Role of Chemical Reagents in the Activation of Rat Hepatic Glucocorticoid-Receptor Complex*

Mohammed Kalimt and Karen Love
From the Department of Biochemistry, Albert Einstein College of Medicine, Yeshiva University, New York, New York 10461

(Received for publication, December 3, 1979, and in revised form, February 11, 1980)

The effect of sulfhydryl modifying reagents on the activation of hepatic glucocorticoid-receptor complex was studied. Unactivated (preincubated at 0°C) [3H]-dexamethasone-receptor complex preincubated with N-ethylmaleimide or iodoacetamide at 0°C and then treated at 25°C was unable to bind to rat liver nuclei. On the other hand, [3H]-dexamethasone-receptor complex first incubated at 25°C and then treated with N-ethylmaleimide or iodoacetamide showed marked binding to nuclei, comparable to that obtained with activated cytosol. The results suggest that sulfhydryl modifying reagents act at the level of unactivated glucocorticoid-receptor complex, blocking its activation and binding to nuclei.

It was previously shown that pyridoxal phosphate inhibits the binding of "activated" dexamethasone-receptor complexes to DNA-cellulose (Calkins, M. H., DiSorbo, D. M., and Litwack, G. (1978) J. Biol. Chem. 253, 4886-4891). We have characterized both the N-ethylmaleimide-treated and the pyridoxal phosphate-treated [3H]-dexamethasone-receptor complexes by agarose gel filtration and sucrose-gradient centrifugation; we found that whereas pyridoxal phosphate-treated cytosol at 0°C showed a considerable amount of a smaller fragment of the receptor that was unable to bind to nuclei, N-ethylmaleimide-treated cytosol was totally devoid of this smaller fragment, and resembled untreated, unactivated cytosol. The sulfhydryl modifying reagents would appear to lock the receptor into an intact unactivated form as opposed to pyridoxal phosphate that degrades the molecule into smaller units. Thus, N-ethylmaleimide treatment may be valuable in the purification of the unactivated form of the receptor to homogeneity.

The formation of the glucocorticoid-receptor complex in the cytoplasm of the target cells for the hormone, and its activation and translocation to the nuclear acceptor sites, is believed to be of primary importance in the induction of certain enzymes in hepatoma and liver cells (1-3). We have shown previously that the binding of glucocorticoids to the receptor proteins is markedly inhibited by treatment of the rat liver cytosol with various sulfhydryl modifying reagents (4). Subsequently, Young et al. (5), using mouse mammary tumors and hamster kidney (BHK) cell lines, observed that sulfhydryl modifying reagents not only inhibit the binding of glucocorticoids to the receptor proteins but also inhibit the binding of preformed hormone-receptor complex to DNA. It was not clear, however, whether sulfhydryl reagents block the activation of glucocorticoid-receptor complex or its subsequent binding to nuclear receptor sites. Although some understanding has been achieved concerning the phenomenon of temperature-dependent activation of rat liver glucocorticoid-receptor complex, the precise mechanism of in vitro activation is not yet known (6-10). The present paper reports evidence suggesting that sulfhydryl reagents block the activation of the glucocorticoid-receptor complex (per se) and not its subsequent binding to nuclear acceptor sites.

**EXPERIMENTAL PROCEDURES**

**Materials**—[1,2,4-3H]Dexamethasone (31 Ci/mmol) was obtained from New England Nuclear, with radiochemical purity periodically checked by thin layer chromatography as described previously (11). Nonradioactive dexamethasone, pyridoxal 5'phosphate, β-mercaptoethanol, and iodoacetamide were purchased from Sigma. N-ethylmaleimide was obtained from Fluka AG Chemische Fabrik, Switzerland.

Male Sprague-Dawley rats (from Charles River Breeding Laboratories), 150 to 200 g, were adrenalectomized bilaterally 7 to 9 days before use and were maintained on standard Purina Chow, 0.9% NaCl solution, and water ad libitum.

**Preparation of Cytosol**—Animals were killed by cervical dislocation, and the livers were perfused in situ with ice-cold TBS buffer (10 mM Tris-HCl, 0.15 M NaCl, pH 7.5) and homogenized in a solution (1:2, w/v) of the same buffer. The homogenates were filtered through cheesecloth and centrifuged at 2,000 × g in a Sorvall SS 34 rotor at 2°C for 10 min to sediment a crude nuclear pellet. The cytosol fraction was prepared by centrifuging the homogenate at 240,000 × g for 60 min at 0-4°C. The upper fatty layer was discarded and the cytosol was carefully removed and used for steroid binding studies. The cytosol was diluted to yield a protein concentration of approximately 20 mg/ml. The cytosol was then incubated with 5 × 10-7 M [3H]-dexamethasone in the presence or absence of a 1000-fold excess of nonradioactive dexamethasone. After incubation for 2 h at 0°C to allow maximal binding, the specific macromolecular-bound fraction of steroid was determined using the charcoal-dextran technique (12).

**Treatment of Cytosol with Chemical Reagents**—Pyridoxal 5'-phosphate, iodoacetamide, and N-ethylmaleimide were prepared in concentrated stock solutions (100 mM, pH 7.5) prior to use in the experiments. Cytosol containing pyridoxal 5'-phosphate, iodoacetamide, or N-ethylmaleimide was incubated for 30 min at 0°C in the dark.

**Nuclear Binding Assay**—Nuclei were purified from the 2,000 × g pellet using 1.8 M sucrose in 10 mM Tris, pH 7.5, containing 0.025 M KCl and 3 mM MgCl2, as previously described (13). The washed nuclei were suspended in homogenization buffer. Aliquots of the nuclear suspension containing 50 μg of DNA were centrifuged at 2,000 × g for 5 min at 0°C and the supernatant fractions were discarded. [3H]-Dexamethasone-treated cytosol (0.2 ml) was added and a final volume of 1 ml of reaction mixture was made by addition of homogenization buffer. The nuclei were resuspended by gently stirring with a Vortex mixer, and the suspension was incubated for 30 min at 0°C. Following incubation, the nuclei were removed by centrifugation at 4,687
Activation of Rat Hepatic Glucocorticoid-Receptor Complex

RESULTS

Table I shows that the [3H]dexamethasone-receptor complex of rat liver cytosol preincubated at 0°C bound poorly to nuclei. However, warming the preformed complex at 25°C for 30 min resulted in the activation of the complex as shown subsequently by markedly increased binding of [3H]dexamethasone-receptor complex to the nuclei.

Cytosol [3H]dexamethasone-receptor complex, preformed at 0°C, when incubated with either N-ethylmaleimide or iodoacetamide and subsequently activated, was unable to bind to nuclei. Addition of a 2-fold excess of \( \beta \)-mercaptoethanol to the N-ethylmaleimide- or iodoacetamide-treated cytosol did not prevent the inactivation of binding of [3H]dexamethasone-receptor complex to nuclei which occurs with these reagents.

In contrast, cytosol [3H]dexamethasone-receptor complex, first activated at 25°C for 30 min and then treated with either N-ethylmaleimide or iodoacetamide for 30 min at 0°C, did bind readily to nuclei. Extending the treatment time to 90 min at 0°C gave similar results.

As determined by charcoal-dextran assay, the [3H]dexamethasone-receptor complex remained intact on treatment with N-ethylmaleimide or iodoacetamide either prior to or after activation of [3H]dexamethasone-receptor complex. This implies that N-ethylmaleimide and iodoacetamide inactivation of [3H]dexamethasone-receptor complex binding to nuclei was not due to the dissociation of [3H]dexamethasone from the receptor. More likely, N-ethylmaleimide and iodoacetamide act by preventing the temperature-dependent activation of [3H]dexamethasone-receptor complex.

The time course of sulphydryl modifying reagent-induced inactivation showed that the maximum level of inactivation was achieved in 30 min. The extent of inactivation was dependent on the concentration of N-ethylmaleimide or iodoacetamide used. Maximum inactivation was obtained at 5 mM N-ethylmaleimide and 8 mM iodoacetamide (data not shown).

Physiochemical changes characterizing [3H]dexamethasone-receptor inactivation by N-ethylmaleimide and pyridoxal phosphate were as follows: Cake et al. (17) have reported that pyridoxal phosphate inhibits the binding of activated dexamethasone-receptor complexes to DNA-cellulose. Subsequently, Cidlowski and Thanassi (18) reported that the molecular properties of rat thymocyte dexamethasone-receptor complexes are markedly affected by pyridoxal phosphate, as shown by both sucrose gradients and Sephadex G-100 gel filtration. We thought it of interest to examine whether similar alterations occur in the molecular properties of rat hepatic glucocorticoid-receptor complexes, and to determine whether sulphydryl modifying reagents (e.g., N-ethylmaleimide) also inactivate the nuclear binding of [3H]dexamethasone by similar mechanisms.

Unactivated cytoplasmic [3H]dexamethasone-receptor complex showed a peak of radioactivity in the 4 to 5 S region in high salt sucrose density gradients (Fig. 1). The N-ethylmaleimide treatment of unactivated cytosol showed a similar radioactivity peak in the 4 to 5 S region. However, treatment of unactivated or activated [3H]dexamethasone-receptor complex with pyridoxal phosphate resulted in appearance of two peaks of radioactivity, a large peak in the region of 3 S and a smaller peak in the 4 to 5 S region.

The molecular properties of [3H]dexamethasone-receptor complex treated with chemical reagents were also examined by agarose gel filtration. Fig. 2 shows the elution profile for unactivated cytoplasmic dexamethasone-receptor complex. Two macromolecular-bound radioactivity peaks were observed; a major peak (I) occurred between Fractions 22 and 30, and a minor peak (II) occurred between Fractions 31 and 38 (we are currently characterizing these two peaks in detail). Unbound dexamethasone eluted after 60% fractions.
myoglobin and bovine serum albumin.

Methods.

X.

ethylmaleimide; 120 min at 0°C with either 5 mM N-ethylmaleimide or 5 mM pyridoxal phosphate. One milliliter of activated cytosol plus pyridoxal 5'-phosphate. One milliliter of unactivated cytosol plus N-ethylmaleimide; ∆, unactivated cytosol plus pyridoxal 5'-phosphate; ×, activated cytosol plus pyridoxal 5'-phosphate.

Fig. 2. Agarose gel filtration of reagent-treated [3H]dexamethasone-receptor complexes. The cytosol, pretreated with [3H]dexamethasone at 0°C for 2 h, was incubated for 30 min at 0°C with N-ethylmaleimide or pyridoxal phosphate. One milliliter of each was applied on the column and eluted with homogenization buffer in order to remove the salt. The macromolecular peaks were pooled, adjusted to identical count, and incubated for 30 min at 25°C. Duplicate aliquots (25 to 150 µl) of these [3H]dexamethasone-receptor complexes were incubated with 0.2 ml of nuclei. The reaction mixture was adjusted to 0.5 ml by the addition of homogenization buffer and incubated for 30 min at 0°C. The nuclear fraction was washed and counted as described under "Methods." Values shown are averages of three determinations, each made in triplicate.

Fig. 3. Nuclear binding of macromolecular-bound radioactivity peaks obtained on agarose gel filtration. Pooled fractions from Peaks I, II, and III of agarose gel filtrations were applied to a Sephadex G-25 column and eluted with homogenization buffer in order to remove the salt. The macromolecular peaks were pooled, adjusted to identical count, and incubated for 30 min at 25°C. Duplicate aliquots (25 to 150 µl) of these [3H]dexamethasone-receptor complexes were incubated with 0.2 ml of nuclei. The reaction mixture was adjusted to 0.5 ml by the addition of homogenization buffer and incubated for 30 min at 0°C. The nuclear fraction was washed and counted as described under "Methods." Values shown are averages of three determinations, each made in triplicate.

Discussion

The results obtained show that treatment with either N-ethylmaleimide or iodoacetamide, prior to activation induced by elevated temperature, completely prevents the increased binding of [3H]dexamethasone-receptor complex to nuclei that occurs in the absence of such treatment. However, these reagents are ineffective in preventing binding to nuclei when added to preactivated [3H]dexamethasone-receptor complex. These observations indicated that sulfhydryl modifying reagents act by inhibiting activation of the [3H]dexamethasone-receptor complex rather than by inhibiting binding to nuclei.

The chemical nature of the "activation" of the steroid-receptor complex, which enables it to interact with nuclear acceptor sites, remains unclear. It has previously been shown that activation generates an increased accessibility of cation sites on the glucocorticoid-receptor complex (6, 7). Recently, Cade et al. (17) reported that pyridoxal phosphate inhibits the binding of activated dexamethasone-receptor complex to...
DNA-cellulose in vitro. They postulated that pyridoxal phosphate acts by forming a Schiff intermediate with an NH$_2$ group of lysine which may be a residue appearing on the surface of the receptor complex upon activation. They observed that Tris buffer and certain amino acids form Schiff bases with pyridoxal phosphate and facilitate the number of steroid receptor molecules binding to DNA-cellulose as compared to other buffers. Accordingly, pyridoxal phosphate inhibition of steroid-receptor complex binding to DNA-cellulose occurred only in buffers which do not form Schiff bases with pyridoxal phosphate, such as with borate buffer. In contrast to their finding, we have observed the pyridoxal phosphate inhibition of steroid receptor complex binding to nuclei using Tris buffer. In fact, in the present study, we have detected the presence of large quantities of smaller fragments of [H]dexamethasone-receptor complex on agarose gel filtration and on sucrose density gradients following treatment of cytosol with pyridoxal phosphate. This form of the receptor was unable to bind to nuclei and could explain the inhibitory action of pyridoxal phosphate-treated [H]dexamethasone-receptor complex on nuclear binding. Precise mechanism(s) of pyridoxal phosphate cleavage of rat hepatic glucocorticoid-receptor complex into smaller fragments will only be obtained using a purified receptor protein. In contrast, N-ethylmaleimide inhibits the receptors activation without any detectable change in the size of the receptor molecule. Thus, pyridoxal phosphate-induced inhibition of activation seems to be different from that which involved N-ethylmaleimide.

Even though the precise mechanism of the action of sulphydryl blocking reagents on [H]dexamethasone-receptor complex activation is difficult to interpret in a crude cytosol preparation, two possibilities appear most likely. (a) A sulphydryl group is directly involved in the activation of the receptor, and blocking of the sulphydryl group by N-ethylmaleimide or iodoacetamide induces conformational changes in such a way that the resulting steroid-receptor complex is unable to be activated; or (b) N-ethylmaleimide or iodoacetamide could be inactivating a second protein(s) or protease(s) in the cytosol which is essential for the activation of the complex. Currently, we are studying these possibilities with radioactive N-ethylmaleimide.

Finally, we have observed that sulphydryl blocking reagents maintain the [H]dexamethasone-receptor complex in the unactivated form with considerable stability in vitro. This should allow us to understand more precisely the mechanism of in vitro steroid-receptor activation.

REFERENCES