Characterization of 3-Methylcholanthrene Effects on the Rat Glucocorticoid Receptor in Vivo

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ABSTRACT

The effects of 3-methylcholanthrene and phenobarbital treatment on the adult rat hepatic cytosolic glucocorticoid (GRc) receptor were investigated. Analyses of sucrose gradient profiles and equilibrium binding data of [3H]dexamethasone bound to the GRc revealed that administration of 3-methylcholanthrene (20–40 mg/kg daily i.p. for 2 days) to adult female Fischer F344 rats led to a significant decrease in the maximal binding capacity of the 5-75 GRc [Bmax = 209 ± 3 (SE) fmol/mg of protein] compared to the vehicle-treated controls (Bmax = 277 ± 13 fmol/mg) but had no significant influence on the affinity of the GRc (Kd = 0.9 ± 0.1 nM). This response was not dependent upon the sex or rat strain (female F344 versus Sprague-Dawley). Phenobarbital treatment (80 mg/kg daily i.p. for 4 days) decreased Bmax and Kd values compared to the vehicle-treated controls (P < 0.05). 3-Methylcholanthrene treatment did not significantly alter the equilibrium parameters of [3H]methyltrienolone bound to the hepatic androgen receptor indicating that the effect was specific to the hepatic GRc. Our data suggest that carcinogens and tumor promoters cause a functional decrease of the cytosolic glucocorticoid receptor in vivo.

INTRODUCTION

The glucocorticoid receptor is a soluble intracellular protein which specifically binds functional hormonal ligands such as circulating glucocorticoids. Occupation of this receptor by its ligand leads to enhanced induction of specific glucocorticoid-sensitive gene expression through its interaction with specific genomic glucocorticoid-response elements (1). It has been suggested that modification of this and other steroid receptor pathways can alter intracellular mechanisms responsible for cell growth, homeostasis, and carcinogenesis (2).

Studies by different laboratories have shown that the glucocorticoid receptor pathway is influenced by a variety of environmental contaminants including those which have potent carcinogenic and tumor-promoting capabilities. Animal studies have shown that chemically stimulated hepatocarcinogenesis is associated with marked decreases in cytosolic glucocorticoid receptor content (3). In addition, a variety of hepatic and nonhepatic carcinogens decrease rat nuclear glucocorticoid receptor binding in vivo (4) as well as rat liver glucocorticoid-stimulated TAT2 and tryptophan pyrolyase activities (5, 6). Neal and associates (7) have shown that administration of TCDD, an hepatic carcinogen and tumor promoter (8–10) led to a decrease in rat liver hydrocortisone-induced TAT activities. These results are consistent with the recent observations that TCDD significantly decreased hepatic cytosolic glucocorticoid receptor binding in adrenalectomized and adrenal-intact adult female rats (11, 12). A similar decrease in hepatic glucocorticoid receptor binding was also observed in pregnant female CSBL6 mice treated with 2,3,4,7,8-pentachlorodibenzofuran or the 1,2,3,4,7,8-hexachloro-congener (13). In addition to these effects, TCDD decreases glucocorticoid receptor binding concentrations in rat skeletal muscle (14), as well as hepatic and uterine estrogen receptor content (15).

The glucocorticoid receptor system is also sensitive to tumor promoters such as TPA and mezerein in the mouse skin tumorigenesis model (16). The exact mechanism by which these carcinogenic and tumor-promoting compounds may modify steroid receptor binding and the receptor-related sequence is not clear. The present report describes studies which further examine the mechanism by which carcinogens and tumor promoters may be acting on the hepatic glucocorticoid receptor pathway, by comparing the effects of two structurally different compounds; the carcinogen 3-MC, and the tumor promoter PB on radiolabeled dexamethasone binding to the rat cytosolic glucocorticoid receptor.

MATERIALS AND METHODS

Chemicals. [1,2,4,6,7-3H]Dexamethasone (50 Ci/mmol, radiochemical purity >96%) was purchased from Amersham International-plc (Little Chalfont Buckinghamshire, England), whereas nonradioactive and [17α-methyl-3H]methyltrienolone (R1881; 87 Ci/mmol) were obtained from Du Pont de Nemours Int. SA. (Regensdorf, Switzerland). Optiphase Hi-safe scintillation fluid was bought from Pharmacia-pdp (Dubendorf, Switzerland). All other chemical reagents were obtained from Sigma Chemical Co. (St. Louis, MO). All reagents and solvents were of the highest grades of purity available. All steroids were routinely checked for purity using thin-layer chromatography as described in the product specifications. Glass-distilled water was used for the preparation of all aqueous solutions.

Animals and Treatments. Adult male (250–300 g) and (150–170 g) female Sprague-Dawley and Fischer F344 rats were obtained from Iffa-Credo S. A. (L’Arbresle, France) and were housed in groups of three in plastic cages under standard laboratory conditions. All animals were fed Nafag 890 rat chow (Nafag S.A., Gossau, Switzerland) and tap water ad libitum. In some experiments, animals were injected i.p. with PB (80 mg/kg daily i.p. for 4 days) in a 0.9 g NaCl/100 ml water solution or 3-MC (10–40 mg/kg daily i.p. for 2 days) in corn oil, whereas the control animals received the equivalent volume of the vehicle on a body weight basis. On the day of the experiment, animals were killed by carbon dioxide asphyxiation followed by exsanguination. All animal experiments were done at 18–20 h following the last injection.

Hepatic Subcellular Fractionation. Livers were quickly dissected, weighed, and the cytosolic and microsomal fractions were prepared according to Sunahara et al.1 In brief, liver minces were homogenized in a freshly prepared ice-cold TEDGM buffer (10 mM Tris-HCl pH 7.4; 2.5 mM EDTA; 5 mM DTT; 10% v/v glycerol; 20 mM sodium molybdate), unless otherwise stated. All procedures were carried out at 4°C. The homogenate was centrifuged at 10,000 × g for 20 min, and the resulting supernatant was recentrifuged at 105,000 × g for 65 min.

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3 To whom requests for reprints should be addressed, at Nestlé Research Centre, Vers-chez-les-Blanc, CH-1000 Lausanne 26, Switzerland.
4 The abbreviations and trivial names used are: TAT, tyrosine aminotransferase; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; BSA, bovine serum albumin; dexamethasone, 1,4-pregnadien-9-fluoro-16a-methyl-11β,17α,21-triol-3,20-dione; 3-MC, methylcholanthrene [1,2-dihydro-3-methylbenz(a)anthracene]; PB, phenobarbital sodium [3-ethyl-5-phenyl-2,4,6-(1H,3H,5H)pyrimidinetrione monosodium salt]; R1881, methyltrienolone (17β-hydroxy-17-methyl Estr-4,9,11-trien-3-one); BPH, benzo(a)pyrene hydroxylase; NSB, nonspecific bound; TPA, 12-O-tetradecanoylphorbol-13-acetate; DTT, dithiothreitol; HPLC, high-performance liquid chromatography.
The clear supernatant (containing cytosol) was recovered by aspiration and stored in 2-ml aliquots at -70°C until analysis, unless otherwise stated.

Microsomes were prepared separately following a similar method as described above, except that iced-cold 150 mM KCl-50 mM Tris-HCl (pH 7.4) buffer was used for the homogenization step. Following the differential centrifugation steps, the supernatant was discarded, and the final pellet was washed and resuspended in a 250 mM sucrose-100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.6) buffer. The microsomal suspension was stored in 0.25-ml aliquots at -70°C until analysis.

Receptor Assays. The cytosolic glucocorticoid and androgen receptor binding assays were carried out under equilibrium conditions according to previous reports (13, 17). The amount of [3H]dexamethasone and [3H]R1881 displaced (or specific binding) was calculated as the difference between total and nonspecific bound fractions for each cytosolic preparation. Equilibrium binding data were analyzed using Scatchard coordinates (18) and the linear descending curve of the Scatchard plot was fitted by the least squares method for linear regression. The apparent equilibrium dissociation constant (Kd) and apparent maximum binding capacity (Bmax) were expressed as nM and fmol per mg of cytosolic protein, respectively. Protein content was determined according to Bradford (19), using BSA as the standard.

Sucrose Gradient Ultracentrifugation. Fixed angle rotor gradients of 5 to 20 g sucrose per 100 ml TEDG buffer (pH 7.4) were prepared in Beckman polyyallomer bell-top Quick Seal ultracentrifugation tubes (16 x 45 mm) as described by others (13, 20, 21). Methyl-14C-BSA (37 μCi/mg protein; Sigma Chemical Co., St. Louis, MO) was used as an internal standard. Fractions (about 0.4 ml) were collected by piercing the bottom of the tube and were assayed for either radioactivity or protein content. The gradients were very reproducible and were run with respect to density, as evidenced by increasing (n20d) values from 1.35 to 1.36 as determined by refractive index, and the appearance of the 14C-BSA peak. As a further control to each experiment, sedimentation coefficients in Svedberg (S20,w) units of the hepatic [3H]dexamethasone glucocorticoid receptor complex were determined according to the method of Martin and Ames (22) using nonlabeled protein standards. These conditions consistently yielded linear standard curves in the range of 1.9 to 11.2S. As anticipated, the radiolabeled and nonlabeled BSA cofractionated. The sedimentation coefficients of the radiolabeled dexamethasone glucocorticoid receptor complex were estimated using extrapolation. Sucrose gradient profiles of the hepatic cytosolic glucocorticoid receptor complex shown in the figure are representative of three or more replicate experiments.

BPH Assay. This fluorimetric assay was carried out according to the method of Nebert and Gelboin (23). The authentic metabolite was obtained from the Midwest Research Institute (Kansas City, MO) and was used as the standard. The BPH activities were measured as picomoles 3-hydroxybenzo[a]pyrene formed per min per mg microsomal protein.

HPLC Analysis of Cytosolic Dexamethasone Metabolism in Vitro. The rat liver cytosol was prepared (4 mg protein/ml) and incubated with 4 nM [3H]dexamethasone in the presence of absence of competitor, as described above. Following incubation at 4°C for 18–20 h, and ethanol extraction, the resulting suspension was centrifuged at 10,000 x g for 10–15 min. The supernatant containing the extracted steroids was decanted and stored at -70°C until analysis. Analysis was carried out using an HP Model 1090M HPLC equipped with a Supelco C18 reversed-phase column (5-μm particle size, 4.6 mm i.d. x 25 cm) and UV detector set at 254 nm. The extracted steroids were eluted using a linear gradient from 50 to 100% methanol over 20 min at a flow rate of 1 ml/min. The aqueous phase contained 50 mM ammonium acetate. These conditions yielded >95% recovery of injected sample. In all cases, 1-min fractions were taken and analyzed for radioactivity.

Statistical Analysis. Data were expressed as the mean ± SE, the numbers in parentheses denote the number of animals analyzed. Differences between control and test groups were analyzed using the Student's t test for unpaired data and were considered significant at P < 0.05. All samples were analyzed in duplicate or triplicate, individual data presented are representative examples from at least two or more experiments.

RESULTS

It is well known that 3-MC administration leads to significant alterations in rat liver biochemistry. In the adult female F344 rat, 3-MC treatment (20 mg/kg daily, i.p. for 2 days) significantly increased the liver to body weight ratios (%) from 3.3 ± 0.1 in the corn oil treated control to 4.0 ± 0.2 in 3-MC treated groups (data not shown). In addition, arylhydrocarbon hydroxylase activities were greatly increased by 3-MC treatment, as evidenced by the induction of hepatic microsomal BPH activities [corn oil controls, 62 ± 8 pmol/mg/min; 3-MC treated, 827 ± 13 pmol/mg/min (P<0.05)]. These results are consistent with the known effects of 3-MC and are similar to those caused by TCDD administration (11). The following studies describe our attempts to elucidate the mechanisms underlying the early biochemical effects of 3-MC on the glucocorticoid receptor system, and how they may be related to carcinogenesis.

The effect of 3-MC on the [3H]dexamethasone bound to the glucocorticoid receptor in vivo was investigated using sucrose density gradients. Representative profiles are shown in Fig. 1. The 5–7S glucocorticoid receptor binding peak in both control and treated animals appeared between fractions 6 and 11. Sucrose gradients were highly reproducible within and between experiments as evidenced by the consistent appearance of the 5–7S glucocorticoid receptor binding peak and the quantitatively similar amounts of 14C-BSA (4.2S) which was used as the internal standard. In this representative case, 3-MC administration decreased radio labeled dexamethasone bound to the 5–7S...
compared to the total bound fractions, suggesting that most of control and 3-MC-treated animals was similar and negligible the radioactivity bound to the 5-7S glucocorticoid receptor nonspecifically bound dexamethasone in cytosols taken from glucocorticoid receptor peak by more than 35% compared to controls (0.8 \text{ UM}; 309 \text{ fmol/mg protein}) and 3-MC treated (0.7 \text{ nM}; 200 \text{ fmol/mg}).

Within these fractions, the binding parameters ($K_i$, $B_{max}$) in the following treatment groups: corn oil treated control, multiple experiments show no significant difference to the free dexamethasone concentration. Although in this case, the NSB of a 3-MC-treated animal was less than that of a corn oil control, multiple experiments show no significant difference between the NSB levels of control and 3-MC-treated (20 mg/kg) animals. Preliminary studies indicate that when cytosol was incubated with 0.5 nM labeled dexamethasone, the NSB for the glucocorticoid receptor was due to increased dexamethasone metabolism. Another possibility was investigated. A release of proteases into the cytosol during preparation would account for decreased receptor concentrations; however, proteolysis does not seem to be a problem because experiments have indicated that addition of the mixture of protease inhibitors aprotinin: leupeptin: phenylmethylsulfonyl fluoride (100 units:1 \mu{g}:10 \mu{g}, per ml TEDGM buffer, respectively) did not increase glucocorticoid receptor binding concentrations in 3-MC-treated animals towards those of the controls (data not shown).

Saturation binding studies under equilibrium conditions were undertaken to determine whether the 3-MC-mediated decrease in the hepatic 5–7S glucocorticoid receptor binding complex was related to an alteration in this receptor’s affinity or the number of binding sites, or both. Representative saturation profiles (Fig. 2A) and corresponding Scatchard plots (Fig. 2B) of the equilibrium binding data showing the effects of 3-MC on the glucocorticoid receptor are illustrated. In Fig. 2A, the NSB from treated and control animals increased linearly with respect to the free dexamethasone concentration. Although in this case, the NSB of a 3-MC-treated animal was less than that of a corn oil control, multiple experiments show no significant difference between the NSB levels of control and 3-MC-treated (20 mg/kg) animals. Preliminary studies indicate that when cytosol was incubated with 0.5 nM labeled dexamethasone, the NSB for controls was 4.2 ± 0.2 fmol/mg, whereas that from 3-MC-treated animals was 5.4 ± 0.9 fmol/mg. These data confirm the results of the sucrose density profiles which also show that the amount of nonspecific binding in control and 3-MC-treated animals are similar (Fig. 1). Differences in glucocorticoid receptor specific binding between treated and control groups would therefore be due to differences in the replaceable or specific binding.

Specific binding isotherms representing radiolabeled dexamethasone bound to the glucocorticoid receptor are shown in Fig. 2A. In the control animal, the dexamethasone binding to the glucocorticoid receptor was saturable and displaceable within the nanomolar range. These values are consistent with previously reported observations (11).

3-Methylcholanthrene treatment caused a marked decrease in specific dexamethasone binding to the glucocorticoid receptor compared to the corn oil-treated control. This effect can be seen at 5 nM free dexamethasone, at which 3-MC treatment led to a 23% decrease in glucocorticoid receptor binding compared to controls. Analysis of the Scatchard plots (Fig. 2B) for these data revealed the following apparent equilibrium binding parameters ($K_i$ in nM, $B_{max}$ in fmol/mg) for the control (0.8, 309)
and 3-MC-treated (0.7, 200) animals. In this example, 3-MC treatment caused a marked decrease in the maximal binding capacity of the glucocorticoid receptor and a slight decrease in the equilibrium dissociation constant, $K_d$ (an indication of the receptor affinity to its endogenous ligand), compared to the control.

The effects of 3-MC on adult female rat hepatic glucocorticoid receptor equilibrium binding parameters from replicate experiments are summarized in Table 1. 3-Methylcholanthrene treatment (20 mg/kg daily i.p. for 2 days) of adult female F344 rats significantly decreased the maximal binding capacity ($B_{max}$) of the glucocorticoid receptor by 25% compared to the corn oil-treated controls. This effect was not associated with liver weights or cytosolic protein content (data not shown). The average $B_{max}$ value of the 3-MC treatment group was 209 ± 3 fmol/mg protein compared to the control group of 277 ± 13 fmol/mg. There was no significant alteration in the equilibrium dissociation constant ($K_d$) of the glucocorticoid receptor between the 3-MC-treated (0.9 ± 0.1 nM) and control groups (0.9 ± 0.1 nM).

Since the Fischer F344 rat is an inbred strain, the effect of 3-MC administration on the rat hepatic glucocorticoid receptor in vivo was further investigated using an out-bred strain of rat. Adult female Sprague-Dawley rats were treated with different doses of 3-MC (from 10 to 40 mg/kg daily, i.p.) for varying periods of time. Table 2 shows that after 2 days of treatment, 3-MC caused a dose-dependent decrease in apparent $B_{max}$ of the glucocorticoid receptor of adult female Sprague-Dawley rats. Maximal and significant effects were detected in the treatment groups receiving 3-MC doses greater than or equal to 20 mg/kg (20 and 40 mg 3-MC/kg; $B_{max}$ = 130 ± 12 and 195 ± 16 fmol/mg, respectively) compared to the vehicle-treated control groups (228 ± 35 and 260 ± 21 fmol/mg, respectively). The 3-MC-mediated decrease in glucocorticoid receptor maximum binding capacity was not accompanied by significant alterations in the receptor affinity (range of average $K_d$ values: controls, 0.8–1.5 nM; 3-MC-treated, 0.8 nM). The low dose of 3-MC had no significant effect on the glucocorticoid receptor equilibrium binding parameters ($K_d$ = 1.3 ± 0.1 nM; $B_{max}$ = 204 ± 16 fmol/mg) compared to controls. The 3-MC-mediated decrease in the glucocorticoid receptor concentration is similar to that in the adult F344 rat. These results demonstrate that the 3-MC-mediated decrease in the hepatic glucocorticoid receptor binding concentrations is not strain dependent. After 1 day of treatment, 3-MC did not significantly alter the glucocorticoid receptor equilibrium binding parameters (data not shown). For example, 3-MC treatment groups receiving 10 and 40 mg/kg had the following glucocorticoid receptor binding parameters ($K_d$ = 2.4 ± 0.4 nM; $B_{max}$ = 188 ± 12 fmol/mg) and ($K_d$ = 2.0 ± 0.3; $B_{max}$ = 206 ± 16), respectively compared to corn oil controls ($K_d$ = 1.7 ± 0.2; $B_{max}$ = 222 ± 28).

To test the specificity of the glucocorticoid receptor response to 3-MC, hepatic cytosolic glucocorticoid receptor binding experiments were done using animals treated with PB, a 5-ethyl, 5-phenyl barbiturate, and a cocarcinogen (25). A summary of the effects of PB on the adult female F344 rat liver glucocorticoid receptor in vivo is found in Table 1. Our studies show that similar to the effects of 3-MC on the glucocorticoid receptor described above, administration of PB (80 mg/kg daily i.p. for 4 days) elicited a significant decrease (30%) in the glucocorticoid receptor apparent maximal binding capacity in the phenobarbital-treated (301 ± 23 fmol/mg) compared to the 0.9 g NaCl in 100 ml water-treated adult female F344 controls (404 ± 37 fmol/mg). In contrast to 3-MC, PB treatment also induced a significant decrease in the equilibrium dissociation binding constant between the control (1.2 ± 0.1 nM) and the PB-treated (0.7 ± 0.1 nM) groups. The apparent decrease in receptor binding to dexamethasone was not due to competition between 3-MC or PB and the radiolabeled ligand for the receptor site. Preliminary displacement studies indicate that neither compound (up to 1 μM) specifically binds to the glucocorticoid receptor in vitro. These results suggest that despite their differences in molecular structure PB and 3-MC both modify the binding properties of the hepatic glucocorticoid receptor in vivo by causing a decrease in the number of binding sites, and for PB but not 3-MC the receptor affinity is altered.

To test whether the 3-MC and PB-mediated decrease in hepatic glucocorticoid receptor was dependent upon the gonadal status, age-matched adult male F344 rats were treated with 3-MC or PB using the same schedule as above. As shown in Table 1, administration of 3-MC and PB caused a significant decrease in the apparent maximal binding capacity of the adult male F344 rat hepatic glucocorticoid receptor (3-MC treated, 257 ± 16 fmol/mg; PB treated, 248 ± 8 fmol/mg) compared to vehicle-treated controls (corn oil treated, 345 ± 9 fmol/mg; 0.9 g NaCl/100 ml aqueous solution treated, 320 ± 7 fmol/mg). These responses are similar to the earlier observations using the female rat. Like the female rat, PB treatment to the males resulted in a significant decrease in the $K_d$ of the glucocorticoid receptor (0.6 ± 0.0 nM) compared to the control groups (0.8 ± 0.0 nM). It is difficult to determine whether these differences reflect an alteration in the nature of receptor binding or the generation of a different population of glucocorticoid receptor. Assuming the latter, the biological significance of this change

### Table 1 Comparison of 3-methylcholanthrene and phenobarbital effects on cytosolic steroid receptor equilibrium binding parameters in the adult F344 rat liver

<table>
<thead>
<tr>
<th>Sex</th>
<th>Treatment group</th>
<th>Glucocorticoid receptor</th>
<th>Androgen receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$K_d$ (fmol/mg)</td>
<td>$B_{max}$ (fmol/mg)</td>
</tr>
<tr>
<td>Females</td>
<td>Corn oil control</td>
<td>0.9 ± 0.1*</td>
<td>277 ± 13 (7)</td>
</tr>
<tr>
<td></td>
<td>3-MC treated</td>
<td>0.9 ± 0.1*</td>
<td>209 ± 3' (6)</td>
</tr>
<tr>
<td></td>
<td>Corn oil control</td>
<td>0.6 ± 0.00</td>
<td>345 ± 9 (8)</td>
</tr>
<tr>
<td>Males</td>
<td>3-MC treated</td>
<td>0.5 ± 0.00*</td>
<td>257 ± 16' (8)</td>
</tr>
<tr>
<td></td>
<td>0.9% NaCl solution control</td>
<td>1.2 ± 0.2</td>
<td>404 ± 37 (7)</td>
</tr>
<tr>
<td></td>
<td>PB treated</td>
<td>0.7 ± 0.1*</td>
<td>301 ± 23' (9)</td>
</tr>
<tr>
<td></td>
<td>0.9% NaCl solution control</td>
<td>0.8 ± 0.0</td>
<td>320 ± 7 (4)</td>
</tr>
<tr>
<td></td>
<td>PB treated</td>
<td>0.6 ± 0.00*</td>
<td>248 ± 8' (4)</td>
</tr>
</tbody>
</table>

* Data are expressed as average ± SE; (n) = no. of animals analyzed.

No significant difference between control and test group.

** Significant difference ($P < 0.05$) between control and test groups, using the Student’s $t$ test.

ND, not determined.
is probably minor since circulating glucocorticoids in the rat would occupy both receptors to the same extent.

To further characterize the specificity of the glucocorticoid receptor response to 3-MC in vivo, another rat hepatic cytosolic steroid receptor, the androgen receptor was also assayed. Fig. 3 shows Scatchard plots of radiolabeled methyltrienolone binding to the hepatic cytosolic androgen receptor in a corn oil-treated male control animal and in a rat receiving 20 mg/kg 3-MC for 2 days. Analysis of this plot revealed the following equilibrium binding parameters (control animal: \( K_d = 0.2 \text{ nM} \), \( B_{\text{max}} = 19 \text{ fmol/mg} \); 3-MC treated: \( K_d = 0.2 \text{ nM} \), \( B_{\text{max}} = 16 \text{ fmol/mg} \). In this experiment, 3-MC treatment caused a 19% decrease in androgen receptor binding concentrations and no effect on receptor affinity, compared to controls. Replicate experiments, however, demonstrate that in contrast to the effects of 3-MC on the glucocorticoid receptor described above, 3-MC did not have a significant effect on the androgen receptor (\( K_d = 0.2 \pm 0.0 \text{ nM} \); \( B_{\text{max}} = 18 \pm 0 \text{ fmol/mg} \)) compared to controls (\( K_d = 0.2 \pm 0.0 \text{ nM} \); \( B_{\text{max}} = 18 \pm 2 \text{ fmol/mg} \)). The androgen receptor is not present in the adult female rat (17), and was not measured. Taken together, these data suggest that the mechanism by which 3-MC modifies glucocorticoid receptor binding concentrations in vivo following 3-MC administration may be different than that which regulates the androgen receptor. Furthermore, this effect may represent a receptor-selective response.

The mechanism underlying the 3-MC-mediated decrease in glucocorticoid receptor binding concentrations is intriguing. Studies were then continued to explore the role of other constituents which may modify glucocorticoid receptor binding. Our glucocorticoid receptor binding studies routinely included the addition of DTT to the preparation and incubation media since it is thought that DTT and other sulphydryl reducing agents help to stabilize the glucocorticoid receptor (26, 27). A decrease in glucocorticoid receptor binding such as that caused by 3-MC administration may be due to suboptimal concentrations of these reducing agents in vitro. Preliminary analyses of sucrose gradient profiles showed that addition of 5 and 10 mM DTT to hepatic cytosol taken from vehicle or 3-MC-treated animals did not increase the amount of displaceable \( [^{3}H] \) dexamethasone binding to the 5-7S glucocorticoid receptor complex indicating that the DTT concentration in vitro was optimal for dexamethasone binding to the glucocorticoid receptor.

### Table 2

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Number of animals used (n)</th>
<th>Glucocorticoid receptor Bmax (fmol/mg)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn oil control A</td>
<td>4</td>
<td>228 ± 35*</td>
<td>(100)</td>
</tr>
<tr>
<td>3-MC treated</td>
<td>4</td>
<td>204 ± 16*</td>
<td>(92)</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>4</td>
<td>130 ± 12'</td>
<td>(57)</td>
</tr>
<tr>
<td>20 mg/kg</td>
<td>8</td>
<td>260 ± 21</td>
<td>(100)</td>
</tr>
<tr>
<td>Corn oil control B</td>
<td>8</td>
<td>195 ± 16'</td>
<td>(75)</td>
</tr>
<tr>
<td>3-MC treated (40 mg/kg)</td>
<td>8</td>
<td>204 ± 35*</td>
<td>(100)</td>
</tr>
</tbody>
</table>

* Data are expressed as average ± SE.

No significant difference between treated and control groups.

Significant difference (P < 0.05) between control and test groups, using the Student's t test.

### DISCUSSION

The influence of 3-MC and PB on the rat liver cytosolic glucocorticoid receptor in vivo was investigated. Analysis of sucrose gradient profiles and equilibrium binding data revealed that 3-MC treatment significantly decreased the apparent maximal binding capacity (\( B_{\text{max}} \)) of radiolabeled dexamethasone bound to the adult rat hepatic cytosolic glucocorticoid receptor. 3-Methylcholanthrene administration did not significantly influence the apparent equilibrium dissociation binding constant (\( K_d \)) of the glucocorticoid receptor in adult female rats. The effect 3-MC was dose dependent after 2 days and was confirmed using two strains of adult male and female rats. This effect was similar to that reported to be caused by TCDD and selected polychlorinated dibenzofurans (11–13). In addition, the present studies also showed that phenobarbital treatment significantly decreases cytosolic glucocorticoid receptor binding. However, PB administration to adult male and female rats caused significant decreases in \( K_d \) which suggests that this xenobiotic has in addition, the potential of modifying receptor affinity. We also examined the effects of 3-MC on the hepatic cytosolic androgen receptor. The equilibrium binding parameters of displaceable radiolabeled methyltrienolone bound to the hepatic cytosolic androgen receptor were not significantly altered by 3-MC treatment compared to controls which indicates that 3-MC was not
having a general effect on hepatic steroid receptors. The underlying mechanism by which these xenobiotics influence the hormonal binding characteristics of the hepatic glucocorticoid receptor remains speculative but these glucocorticoid receptor changes may reflect some of the early biochemical events in carcinogenesis which may be important in modulating cell proliferation.

There are numerous reports describing decreased glucocorticoid receptor binding concentrations following physiologically and chemically stimulated cell proliferation. Partial hepatectomy, a potent mitogenic stimulus causes acute effects on the cytosolic liver glucocorticoid receptor. Partial hepatectomy causes a time-dependent biphasic response on dexamethasone binding to the hepatic cytosolic glucocorticoid receptor in both intact and adrenalectomized rats (28, 29). In particular, the glucocorticoid receptor binding concentrations rapidly decrease to about 30% of laparatomized controls at 1.5 days postsurgery, followed by a slower recovery to, and augmentation above, control levels at 6 days following 70% hepatectomy. It was also noted that the early decrease in glucocorticoid receptor binding corresponded to the initial period of DNA synthesis following partial hepatectomy when hydrocortisone-stimulated tryptophan oxygenase activities were depressed. The mechanism underlying the 3-MC and PB-mediated decrease in glucocorticoid receptor concentration may be related to mechanisms(s) regulating a similar decrease following partial hepatectomy. In our studies we used intact animals and found that a longer time latency was required before 3-MC or PB treatment could significantly decrease rat liver glucocorticoid receptor concentrations. If the decrease in glucocorticoid receptor levels by 3-MC, PB, and liver regeneration was through the same mechanism, then one would expect a similar time to response profile. This did not seem to be the case, and that perhaps the slower receptor response to 3-MC and PB observed in our studies compared to the relatively rapid effect of partial hepatectomy described above may be due to the formation of active metabolites and/or to a different time course with respect to the induction of hepatic DNA synthesis.

Carcinogens influence the glucocorticoid receptor pathway in both hepatic and nonhepatic tissue. A variety of rat carcinogens including 3-MC, significantly decreased the binding capacity and dissociation constant of the hepatic nuclear glucocorticoid receptor complex in adrenalectomized rats (4). These results would imply that the effects of carcinogens on the glucocorticoid receptor are not entirely dependent on the presence of endogenous glucocorticoids. Using the same dose range of 3-MC as in our study, these workers reported a 60% decrease in both \( B_{\text{max}} \) and \( K_d \) values of the hepatic nuclear-glucocorticoid receptor complex. Collectively, the effects of 3-MC on the cytosolic glucocorticoid and nuclear receptor complexes are similar to the TCDD-mediated decreases in mouse and rat glucocorticoid receptor binding concentrations and nuclear uptake of glucocorticoids (11, 13).

The 3-MC-mediated decrease in liver cytosolic glucocorticoid receptor binding concentrations may be similar to the effects of other xenobiotics on the glucocorticoid receptor in nonhepatic tissue. TCDD causes a significant diminution in rat skeletal muscle glucocorticoid receptor binding concentrations (14). An earlier report by Csaba and Inczefi-Gonda (30) described that benzo(a)pyrene, another carcinogen of the polycyclic aromatic hydrocarbon family and a widely spread environmental pollutant, decreased adult rat thymic glucocorticoid receptor binding in animals treated as neonates or at weaning. It would not be surprising therefore, that the glucocorticoid receptor response to 3-MC may be similar to those of TCDD and related compounds since these xenobiotics also share other similar biochemical responses. Their effects on the glucocorticoid receptor may represent a general phenomenon. Recent studies have shown that TCDD decreased liver cytosolic glucocorticoid receptor binding concentrations in intact and adrenalectomized adult female rats (11). In the latter animals, these effects were not associated with TCDD-stimulated arylhydrocarbon hydroxylase activities. The synthesis of the latter enzyme involves the occupation of an intracellular protein called the Ah receptor (31). The dissociation of the TCDD dose-response between the decrease in glucocorticoid receptor binding levels and induction of arylhydrocarbon hydroxylase activities weakens the hypothesis that the TCDD-mediated effects on the glucocorticoid receptor directly involves the Ah receptor. The present studies further examine this mechanism by comparing the glucocorticoid receptor responses following 3-MC or PB treatment. Phenobarbital is a structurally different xenobiotic compared to 3-MC or TCDD.

3-Methylcholanthrene and TCDD but not phenobarbital bind to the Ah receptor (32). If the 3-MC-mediated decrease in glucocorticoid receptor is solely mediated by an Ah receptor mechanism, then PB should not invoke a decrease in glucocorticoid receptor levels. Surprisingly, PB significantly decreased glucocorticoid receptor binding concentrations in vivo and would support the hypothesis that the 3-MC-mediated effects on the glucocorticoid receptor cannot be explained exclusively by the occupation of the Ah receptor. It is also likely that several distinct mechanisms lead to a decrease in glucocorticoid receptor binding concentrations.

It is interesting to note that PB-mediated decrease in glucocorticoid receptor binding concentrations is similar to the decrease in mouse epidermal glucocorticoid receptor binding concentrations following repetitive topical application of the potent tumor promoter TPA (16). Since TPA is a potent activator of protein kinase C, perhaps protein kinase C may be involved in modifying the glucocorticoid receptor binding. It would seem therefore, that despite the vast apparent differences in molecular structure, potent pleiotropic agonists cause similar effects on the glucocorticoid receptor. Alterations in the regulatory mechanisms governing cell proliferation, including the glucocorticoid receptor system may help explain why potent growth stimuli such as partial hepatectomy, PB, and TCDD influence rat hepatocarcinogenesis. Kraujalis et al. (3) reported a decrease in cytosolic glucocorticoid receptor binding content in diethyl-nitrosamine-induced rat hepatocarcinogenesis, an effect which was associated with a twofold decrease in this receptor’s binding to chromatin and agarose-DNA. In addition, this response was negatively correlated with the hepatoma growth rate. Whether the mechanism underlying the response of the glucocorticoid receptor to a variety of tumor initiators and promoters is involved during chemically induced rat hepatocarcinogenesis remains speculative.

Carcinogens and hepatic tumor promoters may influence the glucocorticoid receptor at different steps along this receptor’s response pathway (2). The possibility that 3-MC, PB, and/or their metabolites may bind to the glucocorticoid receptor, thereby leading to an apparent decrease in \([^{3}H]\)dexamethasone binding in vitro seems unlikely due to their apparent differences in molecular structure compared to that of the glucocorticoid ligand (also see “Results”). Our HPLC studies show that dexamethasone is not metabolized in vitro by cytosols taken from vehicle-treated control, 3-MC, or PB treated animals. These results would suggest that the 3-MC-mediated decrease in glucocorticoid receptor binding concentrations in vivo was probably not due to a decrease in the free radiolabeled dexametha-
sonic concentration in vitro. Furthermore, addition of protease inhibitors to the cytosol did not increase the glucocorticoid receptor binding concentrations obtained from the 3-MC treated rats towards those of the control rats indicating that general proteases were not liberated into the cytosolic mixture. This conclusion is supported by the evidence that 3-MC treatment did not significantly alter the hepatic cytosolic androgen receptor binding properties.

The exact mechanism of how carcinogens such as 3-MC and hepatic tumor promoters such as TCDD and PB can modify glucocorticoid receptor function remains an enigma. Further studies are being carried out in our laboratory to examine the role of endogenous factors (sulfhydryl reducing compounds such as glutathione, thioredoxin, etc.) as well as the glucocorticoid receptor mRNA (1, 2, 26) which may modify the glucocorticoid receptor function during hepatocarcinogenesis. Such possibilities open new vistas of research on how the glucocorticoid receptor system may be involved during carcinogenesis.

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REFERENCES

Characterization of 3-Methylcholanthrene Effects on the Rat Glucocorticoid Receptor in Vivo

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