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Short Communication

LIGANDS OF FOUR RECEPTORS IN THE NUCLEAR STEROID/THYROID HORMONE SUPERFAMILY INHIBIT INDUCTION OF RAT CYTOSOLIC ALDEHYDE DEHYDROGENASE-3 (*ALDH3c*) BY 3-METHYLCHOLANTHRENE

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Abstract—Using six ligands that bind to four different receptors in the nuclear steroid/thyroid hormone superfamily, we have examined the effects of these chemicals on induction of the cytosolic aldehyde dehydrogenase (ALDH3c) activity by 3-methylcholanthrene (3MC) in rat liver and uterus. In contrast to negligible activities in the untreated rat, ALDH3c enzyme activities are induced after a single dose of 3MC. Hepatic ALDH3c induction is decreased 60% to 90% when 3MC is administered together with any of the following ligands: estradiol, testosterone, progesterone, hydrocortisol, diethylstilbestrol, or tamoxifen. None of these same doses of chemicals, administered alone, affects ALDH3c enzyme activity. In addition, when these ligands are injected 2 days after 3MC, no changes are observed in liver or uterus ALDH3c induction. These results suggest that ligands that bind to different receptors in the nuclear steroid/thyroid hormone superfamily might inhibit the ALDH3c induction process by polycyclic aromatic hydrocarbons; the molecular mechanism(s) of this inhibitory effect is not yet understood.

Key words: aldehyde dehydrogenase induction; nuclear steroid/thyroid hormone receptor ligands; 3-methylcholanthrene; rat liver; rat uterus

Two rat liver cytosolic aldehyde dehydrogenases, ALDH1 and ALDH3c,‖ are inducible by different classes of foreign chemicals [1–5]. Phenobarbital-type inducers increase ALDH1 enzyme activity as much as 20-fold in rats having the "responsive" genotype *R/R*, but only 2-fold in rats having the "nonresponsive" genotype *r/r* [1, 2]. Dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD) and polycyclic hydrocarbons, such as benzo[a]pyrene and 3MC increase ALDH3c enzyme activity.

The *ALDH3c* gene is a member of the aromatic hydrocarbon-responsive [*Ah*] gene battery. TCDD and polycyclic hydrocarbons coordinately up-regulate the transcription of genes in the [*Ah*] battery, which, in addition to *ALDH3c*, also includes three other "Phase II" genes—*GSTA1*, *UGT1*06*, and *NMO1*—and at least two cytochrome P450 genes, *CYP1A1* and *CYP1A2* [6–9]. This induction process requires a functional cytosolic Ah receptor (AHR) [10]. Following binding to the ligand, the inducer-AHR complex translocates into the nucleus, forms a heterodimer with the Ah receptor nuclear translocator (ARNT) protein, and binds to one or more aromatic hydrocarbon-response elements (AhREs) that have been identified upstream of all six of the above-mentioned mammalian [*Ah*] battery genes. Although the interrelationship and "cross-talk" amongst [*Ah*] battery genes have been worked out extensively in the mouse [9], it is presumed that similar [*Ah*] gene interactions exist in the rat and human.

ALDH1 and *ALDH3c* encode enzymes having differences in substrate preference, coenzyme requirements, and sensitivity to inhibitors [5]. The ALDH1 enzyme activity is best measured with propionaldehyde or phenylacetaldehyde as substrate and NAD^+ as coenzyme (P/NAD), whereas measurement of ALDH3c enzyme activity is best carried out with benzaldehyde and $NADP^+$ (B/NADP) [11]. Specificity of ALDH3c expression can be determined from the ratio of B/NADP- to P/NAD-dependent ALDH activities. Under physiologic conditions, or following phenobarbital treatment, the B/NADP to P/NAD ratio is <1.0, whereas treatment of the rat with AHR ligands such as benzo[a]pyrene, 3MC, or TCDD produces a ratio of >1.0 [12, 13].

There have been reports suggesting that dexamethasone or estrogen can affect induction of drug-metabolizing enzymes by polycyclic hydrocarbons or TCDD [14–20]. In the present study, we have examined the effects of six ligands—which bind to four different receptors in the nuclear steroid/thyroid hormone superfamily [21]—on the inducibility of ALDH3c activity by 3MC. We show that each of these chemicals inhibits ALDH3c induction by 3MC, suggesting a possible role of these ligands in the ALDH3c induction process by 3MC.

Materials and Methods

Treatment of the animals. Male albino rats (weighing 200–250 g) of the Wistar/Mol/Io/rr substrain [2] were used. The rats

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‖ Abbreviations: ALDH3c, the polycyclic hydrocarbon-inducible cytosolic "Class 3" aldehyde dehydrogenase; GSTA1, glutathione S-transferase (Ya or class α); NMO1, NAD(P)H: menadione oxidoreductase, [NAD(P)H:quinone acceptor oxidoreductase, azo dye reductase, quinone reductase, DT-diaphorase]; UGT1*06, uridine diphosphoglucuronic acid glucuronosyltransferase form 1*06; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; 3MC, 3-methylcholanthrene; [*Ah*], aromatic hydrocarbon-responsive gene battery; AHR, Ah receptor; ARNT, Ah receptor nuclear translocator; AhRE, aromatic hydrocarbon response element. By convention, italicized *ALDH3c* represents the gene, whereas nonitalicized ALDH3c represents the mRNA, protein, or enzyme activity.

were kept in plastic cages (Makrolon) with wood chip bedding (*Populus sp.*), and the animals were given free access to tap water and pelleted chow. Intraperitoneal injections of estradiol (1 mg/kg), testosterone (1 mg/kg), progesterone (4 mg/kg), hydrocortisol (1 mg/kg), diethylstilbestrol (1 mg/kg), or tamoxifen (1 mg/kg) were given for 5 days, before killing 24 hr after the last dose. An intraperitoneal injection of 3MC (50 mg/kg) was given 48 hr before killing. Controls received the vehicle only, given on the same time schedule to match the experimental groups.

ALDH enzyme assays. After the rats had been killed by decapitation, the livers were homogenized with a teflon pestle in 3 vol (w/v) of ice-cold 0.25 M sucrose solution. The homogenate was first centrifuged at 10,000 × *g* for 30 min. An equal volume of 0.024 M $CaCl_2$ in 0.25 M sucrose was added to the supernatant fraction. The diluted supernatant fraction was stirred and left on ice for 10 min. The microsomal fraction was then sedimented by centrifugation at 10,000 × *g* for 30 min [22]; the resultant soluble fraction was used for the assays. Determinations of ALDH activity were carried out with a Beckman Model DU-70 spectrophotometer, by monitoring NAD(P)H production at 340 nm and 37°C. To measure the NAD^+-dependent oxidation of propionaldehyde (P/NAD activity), the assay mixture contained sodium pyrophosphate buffer (75 mM, pH 8.0), 1 mM pyrazole (to inhibit alcohol dehydrogenase), 1 mM NAD^+, and 5 mM propionaldehyde. To measure the $NADP^+$-dependent oxidation of benzaldehyde (B/NADP activity), assay conditions were the same except benzaldehyde (5 mM originally in 20% methanol) was substituted for propionaldehyde, and the coenzyme $NADP^+$ (2.5 mM) was used instead of NAD^+. In either enzyme assay, the reaction was started by adding the substrate subsequent to a 5-min preincubation, and a blank was run without the substrate [12]. Protein was measured by the biuret method [23], using bovine serum albumin as standard. Units denote nmoles of NAD(P)H formed per min; specific activities are expressed in units/mg protein. Statistical analyses of the results were performed by Student's two-tailed *t*-test.

Results and Discussion

Pretreatment effect of the ligands of various nuclear steroid-thyroid hormone receptors on ALDH3c inducibility by 3MC in rat liver. Table 1 shows that 3MC treatment induces the B/NADP to P/NAD ratio (ALDH3c activity) from 0.30 to 5.3. Rat liver cytosolic ALDH3c induction of this magnitude, in response to polycyclic aromatic hydrocarbons or TCDD, has been shown previously [3, 11, 13]. This magnitude of ALDH3c induction by 3MC has been found to be in the middle of the dose-response curve, as far as the induction of B/NADP activity is concerned [M. Karageorgou, unpublished data, cited with permission].

Table 1 also shows that pretreatment with estradiol, testosterone, progesterone, or hydrocortisol—endogenous ligands for the estrogen, androgen, progesterone, and glucocorticoid receptors, respectively—each inhibited 3MC induction of ALDH3c activity by more than 75%. Pretreatment with either diethylstilbestrol or tamoxifen—a synthetic agonist and antagonist, respectively, for the estrogen receptor—likewise inhibited by 82% and 63%, respectively, the ALDH3c induction response to 3MC (Table 1). Sole treatment with the same doses of any of these six ligands for receptors in the nuclear steroid/thyroid hormone superfamily had no effect on ALDH3c enzyme activity (data not shown).

Effect of estradiol post-treatment on ALDH3c induction by 3MC in rat liver. To determine if these hormones affect the 3MC-induced ALDH3c enzyme activity *per se*, we examined the effect of estradiol treatment after ALDH3c activity had been induced by 3MC (Table 2). Estradiol did not affect the already-induced ALDH3c enzyme levels. We found similar results with the other five inhibitors shown in Table 1 (data not shown). These results show that, although the six ligands for the four different receptors in the nuclear steroid/thyroid hormone superfamily do not affect basal or 3MC-induced ALDH3c activity, they appear able to inhibit the ALDH3c induction process by 3MC.

Pretreatment effect of the ligands of various nuclear steroid-thyroid hormone receptors on ALDH3c inducibility by 3MC in rat uterus. Induction of ALDH3c enzyme activity or mRNA by 3MC has been shown to occur in a number of extrahepatic tissues, such as lung, spleen, stomach, intestine, and brain [12, 24]. To determine whether these receptor ligands' inhibition of ALDH3c induction by 3MC occurs in tissues other than liver, we examined the effects of estradiol and/or 3MC treatment on rat uterus ALDH3c activity. We chose the uterus because this is a tissue that is rich in sex hormones. 3MC was found to induce ALDH3c activity in this extrahepatic tissue (Table 3); although the fold induction was considerably less than that seen in liver, ALDH3c expression can be concluded from inversion of the B/NADP to P/NAD ratio. To our knowledge, this is the first report of ALDH3c induction in rat uterus; interestingly, basal B/NADP activity in uterus is 3-fold higher than that in liver. Table 3 also shows that estradiol did not affect the basal ALDH3c enzyme activity, but did inhibit ALDH3c induction by 3MC. We conclude that the same effect of estradiol on the 3MC-mediated ALDH3c induction process, which exists in liver, also occurs in uterus.

Possible interpretations of these data. There are three major possible mechanisms as to why these six chemicals—each a ligand for one of four different receptors in the nuclear steroid/

Table 1. Effect of nuclear steroid/thyroid hormone receptor ligand pretreatment on ALDH3c induction by 3MC in rat liver

Treatment	B/NADP	P/NAD	B/NADP to P/NAD Ratio	Percent of ALDH3c induction†
Control	2.2 ± 0.1	7.3 ± 0.3	0.30	(0%)
3MC alone	390 ± 8.3	73 ± 1.2	5.3	100%
+ estradiol	89 ± 40*	18 ± 5.8*	4.9	23%
+ testosterone	77 ± 18*	20 ± 3.4*	3.9	20%
+ progesterone	66 ± 11*	21 ± 5.0*	3.1	17%
+ hydrocortisol	46 ± 5.3*	21 ± 3.5*	2.2	12%
+ diethylstilbestrol	69 ± 8.3*	30 ± 4.9*	2.3	18%
+ tamoxifen	142 ± 53*	23 ± 3.5*	6.2	36%

Animals were treated with each of the four hormones and two synthetic ligands for 5 days. On the fourth day, animals received a single dose of 3MC (50 mg/kg, i.p.), and were sacrificed 48 hr later. Specific activities are expressed as means ± standard deviation ($N = 6$ per group). B/NADP, $NADP^+$-dependent benzaldehyde oxidation. P/NAD, NAD^+-dependent propionaldehyde oxidation. The ratio of B/NADP to P/NAD is >1.00 in all groups except the control group, and reflects expression of ALDH3c activity [12, 13]. Values from all experimental groups are statistically different from those of the controls ($P < 0.001$).

* Statistically significantly different ($P < 0.05$) from the 3MC-group.

† Percent of ALDH3c induction refers to B/NADP activity: 3MC alone = 390 = 100%; 3MC + estradiol = 89 = 23%, etc.

Table 2. Effect of estradiol post-treatment on ALDH3c induction by 3MC in rat liver

Treatment	B/NADP	P/NAD	B/NADP to P/NAD Ratio	Fold induction of ALDH3c activity†
Control	2.5 ± 1.2	6.4 ± 0.4	0.40	1.0
3MC alone	200 ± 23*	36 ± 7.8*	5.7	80
Estradiol alone	3.3 ± 0.7	7.8 ± 1.2	0.40	1.3
3MC + estradiol	190 ± 26*	37 ± 2.2*	5.2	76

Animals were treated with 3MC (50 mg/kg/day) for 2 days. Three days later, the animals started receiving estradiol (1 mg/kg/day) for 4 days. The other groups (control, 3MC, and estradiol) received the corresponding compound at the same times as the combination group. All the animals were killed 24 hr after the last dose of estradiol. Specific activities and abbreviations are the same as those described in Table 1.

* Statistically significantly different from the control ($P < 0.001$).

† Fold induction refers to B/NADP activity with the control values equal to 1.0.

thyroid hormone superfamily—might be causing a diminution of ALDH3c induction by 3MC.

1. The effect is operating via transcriptional factors that bind to regulatory regions of the *ALDH3c* gene.
2. The effect is posttranscriptional, affecting either mRNA translatability or mRNA or protein stability.
3. The results of these chemicals—at relatively high, non-physiologic doses—reflect nonspecific, or indirect effects on the ALDH3c induction process.

Might the effect shown in this study be caused by changes at the transcriptional level? The six inhibitors of 3MC-induced ALDH3c activity shown in Table 1 are all ligands for members of the nuclear steroid/thyroid hormone receptor superfamily. It is well known that 3MC or TCDD does not bind to these steroid/thyroid hormone receptors, nor do these steroid hormones bind to the AHR. The AHR belongs to the Per-ARNT-Sim (PAS) superfamily of transcription factors, evolutionarily unrelated to the nuclear steroid/thyroid hormone receptor superfamily [10, 21]. It is also well known that heterodimers of two different members of the nuclear steroid/thyroid hormone receptor superfamily cooperate in the up- or down-regulation of various genes (e.g. retinoic acid and retinoid X receptors [25], retinoid X and thyroid hormone receptors [26], and peroxisome proliferator-activated and estrogen receptors [27], to name but a few). Whereas the 3MC induction process of ALDH3c does not appear affected by receptors of the nuclear steroid/thyroid hormone superfamily, the AHR is known to be involved [9]. The data in Table 1 might therefore be explained by interactions between members of two different transcriptional factor superfamilies.

Numerous reports have suggested that steroids can affect induction of drug-metabolizing enzymes by polycyclic hydrocarbons or TCDD [14–20]. For example, dexamethasone potentiates CYP1A1 induction by 3MC in cell cultures as well as in the intact animal, at steroid concentrations having little or no effect on CYP1A1 expression in the absence of 3MC [14–17]. On the other hand, dexamethasone has been shown to repress MC induction of GSTA1 and NMO1 activity in adolescent male rats [18]. As mentioned above, changes in enzyme activity alone offer little explanation as to which of several alternative mechanisms might be operating.

Several recent reports suggest some relationship between transcriptional factors of the PAS and the nuclear steroid/thyroid hormone receptor superfamilies. Thomsen *et al.* have shown that restoration of a functional estrogen receptor restores AHR-mediated CYP1A1 inducibility by TCDD in the human breast carcinoma cell line, MCF-7 [19], and that the estrogen receptor antagonist *ICI 164,384* blocks CYP1A1 inducibility by TCDD in these cells [20]. On the other hand, TCDD or 3MC treatment was demonstrated to decrease the rat or mouse estrogen receptor levels in the intact animal and in cell culture lines, suggesting an AHR-mediated mechanism of down-regulation of estrogen receptor gene expression [28–30]. White and Gasiewicz have postulated that the AHR might be implicated in modulation of the estrogen receptor [31]. Our Table 1 data provide evidence that pretreatment with any of four endogenous hormones (as well as a synthetic estrogen agonist or antagonist) inhibits the 3MC inducibility of ALDH3c activity—an induction process known to be AHR-dependent [7–9, 32].

Could these effects on ALDH3c enzyme induction represent post-transcriptional modification of the mRNA or protein? It has been shown, for example, that steroids can alter the translation rates of a number of myelin protein mRNAs, as well as the mRNA encoding the estrogen receptor [33]. This up- or down-regulation operates by way of an element (AGAAGA) found in the 5′-untranslated region of a number of mRNAs [33]. Interestingly, both the mouse and rat *ALDH3c* genes contain the sequence AGAAGG in the 5′-untranslated region [32, 34], in which the last G does not match the consensus. Site-directed mutagenesis studies of the myelin protein cDNA have confirmed the importance of the hexamer AGAAGA in the modulation of mRNA translation by steroids [33]. Because we have not carried out similar studies with the ALDH3c cDNA, at the present time we cannot rule out the possibility of a posttranscriptional modification by the inhibitors listed in Table 1.

Might the inhibition of ALDH3c induction by 3MC be caused by nonspecific, or indirect, effects due to large, non-physiologic doses of these chemicals listed in Table 1? Although this remains a possibility, the fact that these doses had

Table 3. Effect of estradiol pretreatment on ALDH3c induction by 3MC in rat uterus

Treatment	B/NADP	P/NAD	B/NADP to P/NAD Ratio	Fold induction of ALDH3c activity†
Control	6.0 ± 1.3	7.6 ± 0.1	0.80	1.0
3MC alone	18 ± 1.2*	8.5 ± 0.6	2.2	3.0
Estradiol alone	4.0 ± 0.5	6.3 ± 0.9	0.6	0.8
3MC + estradiol	9.2 ± 1.4*	6.8 ± 2.2	1.4	1.5

Treatment of animals, specific activities, and abbreviations are the same as those described in Table 1.

* Statistically significantly different from the control ($P < 0.001$).

† Fold induction refers to B/NADP activity with the control values equal to 1.0.

no effect on basal ALDH3c activity, or on ALDH3c activity already induced by 3MC, is evidence against this hypothesis. In fact, doses of diethylstilbestrol as low as 1.0 μg/kg for 5 days were able to inhibit greater than 50% of the hepatic ALDH3c induction process by 3MC [M. Karageorgou, unpublished data, cited with permission].

Concluding remarks. In summary, the ALDH3c induction process by 3MC in rat liver is inhibited by estradiol, testosterone, progesterone, hydrocortisol, diethylstilbestrol, and tamoxifen; the same effect in rat uterus was found with estradiol treatment. Although the mechanism is presently unknown, it is tempting to speculate that members of the nuclear steroid/thyroid hormone receptor superfamily are capable of interacting with AHR-mediated transcription of the *ALDH3c* gene. Such an interaction might be important in cancer chemotherapy, because several tumor cell lines resistant to cyclophosphamide have been found to exhibit high levels of ALDH3c enzyme activity [35]; if this tumor ALDH3c induction process is AHR-mediated, perhaps one of the ligands studied in the present report might prevent the development of tumor resistance to antineoplastic drugs such as cyclophosphamide. The effects of these hormones—and other ligands for receptors in the nuclear steroid/thyroid hormone superfamily—on the induction process of ALDH3c by 3MC or TCDD are currently under further investigation in our laboratories.

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