Reduced Sulfhydryl Groups Are Required for Activation of Uterine Progesterone Receptor

POSSIBLE INVOLVEMENT OF AN INHIBITOR OF ACTIVATION*

(Received for publication, June 19, 1981)

Richard G. MacDonald and Wendell W. Leavitt†
From the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545

The dependence of uterine progesterone receptor activation on sulfhydryl groups was studied using binding to DNA-cellulose as a measure of activated receptor. Dithiothreitol, thioglycerol, or other sulfhydryl reducing reagents stimulated receptor activation 2- to 3-fold. This effect was produced under mild conditions, i.e. after a 22-h incubation at 0-3 °C with 50 mM dithiothreitol. Other characteristics of the sulfhydryl-dependent stimulation of receptor activation, such as pH-dependence and sensitivity to the sulfhydryl blocking agent iodoacetamide, suggest that the sulfhydryl groups essential for receptor activation are different from those involved in steroid binding. Progesterone receptor activation by sulfhydryl reduction was a reversible process, dependent on addition or removal (by dialysis) of the reducing agent. Optimal receptor activation (up to 75% of total receptor) occurred when cytosol was diluted with buffer in the presence of dithiothreitol, suggesting that dissociation of either a receptor subunit or an inhibitory factor present in cytosol may also be involved in the activation process. This putative inhibitor appears to have a M r ≥ 30,000 since it is not removed from cytosol by dialysis or gel filtration. These results emphasize the importance of sulfhydryl groups or a disulfide bridge, perhaps associated with the DNA-binding domain of the receptor, in a key regulatory step in the mechanism of steroid hormone action: activation and subsequent binding of the steroid-receptor complex to DNA or chromatin.

Activation, an important step in the mechanism of steroid hormone action, is the process by which the steroid-receptor complex acquires the ability to interact with nuclear chromatin or DNA and thereby to alter gene expression in target cells (1, 2). The activated steroid-receptor complex is defined operationally and quantified by its ability to bind in vitro to polyanions such as DNA (3), DNA-cellulose (4, 5), ATP-Sephacore (6) and phosphocellulose (7).

Although the mechanism of receptor activation is still obscure, some knowledge has been gained through the use of chemical reagents which affect this process. Previous studies suggested a role for sulfhydryl groups in the binding of glucocorticoid (8, 9) and progesterone (10) receptors to DNA, but the function of sulfhydryl groups in the general mechanism of steroid receptor activation is not clear.

We discovered and report here that sulfhydryl reducing reagents such as dithiothreitol stimulate activation of progesterone receptor in hamster uterine cytosol. Our data demonstrate that reduced sulfhydryl groups are required for activation. These studies provide new insight into the mechanism of receptor activation, since the sulfhydryl groups or disulfide bridge essential for activation may be associated with the DNA-binding domain of the receptor. Furthermore, reversible oxidation and reduction of sulfhydryl groups may regulate the interaction between the unactivated receptor and an activation inhibitor.

EXPERIMENTAL PROCEDURES

Materials—[1,2-3H]Progesterone (55 Ci/mmol) was purchased from New England Nuclear. Dithiothreitol, iodoacetamide, reduced glutathione, and p-chloromercuribenzenesulfonic acid were from Sigma. N-ethylmaleimide and 2-mercaptoethanol were obtained from Eastman. Adult female golden hamsters were housed and estrous cycles were established in these animals, as previously described (11). All the hamsters for these studies were killed between 0800 and 1100 h on cycle day 4, during the period when the tissue concentration of uterine cytosol Rp is highest (11).

Preparation of Uterine Cytosol Containing [3H]Progesterone Receptor Complex—Uterine cytosol was prepared by homogenization as reported before (11, 12) at a buffer to tissue ratio of 4:1 in phosphate buffer: 50 mM sodium phosphate, pH 7.5 at 22 °C, 10% glycerol. The homogenate was centrifuged for 1 h at 170,000 × g to obtain the cytosol fraction. The cytosol was then incubated for 18-22 h at 0 °C with 20 nm [3H]progesterone (added in ethanol, final ethanol content, 2%, v/v) in the presence of 4 μM unlabeled cortisol. Cortisol was included to block binding of [3H]progesterone to corticosteroid-binding globulin and, at this concentration, does not compete significantly with the labeled steroid for binding to the progesterone receptor (11, 12).

Estimation of Specific [%H]Progesterone Binding in Cytosol—Following the 18-22-h incubation, duplicate 0.1-ml aliquots were taken from each cytosol treatment for measurement of specific [%H] progesterone binding. One set of duplicate samples was incubated at 38 °C for 30 min to destroy receptor binding; whereas the second set was kept at 0 °C. Free and loosely-bound steroids were removed from all samples by treatment with dextran-coated charcoal (11, 12). Specific [%H]-progesterone binding was calculated by subtracting the amount of bound radioactivity present in the heat-treated samples from that in the parallel samples kept in the cold. For only the experiment shown in Fig. 2, nonspecific [%H]-progesterone binding in cytosol was measured by incubating parallel samples with both labeled steroid and 4 μM unlabeled progesterone. The magnitude of the nonspecific steroid binding component (5-10% of total bound steroid) estimated in these ways was found to be the same. The sulfhydryl—

* Supported by Grants PCM 77-29630 from the National Science Foundation and CA-25362, HD-15462, and HD-13152 from the United States Public Health Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom all correspondence and reprint requests should be addressed.

The abbreviations and trivial names used are: Rp, progesterone receptor; progesterone, 4-pregnen-3,20-dione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PCMB, p-chloromercuribenzenesulfonic acid.
modifying reagents employed in these studies had no detectable effect on the amount of nonspecific binding.

DNA-cellulose Binding Assay—Binding of \[^{3}H\]progesterone-receptor complex to DNA-cellulose was used to measure the amount of activated receptor in each treated cytosol. Duplicate aliquots of cytosol (one heat-treated, one control kept in the cold) were incubated with DNA-cellulose in a slurry at 3°C as described before (4, 12). After this incubation, DNA-bound Rp was extracted from the washed DNA-cellulose by two successive 1-h incubations with 0.5 M KCl in phosphate buffer. Radioactivity was measured in these extracts and specific \[^{3}H\]progesterone interaction with DNA was computed as above.

General Methods—Radioactivity was measured in aqueous samples as previously described (12). Values shown in the figures and tables represent single determinations. All experiments were replicated two or three times.

RESULTS AND DISCUSSION

Incubation of uterine cytosol for 19 h with dithiothreitol produced a concentration-dependent enhancement of Rp binding to DNA (Fig. 1). Under equivalent conditions of pH and buffer concentration (pH 7.5, 50 mM), phosphate was superior to HEPES or Tris in supporting dithiothreitol-depending activation of Rp. Maximal Rp activation (30-45% of total Rp in this experiment) occurred with 30-50 mM dithiothreitol in all three buffers. These data suggest that the receptor (or possibly some receptor-modifying agent in cytosol) requires reduced sulfhydryl groups for optimal interaction with DNA. In the absence of added sulfhydryl reducing agents, 10-14% of the receptor is capable of DNA binding (Fig. 1).

Previous work suggested that optimal binding of steroids to their receptors requires reduced sulfhydryl groups (13-15). Conversely, sulfhydryl blocking agents inhibit steroid binding by receptor (8, 13, 16-21). Our data indicate that dithiothreitol and other sulfhydryl reducing agents (Table I) increase the binding of \[^{3}H\]progesterone to uterine Rp. This enhancement is modest, varying from 1-14%. The characteristics of sulfhydryl-dependent increases in Rp activation and steroid binding are considerably different. Activation of Rp by dithiothreitol was optimal between pH 7.5 and 9.0 in phosphate buffer and between pH 8.5 and 9.0 in HEPES (data not shown). Optimal steroid binding in the presence of dithiothreitol occurred at pH 8.5 in each buffer. Moreover, 2-3 mM dithiothreitol or 5-10 mM thioglycerol was sufficient to promote maximal \[^{3}H\]progesterone binding by Rp. These findings suggest that different sulfhydryl groups, either located on the receptor itself or on some interacting molecule, are responsible for these two effects. A similar conclusion was reached by others (8, 10).

Table I shows the effects of the sulfhydryl-blocking reagents PCMB, N-ethylmaleimide, and iodoacetamide on steroid binding to and activation of Rp. At a relatively low concentration (0.5 mM), PCMB and N-ethylmaleimide destroyed steroid binding to Rp. However, at a concentration of 0.5 mM, iodoacetamide caused only a mild decrease in steroid binding and a small reduction in the amount of activated Rp. When 0.5 mM dithiothreitol was included in the DNA-cellulose assay after preincubation of cytosol with iodoacetamide, a slight increase in DNA binding of Rp occurred. The enhancement by dithiothreitol was much smaller in this case than that produced by dithiothreitol in the absence of iodoacetamide (Table I). These data support the hypothesis that different sulfhydryl groups are involved in Rp activation and steroid binding, since iodoacetamide can act selectively to depress activation while having little effect on steroid binding.

In order to study better the interaction between dithiothreitol and sulfhydryl-blocking reagent effects on Rp, the experiment shown in Table II was done. The receptor was first reduced by treatment with dithiothreitol and then the excess reducing agent was removed by dialysis. Note that steroid binding to Rp was exceptionally stable even after prolonged incubation and dialysis. After 5 h of dialysis, a small increase in the amount of activated receptor was evident in the cytosol. Addition of 50 mM dithiothreitol to this dialyzed cytosol caused a slight enhancement of activation, but this is not a significant increase. In contrast to the data in Table I, neither iodoacetamide nor N-ethylmaleimide had a substantial effect on the DNA-binding properties of the reduced-dialyzed receptor, although these compounds did cause a reduction in steroid binding. These findings strongly suggest that the effect of these sulfhydryl-modifying reagents is directly on the activation process itself, rather than on the binding of activated Rp to DNA. When the dithiothreitol-pretreated cytosol is dialyzed for 24 h, a somewhat different picture emerges (Table II). During prolonged dialysis, the previously-reduced sulfhydryl groups on the receptor appear to be oxidized, as is suggested by the marked decrease in the amount of activated Rp in the control cytosol (no additions). Adding back dithiothreitol to this cytosol caused a dramatic recovery of activated receptor, demonstrating that sulfhydryl-dependent activation of Rp is a readily reversible process. As in the experiment shown in Table I, treatment of the 24 h-dialyzed cytosol with iodoacetamide or N-ethylmaleimide reduced the amount of steroid binding to Rp. In this instance, however, the proportion of activated receptor in the cytosol was not changed by these reagents. Collectively, the data in Tables I and II demonstrate that the unactivated (oxidized) form of Rp is more sensitive than the activated (reduced) form to the inhibitory effects of sulfhydryl blocking agents and to the stimulatory effects of sulfhydryl reducing reagents. In particular, iodoacetamide and N-ethylmaleimide have no apparent effect on the ability of previously activated receptor to bind to DNA. These findings suggest that the sulfhydryl groups involved in Rp activation are masked or that alkylation by the sulfhydryl-blocking reagents does not interfere with DNA binding once the receptor is activated. This may result from a conformational change in the receptor molecule which translocates the sulfhydryl groups away from the DNA-binding site (e.g., by dissociation of a subunit or inhibitor). Alternatively, if the

![Fig. 1. Influence of dithiothreitol concentration on activation of uterine progesterone receptor.](http://www.jbc.org/)

Cytosol was prepared (4:1, v/v) in either phosphate, Tris, or HEPES buffers (50 mM, pH 7.5 containing 10% glycerol). Increasing amounts of dithiothreitol were added to 0.5-mL portions of each cytosol followed by incubation with 20 mM \[^{3}H\]progesterone at 0°C for 22 h. Duplicate 0.1-mL aliquots were assayed for specific \[^{3}H\]progesterone binding and for Rp receptor binding to DNA-cellulose using a 1-h assay time. The amount of receptor bound to DNA-cellulose was calculated as a per cent of the total specifically-bound steroid in the corresponding cytosol.

Downloaded from http://www.jbc.org/ by guest on July 9, 2020
TABLE I
Effects of different sulfhydryl modifying reagents on [3H]progesterone binding in uterine cytosol and on binding of cytosol progesterone receptor to DNA-cellulose

Uterine cytosol was prepared in phosphate buffer and incubated with [3H]progesterone, as described under "Experimental Procedures." After 5.5 h of incubation with steroid, additions were made to 0.5-ml aliquots of cytosol (left column). Additions were made from concentrated stock solutions in distilled water adjusted to pH 7.6 and pH 8.1. Following a 16-h further incubation at 0°C, 0.1-

![Image](http://www.jbc.org/)

DNA-binding site were locked in the unactivated, oxidized receptor by a disulfide bond on the DNA-binding domain. Reduction of this bond permitted the molecule to "relax," exposing the DNA-binding site and rendering the molecule activated. The latter situation would be analogous to the case of progesterone binding to uteroglobin (22). In contrast, the sulfhydryl groups associated with steroid binding are available to these reagents in both the activated and unactivated forms of Rp.

All four of the thiol-reducing agents we tested enhanced Rp activation when they were included at high concentrations in cytosol during the incubation with steroid (Table I). These reagents (except for GSH) also stimulated Rp activation when they were added to cytosol only during the 1-h incubation with DNA-cellulose (Table I). At concentrations one-tenth as high as those used during the preincubation period (compensating for the ten-fold cytosol dilution in the assay), activation was stimulated by these three compounds to nearly the same extent as that in the corresponding pretreated cytosols. These findings indicate that some activation of receptor may be occurring during the 1-h DNA-cellulose binding assay.

The time course of binding of dithiothreitol-pretreated and untreated Rp preparations to DNA-cellulose was studied in order to understand better what happens during the assay period (Fig. 2). Binding of Rp to DNA-cellulose occurs in two distinct phases. In phase I, representing the first 4-10 min of cytosol exposure to DNA-cellulose, rapid DNA binding of previously activated Rp takes place. In the slower, more
prolonged phase II, further activation of Rp occurs during the assay. In phase II, the DNA-binding step is not rate-limiting, and binding of Rp to DNA occurs as activated Rp is produced. The above interpretation is borne out by examination of the binding curves in Fig. 2. Treatment of cytosol with 50 mM dithiothreitol prior to the assay increases the level of pre-activated receptor (phase I) 2- to 3-fold over that in untreated cytosol (measured as the difference in Rp binding to DNA between dithiothreitol-pretreated and untreated cytosols at the earliest time, 5 min after cytosol addition to DNA-cellulose). We believe this difference represents a dithiothreitol-group(s) necessary for receptor activation had been fully reduced during the 18-h preincubation and that sulfhydryl groups are reactivated at the beginning of the DNA-cellulose assay (Fig. 2, center curve). Rp is predominantly unactivated early in the assay and the amount of DNA-bound Rp is the same as that in dithiothreitol-untreated cytosol. As dithiothreitol-promoted sulfhydryl reduction progresses, the amount of activated Rp increases. Eventually, this curve converges (between 3 and 4 h) with those of the dithiothreitol-pretreated cytosols. Thus, a change in rate-limiting step appears to occur during this incubation.

It is possible that the ten-fold dilution of cytosol which occurs at the beginning of the DNA-cellulose binding assay results in dissociation of a subunit or an inhibitory factor from the receptor and that this step is necessary for receptor activation. To test this hypothesis, we determined whether dilution of cytosol before the DNA-cellulose assay enhanced Rp binding to DNA. For this purpose, a 10-min exposure of cytosol to DNA-cellulose was used to measure only phase I (pre-activated) receptor binding to DNA (Fig. 3). In the absence of dithiothreitol, dilution with buffer caused a small, but reproducible, increase in the amount of activated Rp in cytosol. Addition of 50 mM dithiothreitol to buffer-diluted cytosol caused a marked increase in activated Rp. Clearly, dilution of cytosol and sulfhydryl reduction are requisites for optimal receptor activation; neither manipulation alone produces the maximal response. Dilution of cytosol with heat-treated, receptor-free cytosol did not cause an increase in activated receptor, either in the presence or absence of dithiothreitol. This finding suggests that cytosol contains a factor(s) which block(s) activation of Rp. Previous work has shown that steroid receptors may be activated by dilution of the cytosol (5, 23, 25), by passage through gel filtration columns (5, 24-26) or by dialysis (25), suggesting that some agent in cytosol acts as an inhibitor of activation. The action of such an inhibitor may explain the rate of DNA binding of Rp during phase II of the DNA binding assay. An alternative possibility is that the receptor may dissociate into subunits upon dilution and thereby become activated. Other proteins in cytosol can interfere with steroid receptor binding to DNA (27), but this is unlikely for the case of uterine Rp, since the dilution-dependent change that stimulates Rp activation seems to occur in pretreatment of the cytosol rather than in
Sulfhydryl-dependent Activation of Progesterone Receptor

the DNA-cellulose binding assay. Finally, the possibility exists that the receptor must undergo a change in conformation upon transition from the activated to unactivated state (28, 29).

Preliminary efforts to separate the putative inhibitor of activation from the receptor have not been successful. The factor is not removed from uterine cytosol by passage through Sephadex G-50 in the presence of 50 mM dithiothreitol (data not shown) or by dialysis (Table II). This would indicate that the inhibitor has an Mr > 30,000 and probably is dissimilar to the inhibitor(s) of glucocorticoid receptor activation present in rat liver cytosol (5, 25). Although activation of Rp appears to be regulated by dissociation of an inhibitor, the possibility that a conformational change occurs upon dissociation of the inhibitor cannot be excluded. Furthermore, it remains to be determined whether the sulfhydryl groups important for activation of Rp are located on the receptor or the inhibitor, or both (perhaps a disulfide bridge links the molecules covalently).

Recently, Hughes et al. (30) showed that purified chick oviduct progesterone receptor subunit A binds in a saturable manner to DNA containing limited nicks, but that binding became nonsaturable as the single-stranded character of the DNA increased. They concluded that the steroid receptor may act as a helix-destabilizing protein. In light of these results, the interaction of steroid receptors with DNA takes on renewed importance in the mechanism of steroid hormone action. Our studies demonstrate that sulfhydryl groups on the DNA-binding domain of the receptor play an essential role in this mechanism.

Acknowledgments—We gratefully acknowledge the excellent technical assistance of William F. Robidoux, Jr. We thank Drs. Earl Baril and David Kupfer for helpful editorial comments.

REFERENCES
1. Buller, R. W., and O’Malley, B. W. (1976) Biochem. Pharmacol. 25, 1-12
2. Gorski, J., and Gannon, F. (1976) Annu. Rev. Physiol. 38, 420-450
3. Rousseau, G. G., Higgins, S. J., Baxter, J. D., Gelfand, D., and Tomkins, G. M. (1975) J. Biol. Chem. 250, 6915-6921
4. Kalimi, M., Colman, P., and Feigelson, P. (1975) J. Biol. Chem. 250, 1080-1086
5. Goidl, J. A., Cake, M. H., Dolan, K. P., Parchman, L. G., and Litwack, G. (1977) Biochemistry 16, 2125-2130
6. Miller, J. B., and Toft, D. O. (1978) Biochemistry 17, 173-177
7. Fleischmann, G., and Beato, M. (1979) Mol. Cell. Endocr. 16, 181-197
8. Young, H. A., Parks, W. P., and Scolnick, E. M. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 3060-3064
9. Kalimi, M., and Love, K. (1980) J. Biol. Chem. 255, 4687-4690
10. Kalimi, M., and Banerji, A. (1981) J. Steroid Biochem. 14, 593-597
11. Leavitt, W. W., Toft, D. O., Strott, C. A., and O’Malley, B. W. (1974) Endocrinology 94, 1041-1053
12. Chen, T. J., MacDonald, R. G., and Leavitt, W. W. (1981) Biochemistry 20, 3405-3411
13. Rees, A. M., and Bell, P. A. (1975) Biochim. Biophys. Acta 411, 121-132
14. Granberg, J. F., and Ballard, P. L. (1977) Endocrinology 100, 1169-1168
15. Sando, J. J., Hammond, N. D., Stratford, C. A., and Pratt, W. B. (1979) J. Biol. Chem. 254, 4779-4789
16. Coty, W. A. (1980) J. Biol. Chem. 255, 8035-8037
17. Banerji, A., and Kalimi, M. (1981) Steroids 37, 469-421
18. Schwarberg, B. P. (1972) Biochim. Biophys. Acta 261, 219-235
19. Watanabe, H., Orth, D. N., and Toft, D. O. (1973) J. Biol. Chem. 248, 7625-7630
20. Gardner, D. G., and Wittliff, J. L. (1973) Biochim. Biophys. Acta 320, 617-627
21. Kobilinsky, M., Beato, M., Kalimi, M., and Feigelson, P. (1972) J. Biol. Chem. 247, 7897-7904
22. Mornon, J. P., Fridiansky, F., Bailly, R., and Milgrom, E. (1980) J. Mol. Biol. 137, 415-429
23. Higgin, S. J., Rousseau, G. G., Baxter, J. D., and Tomkins, G. M. (1973) J. Biol. Chem. 248, 5866-5872
24. Saffran, J., Loeser, B. K., Bohnett, S. A., and Faber, L. E. (1976) J. Biol. Chem. 251, 5607-5613
25. Bailly, A., Sallas, N., and Milgrom, E. (1977) J. Biol. Chem. 252, 858-863
26. Leach, K. L., Deitmer, M. K., Hammond, N. D., Sando, J. J., and Pratt, W. B. (1979) J. Biol. Chem. 254, 11884-11890
27. Chamness, G. C., Jennings, A. W., and McGuire, W. L. (1974) Biochemistry 13, 327-331
28. Sakaue, Y., and Thompson, E. B. (1977) Biochim. Biophys. Res. Commun. 77, 533-540
29. Parchman, L. G., and Litwack, G. (1977) Arch. Biochem. Biophys. 183, 374-382
30. Hughes, M. R., Compton, J. G., Schrader, W. T., and O’Malley, B. W. (1981) Biochemistry 20, 2481-2491
Reduced sulfhydryl groups are required for activation of uterine progesterone receptor. Possible involvement of an inhibitor of activation.
R G MacDonald and W W Leavitt

J. Biol. Chem. 1982, 257:311-315.

Access the most updated version of this article at http://www.jbc.org/content/257/1/311

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/257/1/311.full.html#ref-list-1