Heterogeneity of Binding Sites for Glucocorticoid and the Glucocorticoid-Receptor Complex in Rat Livers

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Abstract—Glucocorticoid binding to cytoplasmic and nuclear fractions and glucocorticoid-receptor complex binding to the nuclear fraction were investigated using rat liver. The glucocorticoid-receptor complex binding to the nuclear fraction was temperature-dependent, saturable, small in amount and of high affinity. The affinity and number of the glucocorticoid-receptor complex binding to the nuclear fraction were altered according to the glucocorticoid. Both the B_{max} of nuclear glucocorticoid-receptor complex binding and the affinity of glucocorticoid to the cytoplasmic fraction were correlated with the relative anti-inflammatory potencies of glucocorticoids reported by Hynes and Murad (1980) and Fried et al. (1958). These results suggest that the number of nuclear binding sites of the glucocorticoid-receptor complex depends on the ligand steroid which is bound to the receptor of the cytoplasmic fraction and may be involved in physiological and pharmacological potencies of the glucocorticoid in addition to the affinity of the glucocorticoid to the receptor.

Glucocorticoids, like other steroid hormones, are thought to act by controlling the rate of synthesis of protein (1). The glucocorticoids react with receptor proteins in the cytoplasm and form a steroid-receptor complex (2–4). The steroid-receptor complex undergoes an energy-dependent activation, enter the nucleus, and bind to acceptor sites, which are thought to trigger nuclear-mediated events that result in the biological responses (1–4). Good correlations have been obtained between relative physiological and pharmacological potencies of the glucocorticoids and their affinities for the receptors of the cytoplasm in tissues *in vitro* and *ex vivo* (5). Non equivalence in glucocorticoid-receptor complex binding to nuclear acceptors was reported (6). Furthermore, a good correlation between the inhibition of nuclear glucocorticoid-receptor complex binding and loss of gene expression of casein in mammary glands *in vitro* was reported recently (7). In the present study, we have characterized the binding of glucocorticoid-receptor complexes

and glucocorticoids to the nuclear fraction in addition to the glucocorticoids binding sites of the cytoplasmic fraction in rat liver and showed the heterogeneity of these binding sites.

Materials and Methods

Male albino rats of the Wistar strain weighing 150–200 g were used throughout the experiments.

Preparation of cytoplasmic fraction: After decapitation, the liver was perfused via the vena cava inferior with 20 ml of ice-cold 0.9% NaCl. The liver was isolated and homogenized with 6 vol. of ice-cold 10 mM Tris-HCl (pH 7.5) buffer containing 1 mM EDTA and 2 mM dithiothreitol. Lipoprotein-removed supernatant was obtained by centrifugation of the homogenate at $100,000\times g$ for 60 min and lipid aspiration from the resultant supernatant, and it was used as the cytoplasmic fraction of rat liver.

Preparation of nuclear fraction: The nuclear fraction of rat liver was prepared according

to the previously reported method (8), with slight modifications. The liver that was perfused with ice-cold 0.9% NaCl was homogenized in 2 vol. of 0.32 M sucrose containing 10 mM Tris-HCl (pH 7.5), 3 mM MgCl_2 and 1 mM glutathione (buffer I) by a Teflon-glass homogenizer. The homogenate was filtered through two layers of silicon-coated gauze. The filtrate was centrifuged at $800\times g$ for 10 min. After removal of the supernatant, the pellet was suspended with 4 vol. of buffer I and centrifuged again at $800\times g$ for 10 min. Then, the resulting pellet was suspended with 3 vol. of buffer I, and 10 ml of this suspension was layered on 18 ml of 1.5 M sucrose containing 10 mM Tris-HCl (pH 7.5), 3 mM MgCl_2 , 25 mM KCl and 1 mM glutathione. Following centrifugation at $20,000\times g$ for 30 min, the pellet was obtained and suspended with buffer I at the original wet weight volume. This suspension was used as the nuclear fraction of liver for nuclear acceptor and receptor assay.

Assay of glucocorticoid binding to cytoplasmic fraction: The binding was carried out as previously reported (9). Three hundred μl of cytoplasmic fraction was incubated with tritiated glucocorticoid at 0°C for 2 hr. The reaction was terminated by addition of 500 μl of activated charcoal solution (1% charcoal and 0.1% dextran (MW 60,000–90,000)/10 mM Tris-HCl (pH 7.5)) and was then mixed for 15 sec. The mixture was centrifuged at 3,000 rpm for 10 min at 2°C . From the supernatant, 0.2 ml was transferred into a counting vial containing PCS-xylene (1:1) scintillation counting fluid. The radioactivity found in the presence of 10 μM nonlabeled glucocorticoid was considered to be nonspecifically bound tritiated glucocorticoid and was subtracted from each experimental value found in the absence of the nonlabeled glucocorticoid (total binding) to obtain the amount of specifically bound tritiated glucocorticoid. As nonlabeled glucocorticoids, HC, DM and triamcinolone acetonide were used for the determination of specific binding of ^3H -HC, ^3H -DM and ^3H -triamcinolone acetonide, respectively. Five nM tritiated glucocorticoids were used for the measurement of the affinities of steroids. Specific bindings of these tritiated

glucocorticoids were found to be 90–95% of the total bindings. These specific binding values increased linearly with the protein concentration up to 3 mg per assay and reached equilibrium within 100–120 min under these experimental conditions. The inhibition constants (K_i) were determined by means of the equation proposed by Chen and Prusoff (10).

Assay of glucocorticoid binding to nuclear fraction: The binding activity of glucocorticoid to nuclear fraction was carried out by the exchange method of Anderson et al. (11). Five hundreds μl of nuclear fraction was incubated with tritiated glucocorticoid at 0 and 30°C for 90 min unless otherwise noted. Following standing for 10 min at 4°C , 2 ml of 10 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 10% glycerol (buffer II) was added and mixed immediately. The mixture was centrifuged at $800\times g$ for 10 min, and the supernatant was discarded. This procedure was repeated twice. The pellet was suspended with buffer II and filtered through a Millipore HAWP (0.45 μm) filter under reduced pressure. The filter was placed in a counting vial containing PCS-xylene scintillator. To obtain the specific binding of tritiated glucocorticoid, the radioactivity found in the presence of 10 μM nonlabeled glucocorticoid was subtracted from the value of total binding as described above. The specific bindings of ^3H -HC and ^3H -DM to nuclear fraction were approximately 95 and 85%, respectively, of the total binding at both 0 and 30°C . These specific binding values increased linearly with the DNA concentration up to 160 μg per assay.

Assay of glucocorticoid-receptor complex binding to nuclear fraction: Tritiated glucocorticoid-receptor complex was obtained by the incubation of 500 nM ^3H -HC, ^3H -DM and ^3H -triamcinolone acetonide with the cytoplasmic fraction as described above. Then, 60–300 μl of ^3H -glucocorticoid-receptor complex were incubated with nuclear fraction (approximately 40 μg DNA) for 60 min at 0 and 30°C in 500 μl (final volume) unless otherwise noted. The specific binding of ^3H -glucocorticoid-receptor complex was determined by subtracting the value of nonspecifically bound

tritiated glucocorticoid-receptor complex. The nonspecific binding of the complex was determined by the incubation of the nuclear fraction with the complex obtained in the presence of $10 \mu\text{M}$ nonlabeled glucocorticoid. The specific bindings of tritiated glucocorticoid-receptor complex were approximately 50 and 70% of the total binding of ^3H -HC- and ^3H -DM-receptor complex, respectively, at 30°C ; and the specific binding of these complexes were 40 and 60%, respectively, at 0°C . For triamcinolone acetonide, specific binding was approximately 60% of the total binding both 0 and 30°C . These specific binding values increased linearly with DNA concentration up to $80 \mu\text{g}$ per assay.

Protein concentration was determined by the method of Lowry et al. (12) using bovine serum albumin as the standard. DNA concentration was assayed according to the method of Burton (13). The radioactivity was determined with a Packard model 3255 liquid scintillation spectrometer at a counting efficiency of 38–44%. The dissociation constant (K_d) and maximum number of the binding sites (B_{max}) were determined by Scatchard analysis (14).

Drugs and materials: HC was obtained from Roussel UCLFA, and DM and triamcinolone acetonide were obtained from Sigma. 1, 2, 4- ^3H -DM (S. A.: 46 Ci/mmol) and 1, 2, 4 (n)- ^3H -triamcinolone acetonide (S. A.: 22 Ci/mmol) were obtained from RCC Amersham, and 1, 2- ^3H -HC (S. A.:

50.7 Ci/mmol) was obtained from New England Nuclear. PCS scintillator was obtained from Amersham/Searle.

Results

^3H -Glucocorticoid binding to the cytoplasmic fraction of rat livers: Scatchard plots of ^3H -DM and ^3H -triamcinolone acetonide bindings consisted of a single straight line (Fig. 1). The B_{max} and K_d values of ^3H -DM and ^3H -triamcinolone acetonide were approximately equal. On the other hand, Scatchard analysis of ^3H -HC binding revealed the presence of two different HC affinity binding sites (Fig. 1). The B_{max} and K_d values of the high affinity binding site of ^3H -HC was significantly smaller than those of ^3H -DM and ^3H -triamcinolone acetonide binding sites. The K_d value of the low affinity binding site of ^3H -HC was larger than those of ^3H -DM and ^3H -triamcinolone acetonide, while the B_{max} of the ^3H -HC binding site was approximately equal to the above two steroids.

Inhibition of specific ^3H -glucocorticoid binding to the cytoplasmic fraction by DM, triamcinolone acetonide and HC: ^3H -DM binding was completely inhibited by DM, triamcinolone acetonide and HC in a dose-dependent manner (Fig. 2). Similarly, ^3H -triamcinolone acetonide binding was inhibited by these glucocorticoids. In contrast, ^3H -HC binding was not completely inhibited by DM and triamcinolone acetonide, except for HC (Fig. 2). Synthetic glucocorticoids,

Fig. 1. Scatchard plot of ^3H -HC, ^3H -DM and ^3H -triamcinolone acetonide binding to cytoplasmic fraction. Between 1 to 60 nM tritiated glucocorticoids were used. Each point represents the mean obtained from 2 to 6 separate experiments performed in duplicate.

DM and triamcinolone acetonide, did not inhibit the ^3H -HC binding completely. These inhibitory effects reached a plateau at 100 nM with 45% inhibition (Fig. 2).

^3H -Glucocorticoid-receptor complex binding to the nuclear fraction: ^3H -DM- and ^3H -HC-receptor complexes bound to the nuclear fraction time- and temperature-

dependently (Fig. 3). Within 30 min, the glucocorticoids-receptor complexes were saturated at both 0 and 30°C. The amount of the ^3H -DM-receptor complex binding was equal to that of the ^3H -HC-receptor complex at 0°C, while at 30°C, the binding of the ^3H -DM complex was double the value of ^3H -HC complex. The B_{max} of ^3H -DM- and ^3H -

Fig. 2. Inhibition of specific ^3H -HC, ^3H -DM and ^3H -triamcinolone acetonide binding to cytoplasmic fraction by various glucocorticoids. Each point represents the mean obtained from 3 to 5 separate experiments. Specific bindings of ^3H -HC, ^3H -DM and ^3H -triamcinolone acetonide were 111.5 ± 11.5 , 131.1 ± 9.0 and 147.0 ± 8.5 fmoles/mg prot., respectively, when 5 nM tritiated glucocorticoids were used. K_i values of HC, DM and triamcinolone acetonide were 49.3, 4.9 and 6.9 nM, respectively, for ^3H -DM binding and 40, 12 and 12 nM, respectively, for ^3H -triamcinolone acetonide binding. K_i value of HC for ^3H -HC was 27.5 nM. These values represent the mean of 3 to 5 separate experiments.

Fig. 3. Time and temperature dependency of ^3H -HC- and ^3H -DM-receptor complex binding to nuclear fraction. As ligands, 1.81 nM ^3H -HC-receptor complex and 1.37 nM ^3H -DM-receptor complex were used.

triamcinolone acetonide-receptor complexes were approximately equal but the value of the ^3H -HC-receptor complex was one-third of those for ^3H -DM and ^3H -triamcinolone,

respectively (Fig. 4). Furthermore, the K_d values of ^3H -DM- and ^3H -triamcinolone acetonide-receptor complexes were equal, but that of HC was about one-third those of

Fig. 4. Scatchard plot of ^3H -HC-, ^3H -DM- and ^3H -triamcinolone acetonide-receptor complex binding to nuclear fraction. An example of some experiments for each glucocorticoid was given. Each point represents the mean of triplicate experiments. K_d and B_{max} values for ^3H -HC-receptor complex, ^3H -DM-receptor-complex and ^3H -triamcinolone acetonide-receptor complex were $0.41 \text{ nM} \pm 0.10$, and $89.6 \text{ fmol}/100 \mu\text{g DNA} \pm 4.4$, $0.88 \text{ nM} \pm 0.06$ and $240 \text{ fmol}/100 \mu\text{g DNA} \pm 30.5$, and $1.02 \text{ nM} \pm 0.2$ and $320 \text{ fmol}/100 \mu\text{g DNA} \pm 40$, respectively. These values represent the mean obtained from 3 separate experiments, performed in triplicate. K_d and B_{max} values for ^3H -HC-receptor complex at 0°C were 0.21 nM and $65 \text{ fmol}/100 \mu\text{g DNA}$, respectively. Between 0.07 to 1.5 nM , 0.30 to 1.67 nM and 0.2 to 0.71 nM were used as the concentrations for the glucocorticoid-receptor complex of ^3H -HC, ^3H -DM and ^3H -triamcinolone acetonide, respectively.

Fig. 5. Time and temperature dependency of ^3H -HC and ^3H -DM binding to nuclear fraction. Five nM tritiated glucocorticoids were used.

Fig. 6. Scatchard plot of ^3H -HC and ^3H -DM binding to nuclear fraction. Between 1 to 100 nM tritiated glucocorticoids were used. Each point represents the mean obtained from 2 to 4 separate experiments performed in duplicate.

the above two synthetic glucocorticoids. ^3H -Triamcinolone acetonide-receptor complex slightly bound to the nuclear fraction at 0°C . The temperature dependency of the glucocorticoid-receptor complex binding to the nuclear fraction was most clearly observed with the triamcinolone acetonide-receptor complex (Fig. 4). There was no clear temperature dependence of ^3H -HC-receptor complex binding.

^3H -Glucocorticoid binding to the nuclear fraction: ^3H -Glucocorticoids binding to the nuclear fraction was also time- and temperature-dependent, while the temperature dependency was not as distinct as that for the glucocorticoid-receptor complex (Fig. 5). There was no difference in the amount of binding between HC and DM to the nuclear fraction. DM and HC bound to the nuclear fraction with a similar pattern at both 0 and 30°C , respectively. The Scatchard analysis showed approximately equal values of $B_{\max}$ for ^3H -HC and ^3H -DM (Fig. 6). These $B_{\max}$ values were significantly larger than that of the glucocorticoid-receptor complex. On the other hand, the affinities of ^3H -HC and ^3H -DM were obviously lower than that of the glucocorticoid-receptor complex. The K_d value of the latter one was about one-hundredth of the former ones. In addition, the specific binding of ^3H -triamcinolone

acetonide to the nuclear fraction was different from those of ^3H -HC and ^3H -DM (Fig. 6).

Discussion

Numerous researchers have subdivided the binding sites of glucocorticoids by chromatographic analyses and suggested that the binding site of synthetic glucocorticoid is just one part of the sites which bind natural glucocorticoids (2, 15–18). Biological potencies of glucocorticoids have been explained by the affinity for the receptor, which is the only site that the synthetic glucocorticoids can bind (5). Certainly, in this study, HC bound to the DM binding site, but DM bound only to a part of the HC binding sites and the affinities of glucocorticoids for the ^3H -DM and ^3H -triamcinolone acetonide binding sites of the cytoplasmic fraction correlated approximately to their physiological and pharmacological potencies (1). On the other hand, there has been a great deal of controversy concerning the demonstration of nuclear acceptor sites. Several investigators have shown that the nuclear binding of the steroid-receptor complex is a saturable phenomenon and suggested the existence of a specific acceptor site (3, 19, 20), while others claimed that a limited number of specific sites does not

exist and that nuclear saturation is an artifact (21–23). Much of this conflict probably stems from the difficulty in detecting a small number of specific sites in the presence of a large number of non specific sites (24). Buller et al. (19) succeeded in demonstrating the saturability, the high affinity of a small amount of sites and tissue specificity of the progesterone-receptor complex binding to nuclei in vitro by limiting the nonspecific binding. Furthermore, recent studies have shown that activation does occur in the cell and is not just an in vitro artifact (25, 26). In this study, we were able to observe the high affinity, saturable, small amount and temperature-dependent glucocorticoid-receptor complex binding by subtracting the non-specific binding. It is wellknown that in mammals, one cell contains approximately 6 pg of DNA (27), and we were able to observe 300 fmoles per 100 μ g DNA as the maximum binding of the DM-receptor complex to the nuclear fraction. From these results, we were able to calculate that one liver cell contains 18×10^{-23} moles of acceptor sites, that is 10,000 molecules of DM-receptor complexes bind to the acceptor per cell. This value was agreed with the binding of the progesterone-receptor complexes to the oviduct nuclei (19). Cidlowski and Munk (6) demonstrated the heterogeneity of nuclear glucocorticoid-receptor complex in the thymus cells. They incubated thymocytes and glucocorticoids, and they measured the distribution and kinetic behavior of the glucocorticoid-receptor complex in the intact cells, which was subjected to differential salt extraction and DNaseI and DNaseII digestion. As for glucocorticoid, they used HC, DM and triamcinolone acetonide and investigated the amount of the glucocorticoid-receptor complex binding to the nuclear fraction. The nuclear distribution of DM- and triamcinolone acetonide-receptor complexes were almost equal, but the HC complex was about half that of either synthetic compound in the salt-extractable and unextractable fraction. Moreover, they indicated that nuclear DM- and triamcinolone acetonide-receptor complexes are significantly more resistant to nuclease attack than the HC-receptor complex. Thus, they

suggested that the natural and synthetic glucocorticoid form different types of nuclear complexes or associate with the nuclei in a different manner. However, in their study, the affinity and the maximum number of binding sites of the glucocorticoid-receptor complex are not clear. In this study, we investigated the complex binding to nuclei using kinetic analysis by incubating the isolated nuclei from rat liver and the glucocorticoid-receptor complex prepared previously by incubating the glucocorticoid with the cytoplasmic fraction of rat liver. We demonstrated that the values for the maximum number of binding sites for the DM- and triamcinolone acetonide-receptor complexes were approximately equal, but that of the HC-receptor complex was one-third of the former ones. In addition, the number of temperature-dependent binding sites for the HC-receptor complex was about 10% of those for DM and triamcinolone acetonide. We have no direct evidence that the numbers of these high affinity, saturable, small amount and temperature-dependent bindings of glucocorticoid-receptor complex are responsible for the biological activity, but these numbers correlated with the biological potency of the glucocorticoids. Temperature-dependent, specific bindings of HC-, DM- and triamcinolone acetonide receptor complex were 24 (89 minus 65), 240 and 320 fmoles/100 μ g DNA, respectively. Anti-inflammatory activity of DM is 25–170 times that of HC (1, 28), and triamcinolone acetonide has approximately the same anti-inflammatory effect as DM (29, 30). Moreover, Majumder et al. (7) indicated recently that there was a good correlation between the level of nuclear binding of the glucocorticoid-receptor complex and casein gene expression in the murine mammary gland in vitro. Putting all the data together, it may be considered that the nuclear binding of glucocorticoid-receptor complex plays an important role and the level of the binding is involved in the efficacy of the glucocorticoid. Free glucocorticoid bindings to the nuclear fraction were observed with a low affinity that was one-hundredth of that of the glucocorticoid-receptor complex, and there were no differences between HC and DM.

In the case of triamcinolone acetonide, however, the specific binding to the nuclear fraction was not observed. So, it seems that the free glucocorticoid binding to the nuclear fraction does not have a significant role in the biological action of glucocorticoid. It has been considered that the affinity of glucocorticoid to the receptor protein in the cytoplasm is the most important factor which is related to the physiological and pharmacological effect of the glucocorticoid (5).

The present study suggests that the number of the binding sites of glucocorticoid-receptor to nuclei may be involved in the efficacy of the glucocorticoid in addition to the affinity of the glucocorticoid to the receptor of the cytoplasm.

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