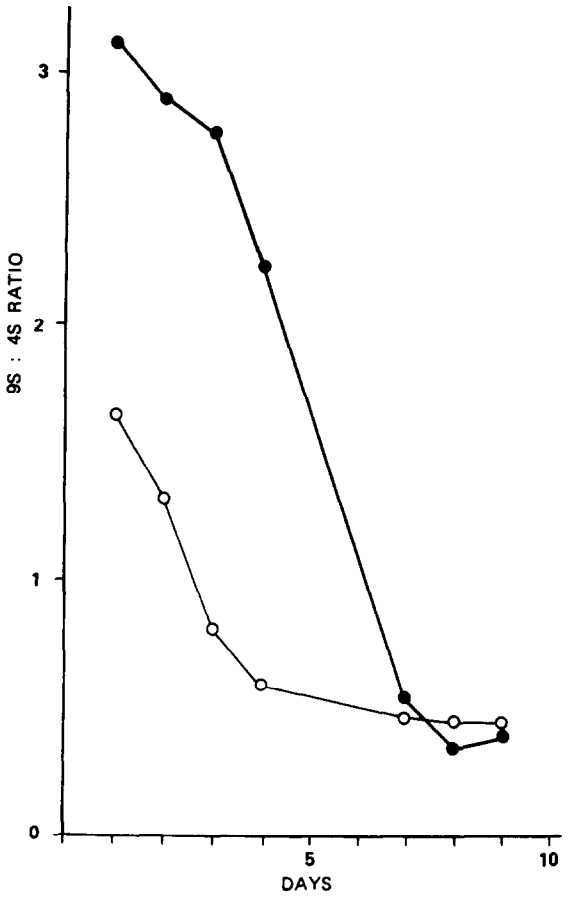

Fig. 3. The long-term effect of molybdate, with or without dithiothreitol, on the stability of the 9 S estrogen receptor during standing at 4°C. The stabilizing agents were added to phosphate-buffered myometrial cytosol to give cytosols equilibrated in PM (○) and PMD (●) buffers. The receptors in these cytosols were labeled with [^{3}H]estradiol by overnight incubation at 4°C. A 2 ml aliquot of the extracts was charcoal treated to remove unbound hormone and chromatographed on Sephacryl S-300 equilibrated in the corresponding buffer. The stability of the 9 S estrogen receptor is expressed as the peak-height ratio (9 S:4 S) of the peaks attributed to the 9 S and 4 S receptor forms.

sulphydryl groups in the receptor molecule [6,7]. Dithiothreitol protects these thiol groups and can reactivate estradiol binding activity lost through oxidation during prolonged storage in the absence of reducing agent at 4°C [8]. Sulphydryl groups are also involved in the binding of progesterone to its receptor [9] and in the formation of glucocorticoid–receptor complexes [10].

The stability of glucocorticoid binding capacity could be dependent not only on the maintenance of a reduced sulphydryl functionality but also on the protection of a phosphorylated component [11]. Both of these conditions were ensured by the joint inclusion of dithiothreitol and the phosphatase inhibitor molybdate in glucocorticoid receptor extracts from rat thymocytes [11]. The results obtained here suggest that similar requirements may exist for the preservation of the 9 S estrogen receptor from human uterus. In all cases examined molybdate and dithiothreitol together afforded the 9 S receptor a degree of protection which significantly exceeded that obtained by the separate stabilizing action of these agents.

The variation in response by the receptor extracts to molybdate–dithiothreitol stabilization may reflect differences in sulphydryl group oxidation during cytosol preparation as well as the overall reducing capacity of the cytosols. This could explain differences in glucocorticoid receptor activation achieved by dithiothreitol in extracts from rat thymocytes and L929 mouse fibroblasts [11].

A number of mechanisms have been proposed for the molybdate-mediated changes in estrogen receptor sedimentation rate. Since molybdate is a very effective phosphatase inhibitor it may prevent dephosphorylation and subsequent cleavage of the receptor [3–5,11,12]. Stabilization of the 9 S estrogen receptor by other known phosphatase inhibitors such as fluoride, glucose-1-phosphate (unpublished) support this concept. A mode of activity in which molybdate precipitates Ca^{2+} and inhibits Ca^{2+}-activated proteolysis of the estrogen receptor has also been suggested [5]. A third proposal involves the inhibition of RNase activity directed toward a 9–10 S receptor which contains RNA [12].

Yet another possibility is that molybdate may act directly on the steroid receptor molecule. The requirement for reduced sulphydryl groups in the system suggests that a thiol(s), suitably located on the receptor, could be interacting with molybdate. Since there is generally very little change in binding affinity or capacity on receptor stabilization with molybdate [2], the reactive sulphydryl group(s) required for 9 S receptor stability may be distinct from those essential for the maintenance of steroid binding capacity. A similar mode of interaction has been proposed for molybdate interference in progesterone receptor activation [13,14]. Molybdate has been shown to form complexes with protein sulphydryl groups and other thiol ligands [15]. Although molybdate did not inhibit the loss of glucocorticoid binding capacity after exogenous phosphatase treatment in rat liver cytosol [16], it allowed restoration of binding activity with dithiothreitol, suggesting that an interaction between molybdate and a sulphydryl group on the receptor may be important for molybdate-mediated stability.

The relationship between the protein structures responsible for the maintenance of 9 S receptor and hormone binding site integrity is not clear. Proteolytic cleavage of the 9 S cytosolic estrogen receptor from a number of target organs produces smaller discrete forms with full retention of the estrogen binding site [1,4,5,12]. Utilizing a cell-free system, a nuclear phosphatase has been described from calf uterus which appears to interact directly with estrogen receptors preventing the binding of hormone to unoccupied sites and releasing receptor-bound steroid by disruption of the receptor–estradiol complex [17].

Failure of molybdate–dithiothreitol to ensure the long-term stability of the 9 S estrogen receptor to prolonged storage at 4°C may indicate a specific 9 S to 4 S receptor conversion not involving dephosphorylation and/or sulphydryl group oxidation [5]. Another cytosolic enzyme, capable of inducing a slow decay of the 9 S receptor to the 4 S unit and which is not inhibited by molybdate, may be involved. Two different receptor modifying enzymes, one inhibited by leupeptin or antipain and the other by phosphoramidon or molybdate, were reported in rat liver [4].

Further research will be required to establish the precise molecular basis for the molybdate-mediated influences on estrogen receptors. This should be facilitated by the purification of these important biological proteins from extracts in which the stabilizing agent has been included.

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