Phorone (diisopropylidene acetone), a glutathione depleter, decreases rat glucocorticoid receptor binding in vivo

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The exact mechanism by which carcinogens and tumor promoters act on the glucocorticoid receptor system in vivo is not known. Based on earlier studies that sulfhydryl-reducing agents stabilize glucocorticoid receptor binding in vitro, some workers have postulated that endogenous reducing factors may be important for glucocorticoid receptor function in vivo. To test whether glutathione (GSH) may serve this purpose, we investigated the effects of phorone, an agent that partially depletes intracellular GSH, on the hepatic cytosolic glucocorticoid receptor (GRc) binding characteristics in intact and 7–10 day adrenalectomized (ADX) adult female Sprague–Dawley rats. Biochemical analysis revealed that a single treatment of phorone (300 mg/kg) to both intact and ADX rats significantly decreased the liver GSH concentration (70–90% of control levels) as well as the GRc maximum binding concentration (30% of control levels). The decrease in GSH levels preceded the reduction in GRc maximum binding concentrations; both effects were reversible after 24 h of treatment. The phorone-mediated decrease of GSH levels was maximum at doses > 75 mg/kg, whereas GRc maximum binding concentrations in vivo appeared dose dependent up to 400 mg/kg. Pretreatment with phorone or the carcinogens mirex and 3-methylcholanthrene significantly decreases GRc binding and nuclear uptake in vivo, as well as diminishes intracellular cytosolic GSH levels. Although a temporal relationship between the GSH levels and the GRc maximum binding concentrations in vivo was observed, there was no quantitative relationship between these two parameters based on our phorone dose–response and the carcinogen pretreatment data. Our findings suggest that during the early phases of carcinogenesis, the hepatocellular GSH does not play a direct role upon the biochemical action of certain carcinogens and tumor promoters on the glucocorticoid receptor binding in the liver.

Introduction

Functional alterations of the glucocorticoid receptor pathway have been associated with early biochemical events during carcinogenesis (1–3). There is considerable evidence that glucocorticoid receptor concentrations are decreased in vivo and in vitro during cell proliferation and following exposure to carcinogens (4–11). It has been recently shown that animals treated with carcinogens such as 3-methylcholanthrene (3-MC*), and co-carcinogens such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and phenobarbital have significantly lower liver cytosolic glucocorticoid receptor maximum binding concentrations compared to controls (2,3). The mechanism underlying how carcinogens and co-carcinogens alter glucocorticoid receptor binding in vivo is not known.

A number of different endogenous factors may modify the glucocorticoid receptor pathway during carcinogenesis (1). The glucocorticoid receptor system has been shown to be sensitive to tumor promoters such as 12-O-tetradecanoylphorbol-13-acetate and mezerein in the mouse skin tumorigenesis model (12). Using this model, others have reported that topically applied glutathione (GSH) inhibited, whereas the treatment with a GSH-depleter diethylmaleate enhanced, murine skin tumorigenesis (13). It was therefore suggested that GSH levels may be important in cancer chemoprevention.

GSH, a ubiquitous tripeptide (γ-L-glutamyl-L-cysteinylglycine) plays a part in the protective mechanism against toxic chemicals and normal oxidative products of cellular metabolism. It acts as a nucleophilic 'scavenger' of different compounds and their metabolites. The GSH pathway is well known for the detoxification of many different carcinogens and co-carcinogens, as well as for other important physiological functions (14–20). It was suggested that intracellular depletion of GSH may predispose the cell to xenobiotic-induced injury and oxidative stress (14). In fact, the role of GSH has been implicated in the molecular mechanism of the prooxidant states of carcinogenesis and tumor promotion (21). Since GSH and other sulfhydryl-reducing reagents are also well known to stabilize glucocorticoid receptor binding in vitro, the possibility of a similar action by endogenous reducing substances in vivo has been proposed (22). We re-evaluated this hypothesis, focusing on endogenous GSH, and wondered whether alterations in its pathway may also be part of the down-regulatory response of the glucocorticoid receptor to certain carcinogens and co-carcinogens in vivo.

For this purpose, we investigated the effects of phorone (diisopropylidene acetone; 2,6-dimethyl-2,5-heptadiene-4-one), a compound that depletes tissue GSH in vivo and in vitro (17,23–26), on hepatic glucocorticoid receptor binding and nuclear uptake in vivo. The effect of phorone on the glucocorticoid receptor was then compared to carcinogens that are known to modify glucocorticoid receptor binding in vivo, such as 3-MC and the chlorinated hydrocarbon pesticide mirex (1,3,4-methenododecachloro-octahydro-2H-cyclobuta[e,d] pentalen).

Materials and methods

Chemicals

[1,2,4,6,7-3H]Dexamethasone (40–50 Ci/mmol, radiochemical purity >96%) was obtained from Amersham International plc (Little Chalfont, Buckinghamshire, UK). Orphiforme Hi-safe II scintillation fluid was bought from Pharmacia-pdf (Dübendorf, Switzerland). Mirex (purity >98% by GC) was obtained from Riedel-de Haen (Germany). All other chemical reagents were obtained either from Sigma Chemical Co. (St Louis, MO) or from Fluka Chemical AG (Buchs, Switzerland). All reagents and solvents were of the highest grades of purity.

*Abbreviations: 3-MC, 3-methylcholanthrene; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; GSH, glutathione; MCT, medium-chain polysaturated triglycerides; ADX, adrenalectomized; DCC, dextran-coated charcoal; GSSG, oxidized glutathione; OPT, o-phthalaldehyde; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; NSB, non-specific binding; DEM, diethyl maleate; GGT, γ-glutamyltranspeptidase; TAT, tyrosine aminotransferase.
available. Glass-distilled water was used for the preparation of all aqueous solutions. Care was taken to avoid the possible effects of peroxides, which may be present in the oil used for autoclaving. The chemical and biochemical parameters of our studies. Analysis indicated that the commercially available synthetic mixture of medium-chain triglycerides (MCT) containing 55–60% C8:0, 35–40% C10:0 and 1–3% C6:0 (Dynavit Noble AG, Germany) had a low peroxide potential of 0.31 meq O2/kg oil and that of commercially available corn oil was 4.3 meq O2/kg oil.

Animals and treatments

Our animal studies were conducted in compliance with the Ethical Guidelines for Animal Experimentation of the Medical Faculty of the University of Groningen (20) and approved by the Cantonal Veterinary Service. Adult female Sprague–Dawley rats (250–300 g; age 70–80 days old) were purchased from Iffa-Credo SA (L’Arbresle, France) and housed in groups of two in plastic cages with autoclaved wood shavings (Laboxex, Scieries des Éplatures, La Chaux-de-Fonds). Environmental conditions were: temperature, 23 ± 2°C; relative humidity, 55 ± 10%; light cycle, on 08:00; off 20:00. In the studies involving 7–10 day adrenalneuterized (ADX) adult animals, surgery was performed by the supplier – 4–5 days before shipment to our facilities; animals were then allowed to acclimatize for another 3–5 days prior to the experiment. All animals were fed ad libitum Nafag 890 rat chow (Nafag SA, Switzerland) and tap water (for the intact) or that containing 0.5% NaCl and 10% glucose (for the ADX animals).

On the day of the experiment, animals were treated i.p. with different doses of phorone (75–400 mg/kg) 3 h prior to being killed, unless otherwise stated. Control animals received an equal volume of the MCT vehicle. These treatments did not significantly alter the liver weights (absolute or relative to body weights, L/BW) compared to vehicle-treated control animals, except in the animals receiving the top dose, which otherwise appeared normal (L/BW of control group: 4.1 ± 0.3 g/100 g body wt; phorone-treated, 400 mg/kg: 3.3 ± 0.2). This effect was not due to differences in tissue water content since dry liver weights in treatment and control groups were similar (data not shown).

In other experiments, animals were treated with 3-MC (40 mg/kg, gavage daily for 2 days), or a single dose of mixe (100 mg/kg, gavage) or the equivalent volume of the corn oil vehicle. 3-MC-treated animals were killed 18–20 h following the last treatment, whereas mixetreated animals were killed 48 h after gavage. These treatment schedules have been shown to decrease glucocorticoid receptor binding in vivo (2,27,28). All animals were killed according to accepted methods, using CO2/O2 anesthesia followed by exsanguination. In some studies, blood samples were used for biochemical analysis. The livers were immediately dissected, scissors minced, weighed and placed in the appropriate ice-cold buffer solution.

For the glucocorticoid receptor nuclear uptake in vivo experiments, ADX animals were injected i.p. with a 1% carboxymethylcellulose suspension containing 10 μCi of [3H]dexamethasone alone or supplemented with a 1000-fold molar excess non-labelled dexamethasone as described by others (29). Accordingly, animals were killed 30 min later, when dexamethasone uptake in vivo was maximal (29).

Hepatic subcellular fractionation

Hepatic cytosols were prepared using differential centrifugation techniques, following a method described earlier (2,3). All manipulations were carried out at 4°C. For the preparation of cytosol, livers were homogenized in a TEDGM buffer (10 mM Tris–HCl, 2.5 mM EDTA, 5 mM DTT, 10% glycerol, 20 mM sodium molybdate, pH 7.4) using a motor-driven Teflon pestle and a Potter–Elvehjem homogenizing tube. The homogenate was centrifuged at 10 000 g for 10 min, and the resulting supernatant was then resuspended at 100 000 g for 65 min. The clear supernatant (containing cytosol) was decanted and frozen at −80°C until analysis.

In vivo nuclear glucocorticoid receptor uptake assay

To determine whether alterations in cytosolic glucocorticoid receptor binding influences nuclear uptake of glucocorticoid in vivo, ADX animals were treated with [3H]dexamethasone 30 min prior to being killed, and hepatic nuclei were isolated using a discontinuous sucrose gradient centrifugation procedure (3). In brief, 3 g of liver minces were gently homogenized in 25 ml of TCMD buffer (20 mM Tris–HCl, 2 mM CaCl2, 1 mM MgCl2, 0.1 mM DTT, pH 7.4) containing 250 mM sucrose, using a douche-type homogenizing tube and a motor-driven Teflon pestle. The homogenate was filtered through a monofilm high-capacity nylon filter (mesh size 150 μm; ZBF, Zurich Bolting Cloth Mfg Co., Switzerland) and centrifuged at 800 g for 10 min. The supernatant was decanted and the pellet suspended with 10 ml of 3M sucrose (pH 7.4) by using a hand-held homogenizer. This mixture was then underlaid beneath 15 ml of TM buffer (containing no sucrose) in ultracentrifuge tubes. The discontinuous sucrose gradient was centrifuged at 90 000 g for 60 min using a fixed-angle rotor, and the resulting pellet (containing nuclei) was carefully resuspended in 10 ml of 250 mM sucrose in TCMD buffer using a small glass pipette. Aliquots of this mixture were analyzed for nuclear incorporation of radioactivity and for DNA content. Glucocorticoid receptor nuclear uptake activities were expressed as nmol dexamethasone/g DNA.

Glucocorticoid receptor binding assay

Cytosolic glucocorticoid receptor binding studies were carried out in vivo under equilibrium conditions as described earlier (2,3) using different concentrations of [3H]dexamethasone (final concentrations: 0.05–4 nM) in the presence (non-specific binding) or absence (total binding) of non-labelled dexamethasone (1000-fold molar ratio excess) as the competitor, unless otherwise stated. Preliminary studies indicated that 1000-fold molar excess of non-radioactive phorone, mirex and 3-MC did not displace [3H]dexamethasone in vitro (data not shown) and suggested that these xenobiotics did not occupy the glucocorticoid receptor in vitro. Alterations in receptor specific binding were therefore due to treatment in vivo. All incubations were done overnight at 4°C, before treatment with 3.75% (w/v) dextan coated charcoal (DCC) and centrifugation at 1000 r.p.m. to separate the bound and free fractions. The DCC-resistant supernatant was analyzed for radioactivity (efficiency 40–50%). The amount of [3H]dexamethasone displaced (or specific binding) was calculated as the difference between total and non-specific bound fractions for each cytosolic preparation. Equilibrium binding data were analyzed using Scatchard coordinates (30) and the linear descending curve of the Scatchard plot was fitted according to the least-squares method for linear regression. The apparent equilibrium dissociation constant (Kd) and apparent maximum binding capacity (βmax) were expressed as nm and fmol of cytosolic protein respectively. Protein content was determined following the procedure described by Bradford (31), and using BSA as the standard.

Other assays and statistical analysis

Hepatic reduced glutathione and oxidized (GSSG) glutathione concentrations were analyzed according to a fluorimetric method (32), using authentic GSH and GSSG as standards and d-phenylaldehyde (OPT) as the fluorescent reagent. Nuclear DNA content was determined by the fluorimetric method of Downs and Wilfinger (33) with calf thymus DNA as the standard. In other experiments, plasma hepatic enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using the Cobas Bio automated centrifugal analyzer (Roche) at 340 nm, using kits purchased from Roche (Basel, Switzerland). These enzyme activities were expressed as U/l, where one unit is the amount of NADH (44 μmoles per min) oxidized at 37°C in vitro.

Data were expressed as the mean ± SEM, unless otherwise stated. Differences between control and test groups were analyzed using Student’s t-test for unpaired data, and 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 has been well recognized that sulphydryl groups are essential for adequate hormone binding to the cytosolic glucocorticoid receptor in vitro, as evidenced by adding sulphydryl-modifying reagents such as dithiothreitol, β-mercaptoethanol, GSH or similarly acting compounds to the incubation mixture. To test whether intracellular cytosolic GSH may also modify the function of glucocorticoid receptor in vitro, time-course and dose–response studies were carried out using intact or 7–10 day ADX adult female rats treated with phorone, a hepatic GSH-depleting agent (17,23–25).

Effect of phorone on hepatic GSH levels and glucocorticoid receptor binding concentrations in vivo

A series of studies were performed to test the possibility that intracellular cytosolic GSH may have a direct role in modifying glucocorticoid receptor binding in vivo. As a first attempt, intact and 7–10 day ADX adult female rats were injected i.p. with phorone (300 mg/kg) and were killed 3 h after treatment. This dose was chosen since it is not hepatoxic and it is known to diminish hepatic GSH content by >90% (23). Phorone treatment significantly decreased hepatic GSH concentrations by >70% compared to the vehicle-treated controls in both intact (vehicle-treated controls = 1.39 ± 0.02 mg/g tissue; phorone-treated 0.24 ± 0.01) and 7–10 day ADX adult female rats (vehicle-treated controls 0.83 ± 0.02; phorone-treated 0.28 ± 0.01; data not shown). The depletion of GSH by phorone treatment in vivo was consistent with other reports (23,25). It was interesting to note that in the vehicle-treated animals, 7–10 day adrenalectomy...
Intracellular glutathione and glucocorticoid receptor

Table I. Effects of phorone, mirex and 3-MC on hepatic glutathione and the glucocorticoid receptor in vivo

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Phorone</th>
<th>Control</th>
<th>Mirex</th>
<th>Control</th>
<th>3-MC</th>
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<tr>
<td>Relative liver weight (g/100 g body weight)</td>
<td>3.49 ± 0.08 (16)</td>
<td>3.32 ± 0.11 NS (16)</td>
<td>3.92 ± 0.10 (14)</td>
<td>4.07 ± 0.13 NS (14)</td>
<td>3.58 ± 0.17 (6)</td>
<td>3.86 ± 0.11 NS (6)</td>
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<tr>
<td>Glutathione content (mg/g tissue)</td>
<td>0.95 ± 0.04 (12)</td>
<td>0.17 ± 0.01 NS (12)</td>
<td>1.16 ± 0.06 (14)</td>
<td>0.60 ± 0.05 NS (14)</td>
<td>1.16 ± 0.09 (6)</td>
<td>0.89 ± 0.11 NS (6)</td>
</tr>
<tr>
<td>Dexamethasone uptake (nmol/g DNA)</td>
<td>0.36 ± 0.05 (13)</td>
<td>0.25 ± 0.02 NS (16)</td>
<td>0.31 ± 0.03 (14)</td>
<td>0.23 ± 0.01 NS (9)</td>
<td>0.26 ± 0.02 (5)</td>
<td>0.19 ± 0.01 NS (6)</td>
</tr>
<tr>
<td>Glucocorticoid receptor binding (fmol/mg protein)</td>
<td>497.0 ± 44 (8)</td>
<td>333.0 ± 13 NS (4)</td>
<td>627.0 ± 33 (4)</td>
<td>481.0 ± 26 NS (4)</td>
<td>514.0 ± 39 (8)</td>
<td>425.0 ± 28 NS (8)</td>
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Seven to 10 day ADX adult female Sprague-Dawley rats received treatments of phorone (300 mg/kg, i.p., 3 h), mirex (100 mg/kg, gavage, for 1 day), 3-MC (40 mg/kg/day, gavage, daily for 2 days) or an equivalent volume of the control oil vehicle. Thirty minutes prior to being killed, animals received an i.p. injection of 10 μCi [3H]dexamethasone. Crude hepatic nuclei were isolated and radioactivity was analyzed; tissue glutathione and DNA concentration were determined, as described in Materials and methods. Simultaneous injection of a 1000-fold excess molar ratio of unlabeled dexamethasone inhibited the uptake of radiolabeled dexamethasone by ~90%, suggesting that nuclear uptake in vivo was mediated by the glucocorticoid receptor. Radioreceptor binding assays were done on liver cytosol from animals pretreated with phorone, mirex, 3-MC or appropriate vehicle as described in Materials and methods. Data are expressed as the mean ± SEM; number of animals in parentheses. *Denotes statistically significant compared to controls (P < 0.05); NS, not significantly different.

Fig. 1. Representative binding isotherms showing the effect of phorone on the intact (circles) and 7—10 day ADX (squares) adult female rat liver cytosolic glucocorticoid receptor binding in vivo. Intact animals and ADX animals were treated with the MCT vehicle (open symbols) or phorone (filled symbols; 300 mg/kg) and were killed 3 h later. Liver cytosol (0.8 mg of protein) in TEDGM buffer was incubated in the presence of varying concentrations of [3H]dexamethasone for 18—20 h at 4°C, as described in Materials and methods. The specific bound fraction was calculated as the total bound (no competitor added) minus the non-specific bound, NSB (1000-fold excess competitor added in parallel) fractions. The figure shows Scatchard plots of the specific binding data shown in the inset. Linear regression analysis revealed the apparent equilibrium binding parameters (K_d expressed as nM, B_max expressed as fmol/mg protein) in the following treatment groups: MCT-treated intact animal (open; 0.9, 258), phorone-treated intact animal (filled; 1.1, 167), MCT-treated ADX animal (open; 0.9, 552), phorone-treated ADX animal (filled; 0.8, 354). For simplicity, only the NSB of the MCT-treated control animal (X) is shown; that of the phorone is almost superimposable over the controls.

Time-dependent effects of phorone on glucocorticoid receptor binding in vivo

Dose—response and time-course studies were then performed to test further the hypothesis that intracellular GSH has a direct role in regulating glucocorticoid receptor function in vivo. Intact adult female rats were injected i.p. with 300 mg/kg phorone or an equivalent volume of the vehicle; the animals were killed at different time periods after treatment. Figure 2 summarizes the results of two studies. Phorone treatment rapidly and significantly lowered GSH levels compared to their intact counterparts.

Radioreceptor studies using [3H]dexamethasone indicated that glucocorticoid receptor binding was specific and saturable (Figure 1, inset), consistent with our earlier reports (2,3); non-specific binding was linear and represented ~5—10% of total dexamethasone binding. Figure 1, which shows the Scatchard plots of the equilibrium binding data, indicates that in the vehicle-treated animals, 7—10 day post-adrenalectomy increased the glucocorticoid receptor B_max by ~2-fold above that of an intact animal (B_max, intact animal = 258 fmol/mg protein; ADX animal = 552 fmol/mg protein). Phorone treatment decreased glucocorticoid receptor B_max by ~36% in both the intact (167 fmol/mg protein) and the ADX animals (354 fmol/mg protein) compared to their respective vehicle-treated controls. Phorone treatment did not seem to influence the equilibrium dissociation constant of the receptor compared to controls. Other experiments were performed to confirm and characterize further the phorone effects on the hepatic cytosolic glucocorticoid receptor equilibrium binding parameters in vivo. Replicate experiments confirmed that in the intact adult female rat, phorone treatment caused a significant decrease (>21%) in the cytosolic glucocorticoid receptor B_max compared to vehicle-treated controls (242 ± 18 fmol/mg protein, n = 6; phorone treated group = 191 ± 5; n = 6; data not shown). On the other hand, phorone treatment had no significant effects on the receptor equilibrium dissociation constant compared to controls (K_d of control group = 0.8 ± 0.1 nM; phorone-treated group = 1.1 ± 0.2 nM). In the ADX animals, phorone treatment significantly decreased the receptor B_max concentrations (~33%; Table I) as well as the equilibrium binding constant (K_d in nM: ADX control group = 0.9 ± 0.1; phorone-treated group = 0.6 ± 0.1; data not shown). These data suggest that the effects of phorone characterized by decreased intracellular GSH and cytosolic glucocorticoid receptor binding are not dependent upon the adrenal status.
decreased GSH levels as early as 1 h post-treatment. This effect remained constant for 3 h, after which GSH levels increased between 6 and 24 h post-treatment. Similar effects were observed after 3 h post-treatment on hepatic cytosolic GSSG concentrations (determinations at 1 and 2 h post-treatment were not done).

Scatchard analysis of the glucocorticoid receptor equilibrium binding data indicated that phorone treatment caused a marked and significant decrease (30–40%) in the $B_{\text{max}}$ concentration of the glucocorticoid receptor as early as 2 h compared to the vehicle-treated controls, which were killed in parallel to the treated group (Figure 2). This observation followed the earlier effects of phorone on GSH concentrations (as described above), in that the receptor $B_{\text{max}}$ concentrations remained maximally decreased for up to 6 h post-treatment. After 24 h, however, phorone treatment did not have a significant effect on glucocorticoid receptor binding levels compared to controls. Phorone treatment also did not have a significant effect on the $K_d$ values of the receptor compared to controls ($K_d$ 0.8 nM), except at 6 h after treatment ($K_d$ 0.7) when a significant decrease was observed. The phorone-mediated reversible decrease in intracellular GSH concentrations preceding the diminution of glucocorticoid receptor $B_{\text{max}}$ concentrations in vivo lends support to the possibility that intracellular GSH may be associated with altered glucocorticoid receptor binding function in vivo. To confirm further this possibility, phorone dose–response studies were carried out.

**Phorone dose–response studies**

The dose-dependent effects of phorone on GSH and glucocorticoid receptor binding were studied using animals treated i.p. with different doses ranging from 75 to 400 mg/kg and killed 3 h post-treatment (Figure 3). Our time-course studies indicated that 3 h post-treatment offered maximal phorone effects on GSH and glucocorticoid receptors without causing significant changes

Fig. 2. Time course of phorone effects on intact adult female rat liver glutathione concentrations and glucocorticoid receptor binding in vivo. Animals were treated with a single dose of phorone (300 mg/kg, i.p.) and were killed at different periods following treatment. In parallel, control animals received an equal volume of the vehicle. Preparation of liver tissue and glucocorticoid radioreceptor binding assays were done as described in Materials and methods. Hepatic reduced glutathione concentrations (GSH, open bars) and oxidized glutathione concentrations (GSSG, hatched bars) were expressed as mg/g tissue, whereas $B_{\text{max}}$ values for the cytosolic glucocorticoid receptor (solid bars) were expressed as fmol [3H]dexamethasone/mg protein. •Significant difference ($P < 0.05$) compared to the control group at the corresponding time of being killed; ns, no significant difference versus controls.

Fig. 3. Dose–response effects of phorone on intact adult female rat liver glutathione (GSH) concentrations and hepatic cytosolic glucocorticoid receptor binding in vivo. Animals were treated with different doses of phorone (75–400 mg/kg, i.p., 3 h) or an equal volume of the vehicle. Preparation of liver tissue and glucocorticoid radioreceptor binding assays were done as described in Materials and methods. Equilibrium binding parameters of the cytosolic glucocorticoid receptor (GRc) binding concentrations were estimated using linear regression, with $K_d$ values in the range of 0.7–0.9 nM. GRc maximum binding concentrations (•) were expressed as fmol [3H]dexamethasone/mg protein and GSH concentrations (O) were as mg GSH/g tissue. *Significant difference ($P < 0.05$) compared to the control group.
on liver weights (described above). While the effect on GSH concentrations was apparently maximum at phorone doses > 75 mg/kg with experimental variability at the 150 mg/kg dose, the glucocorticoid receptor binding concentrations continued to decrease at doses up to 400 mg/kg. The dissociation constant of the receptor was not significantly altered by treatment.

Effects of 3-MC and mirex on the GSH and the glucocorticoid receptor

The effects of phorone on the glucocorticoid receptor pathway were compared to those of carcinogens known to influence glucocorticoid receptor binding in vivo. If compounds such as phorone, mirex and 3-MC decrease cytosolic glucocorticoid receptor maximum binding concentrations in vivo (our results shown above; 2,27,28), then a concomitant decrease in glucocorticoid receptor nuclear uptake in vivo would be expected and a physiological consequence of altered cytosolic glucocorticoid receptor binding would be illustrated. For this purpose, nuclear uptake of radiolabeled glucocorticoid and tissue GSH levels were measured in animals pretreated with phorone, mirex or 3-MC.

ADX adult female rats were treated with a single dose of either phorone (300 mg/kg, i.p., 3 h), mirex (100 mg/kg, gavage, 48 h) or an equivalent volume of vehicle. Mirex treatment did not significantly alter plasma hepatic enzymes such as ALAT and ASAT compared to the vehicle-treated ADX controls (controls: ALAT = 82 ± 8 U/l, ASAT = 155 ± 12; mirex-treated: ALAT = 80 ± 8, ASAT = 145 ± 12), confirming earlier results that this mirex dose was not hepatotoxic (34,35). It was interesting to note that mirex also had no significant effect on liver weights compared to controls (Table I), consistent with the results of Erwin and Yarbrough (35) but in contrast to those reported by Thottassery and Yarbrough (28).

In replicate experiments, phorone consistently and significantly decreased nuclear glucocorticoid receptor uptake in vivo compared to vehicle-treated controls (Table I). In one of these experiments, animals were also treated with a 1000-fold molar excess of dexamethasone competitor in order to estimate the extent of non-specific binding (NSB). Non-specific nuclear uptake of \(^{1}H\)dexamethasone in vivo represented ~10% of total binding in both vehicle-treated control and phorone-treated animals (control ADX rats: not treated with competitor = 0.36 ± 0.05 nmol/g DNA, co-treated with competitor = 0.04 ± 0.01; phorone-treated ADX rats: not treated with competitor = 0.25 ± 0.02, co-treated with competitor = 0.02 ± 0.00). These results could not be explained by alterations in DNA content since the levels were similar between control and treated groups (data not shown) or by occupation of the glucocorticoid receptor (36). A decrease in glucocorticoid nuclear uptake would be consistent with the previously described effects of phorone on the \(B_{\text{max}}\) concentration of cytosolic glucocorticoid receptor (Figures 1–3).

Analysis of the cytosolic preparation taken from 7–10 day ADX adult female rats treated with a single dose of mirex (100 mg/kg, gavage, 48 h) demonstrated that, like phorone, mirex treatment significantly decreased liver GSH concentrations and glucocorticoid receptor nuclear uptake compared to controls (Table I). We observed that the glucocorticoid receptor nuclear uptake in vivo was decreased in mirex-treated compared to controls, an effect that could not be explained by alterations in DNA content (data not shown). In a separate study (Table I), we confirmed the earlier results of Thottassery and Yarbrough (33) that mirex significantly decreased cytosolic glucocorticoid receptor \(B_{\text{max}}\) concentrations in mirex-treated compared to corn oil-treated controls. Taken together, the decrease in glucocorticoid receptor nuclear uptake would be explained by a decrease in its \(B_{\text{max}}\) concentration according to the classical action of steroid hormone receptors.

The carcinogen 3-MC-mediated decrease of cytosolic glucocorticoid receptor binding concentrations in vivo is also associated with alterations in glucocorticoid receptor nuclear uptake and with a decrease in intracellular GSH concentrations. As shown in Table I, 3-MC treatment significantly decreased liver glucocorticoid receptor concentrations in 7–10 day ADX adult female rats, confirming earlier reports (2,4). Also, 3-MC treatment significantly decreased nuclear uptake of \(^{1}H\)dexamethasone in vivo. Cytosolic GSH concentrations were significantly decreased compared to vehicle-treated controls, and was consistent with the report of Docks and Krishna (37).

Discussion

The present studies evaluate the possibility that the binding of the hepatic cytosolic glucocorticoid receptor to its ligand leading to the subsequent nuclear translocation of the receptor—ligand complex in vivo may be associated with the changes in intracellular GSH content. Evidence is provided which demonstrates that depletion of hepatic GSH content is accompanied by a decrease in both liver cytosolic glucocorticoid receptor \(B_{\text{max}}\) concentrations and glucocorticoid receptor nuclear uptake in vivo. The decrease in GSH levels preceded the decrease in the receptor \(B_{\text{max}}\) concentrations. Pretreatment with the carcinogens mirex and 3-MC also decreased GSH content and glucocorticoid receptor function in vivo. Despite an apparent qualitative association, quantitative analysis failed to demonstrate a clear relationship between hepatocellular GSH levels and neither glucocorticoid receptor binding nor nuclear uptake in the different models tested.

Phorone is an \(\alpha,\beta\)-unsaturated compound which by combining with GSH through the action of glutathione S-transferase leads to a 90% depletion of hepatic intracellular GSH concentrations (23). This agent was used in our studies since other \(\alpha,\beta\)-unsaturated carbonyl compounds, such as diethyl maleate (DEM) which also depletes hepatic GSH by a similar mechanism, has a number of secondary and undesirable effects including decreased activity of some liver microsomal drug metabolizing enzymes and stimulation of lipid peroxidation in vivo (17,38). Furthermore, in contrast to phorone, DEM inhibits protein synthesis in different tissues including liver in vivo (39). Since the presence of sulphydryl-protecting agents such as GSH is known to stabilize glucocorticoid receptors in vitro (22), we tested the possibility that GSH may have a similar action on this receptor in vivo. For this purpose, phorone was used as a tool to decrease hepatic cytosolic GSH concentrations in vivo and to relate this effect to the liver cytosolic glucocorticoid receptor equilibrium binding parameters and glucocorticoid receptor nuclear uptake in vivo.

Phorone decreased both hepatic cytosolic GSH concentrations and the glucocorticoid receptor \(B_{\text{max}}\) concentrations in intact animals. A similar response was found in the 7–10 day ADX animals, indicating that the decrease in GSH and glucocorticoid receptor binding was not dependent upon the adrenal status. The decrease in glucocorticoid receptor binding in vivo was accompanied by a reduction in the nuclear uptake of the glucocorticoid receptor in vivo (Table I), as would be expected according to the classical theory of glucocorticoid receptor action.

An unexpected finding was that 7–10 day adrenalectomy significantly decreased GSH levels by 40% compared to the intact controls and would suggest that the GSH content may be
influenced by the adrenal status. Although further study is required, this observation may be explained by a decrease in glutathione peroxidase (GPx) activity following adrenalectomy (40), which might then lead to decreased GSH transport and amino acid uptake, and subsequently to a lowering of intracellular GSH concentrations.

Time-course studies indicated that phorone caused a rapid decrease in hepatic GSH concentrations with significant effects as early as 1 h post-treatment, followed by a reversal to control levels by 24 h (Figure 2). These results are consistent with an earlier report (23). Analysis of the equilibrium binding parameters of the hepatic glucocorticoid receptor indicated that phorone significantly decreased glucocorticoid receptor binding in vivo as early as 2 h post-treatment, an effect that was also reversible after 24 h. These data demonstrate the temporal relationship between the decrease in GSH levels and the decrease in glucocorticoid receptor binding in vivo which follows. A possible cause—effect relationship is further supported by the reversibility of phorone effects on these two parameters. Although phorone caused a significant and consistent decrease in the $B_{\text{max}}$ concentrations of the glucocorticoid receptor, this treatment also upon occasion affected the equilibrium dissociation constant ($K_d$) of the receptor (3 and 6 h post-treatment in ADX and intact animals respectively). Overall, a phorone-mediated decrease in glucocorticoid receptor nuclear uptake in vivo can be better explained by diminished levels of cytosolic glucocorticoid receptor than by transient alterations in receptor affinity. It is doubtful that a phorone-mediated alteration in receptor affinity would induce a new subpopulation of glucocorticoid receptors. Nevertheless, under normal physiological conditions, it would be assumed that these glucocorticoid receptors would be occupied, and therefore the effects of phorone would still be predominantly upon the number of receptors available for ligand binding.

Dose—response studies confirm that the phorone treatment causes a decrease in hepatic GSH levels without obvious liver toxicity. This effect was maximal at doses $> 75$ mg/kg i.p. (Figure 3). Phorone treatment caused a dose-dependent decrease in glucocorticoid receptor binding, but in contrast to its effect on GSH, higher doses of phorone are probably required to decrease to a minimum the receptor binding levels. Further analysis failed to demonstrate a quantitative relationship between decreased GSH content and diminished glucocorticoid receptor binding.

We have also shown that carcinogens such as 3-MC and mirex decrease glucocorticoid receptor equilibrium binding concentrations in vivo, consistent with previous studies (2,8,27,28). Furthermore, this effect is accompanied by decreased receptor nuclear uptake (Table I), which would be expected according to classical theory and confirms the physiological importance of our radioreceptor binding data. The mechanism of this action for these two chemically distinct compounds on the glucocorticoid receptor is not known.

Mirex, a formerly used organochloride insecticide and flame retardant, has potent carcinogenic effects in mice and rats (41,42). It is a potent stimulant of hepatocyte proliferation in vivo as well as microsomal drug metabolizing enzyme activities (36,41,43–45). It is not a mutagen using Ames tester strain—microsome assays nor does it induce sister chromatid exchange or chromosomal aberrations in Chinese hamster ovary assays (42). Mirex decreased hepatic cytosolic glucocorticoid receptor binding concentrations in both intact and ADX rats (27). Recently, Thottassery and Yarbrough (28) demonstrated that this decrease in glucocorticoid receptor concentration was not accompanied by an alteration on glucocorticoid induced tyrosine aminotransferase (TAT) activities. Since the expression of TAT activity may be regulated by numerous factors, we re-examined the action of mirex on the glucocorticoid receptor in comparison to phorone and 3-MC. This involved investigating biological sequelae more proximal to the occupation of the cytosolic glucocorticoid receptor in vivo than distal effects such as TAT gene activation. It was interesting to note that phorone, mirex and 3-MC treatments led to both decreased concentrations of hepatic cytosolic GSH levels as well as reduced glucocorticoid receptor nuclear uptake in vivo (Table I). The hypothesis that mirex and 3-MC may act on the glucocorticoid receptor by a similar mechanism (i.e. via the influence of the GSH pathway) during the early phases of carcinogenesis may seem attractive in its simplicity. However, a direct correlation between the effects of the compounds tested on GSH levels and the down-regular of the glucocorticoid receptor binding was not found. Coupled with our GSH depletion experiments, phorone, mirex and 3-MC decrease glucocorticoid receptor $B_{\text{max}}$ concentrations in vivo as well as glucocorticoid receptor nuclear uptake to approximately the same extent (by about 70% of controls), and yet these xenobiotics decreased GSH levels by 18, 52 and 77% respectively (Table I).

Although a temporal relationship between changes in intracellular GSH content and altered cytosolic glucocorticoid receptor binding was observed, quantitative relationships between these two parameters were not found based on our phorone dose—response and carcinogen pretreatment studies. Due to these inconsistencies, one would conclude that intracellular GSH does not play a direct role in modulating glucocorticoid receptor function. Whether other endogenous factors (1,46–48) are regulating glucocorticoid receptor binding during the early phases of carcinogenesis and tumor promotion still remains speculative.

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