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THE INFLUENCE OF FIVE MONOOXYGENASE INDUCERS ON LIVER CYTOSOL ESTRADIOL RECEPTOR LEVELS IN THE OVARIECTOMIZED ADULT RAT.

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SUMMARY

The intraperitoneal injection of the well known monooxygenase inducers (phenobarbital, β-naphtoflavone, pregnenolone-16α-carbonitrile, benzo(a)-pyrene, methylcholanthrene) elicits a net decrease in the specific binding of estradiol to its cytosol receptor in female rat livers. Amongst the five chemicals tested, only phenobarbital did not exhibit such a phenomenon, but caused a slight increase. This observation was neither due to a competitive inhibition by these compounds, nor to an enhanced metabolism of [^{3}H]-estradiol. Moreover, when this effect was produced by polycyclic hydrocarbons, it was inversely correlated to the activity of aryl hydrocarbon hydroxylase, induced by these same chemicals.

INTRODUCTION

It is well known that the action of steroid hormones is dependent upon a specific receptor in the cytoplasm of the target cells (1). Many investigators believe that monooxygenase inducers act via a similar process. The discovery of a specific binder for dioxine in liver cytoplasm supports this hypothesis (2).

As structural similarities exist between estrogens and various inducers, particularly the polycyclic hydrocarbons (3), we theorized that these sub-

ABBREVIATIONS

AHH	:	aryl hydrocarbon hydroxylase
DES	:	diethylstilbestrol
EDTA	:	(ethylenedinitrilo) tetraacetic acid
ER	:	estrogen receptor
Tris-HCl	:	2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride
BNF	:	β-naphtoflavone
BP	:	benzo(a)pyrene
MC	:	methylcholanthrene
PB	:	phenobarbital
PCN	:	pregnenolone-16α-carbonitrile

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stances might also interact at the level of their respective receptors and thus modify their biological effects.

In this study, we investigated the influence of five well known monooxygenase inducers (phenobarbital (PB), pregnenolone-16α-carbonitrile (PCN), β-naphtoflavone (BNF), benzo(a)pyrene (BP) and methylcholanthrene (MC) on hepatic hormone receptor levels, and particularly, on those of estrogen (ER). We found that the administration of two polycyclic hydrocarbons, BNF and PCN, brought about a significant decrease in the cytoplasmic estrogen receptor levels in the rat liver.

MATERIALS AND METHODS

Chemicals

(11β-Methoxy-^{3}H)-moxestrol (specific activity: 88.0 Ci/mmole) and (2,4,6,7-^{3}H)-estradiol-17β (specific activity: 91 Ci/mmole) and unlabelled moxestrol were obtained from New England Nuclear (Boston, Mass., U.S.A.). Unlabelled estradiol-17β and diethylstilbestrol (DES) were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Purity of labelled and unlabelled steroids was verified by thin layer chromatography and/or Sephadex LH-20 chromatography and the chemicals were shown to be more than 99% pure. Activiated charcoal (carbon decolorizing alkaline - Norit A) was obtained from Fisher Scientific Co. (Fairlawn, New Jersey, U.S.A.). Scintillation liquid Aqua Luma was bought from Lumac Systems (Basel, Switzerland). Benzo-(a)pyrene and methylcholanthrene were purchased from Fluka (Neu-Ulm, F.R.G.), β-naphtoflavone from Aldrich (Beerse, Belgium) and pregnenolone-16α-carbonitrile was a gift from Upjohn (Puurs, Belgium).

Animals

Adult female rats (Wistar strain) were ovariectomized under light anesthesia a week before inducer administration. Animals were ovariectomized both to diminish the dispersion of the results and to increase the estrogen receptor levels in the liver. This castration did not influence the action of the monooxygenase inducers on the estrogen receptors. Inducers (BNF, PB, PCN, BP and MC) were intraperitoneally injected at doses of 60, 30, 30, 60 and 60 mg/kg of body weight, respectively. Animals were sacrificed at the designated times after a single injection of the inducers.

The livers were perfused with 0.9% saline by vena cava, excised and homogenized in 2 volumes of TEMG (0.05 M Tris-HCl, pH 7.4 - 1 mM EDTA - 1 mM mercaptoethanol - 10% glycerol) containing 1.5 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, 0.02% NaN_3 with a Potter Elvehjem tube. The homogenates were centrifuged at 800 x g for 10 min at 4°C, then for 1 hour at 105,000 x g to obtain the cytosol and microsomal fractions. Protein was quantitated by the method of Lowry (4).

Estrogen receptor assays

The incubations were performed in triplicate for one night at 4°C with 200 µl of cytosol in the presence of 20 nM [^{3}H]-estradiol-17β, with and without a 100-fold excess of DES, or 10 nM [^{3}H]-moxestrol, with and without a 100-fold excess of unlabelled moxestrol for confirmation. After incubation, a 200 µl suspension of dextran-coated charcoal (0.5% Norit A, 0.05% dextran in TEMG buffer, w/v) was added to each tube and shaken for 1 hour at 4°C to extract the excess unbound steroid. Then the tubes were centrifuged at 2,000 x g for 10 min at 4°C. The radioactivity of the supernatant (200 µl) was counted by scintillation liquid.

Aryl hydrocarbon hydroxylase (AHH) assay

This assay was performed as previously described by Van Cantfort *et al.* (5) according to an isotopic method.

Lactic dehydrogenase (LDH) assay

LDH assays were carried out using an LDH-UV test 3388 Merck (Darmstadt, F.R.G.).

RESULTS AND DISCUSSION

Contrarily to phenobarbital which elicited a significant increase of about 20%, the four other inducers (BNP, PCN, MC, BP) produced a net decrease of estradiol-specific cytosol binding to its hepatic receptor 24 hours after intraperitoneal injection. This fall in receptor levels varied as a function of the chemicals tested (table 1), and was particularly significant with PCN and MC. This study was pursued with MC. It appeared that the extent of the decrease in the specific binding depended upon the quantity administered (table 1). Similarily, the phenomenon became more pronounced when it was observed as a function of time elapsed since the injection. After 72 hours, it was found to be no more than 20% of the control levels (table 2). However, 72 hours after the injection of PCN (30 mg/kg), the animals recovered their initial liver estrogen receptor levels (data not shown). Could the decrease produced by these chemicals be explained as a competition between the inducers for the binding sites? At 10^{-4} M, neither of the compounds inhibited the specific binding of estradiol, except MC, which brought about a 50% decrease (table 3). Nevertheless, as soon as the MC concentration fell, inhibition *in vitro* was reduced to a small percentage.

TABLE 1

INDUCERS	DOSE mg/kg	ESTROGEN RECEPTOR fmoles/mg prot. m ± S.D. (n)	VARIATION %	p
EXPERIMENT 1				
VEHICLE (OIL)	---	120.5 ± 8.2 (4)	---	---
8-NAPHTOFLAVONE	60	76.7 ± 11.3 (4)	- 36.3*	$p < 0.001$
PHENOBARBITAL	30	140.9 ± 5.6 (4)	+ 16.9	$p < 0.01$
BENZO(A)PYRENE	60	67.5 ± 16.4 (6)	- 44.0	$p < 0.07$
METHYLCHOLANTHRENE	40	57.7 ± 8.1 (4)	- 52.1	$p < 0.001$
CYANOPREGNENOLONE	30	38.9 ± 12.9 (4)	- 67.6	$p < 0.001$
EXPERIMENT 2				
VEHICLE (OIL)	---	102.8 ± 10.8 (4)	---	---
METHYLCHOLANTHRENE	2	100.3 ± 21.0 (4)	- 2.4	N.S.
	10	69.6 ± 11.0 (4)	- 32.3	$p < 0.005$
	40	57.7 ± 8.1 (4)	- 43.9	$p < 0.001$

*- : DECREASE
+ : INCREASE

RATS WERE SACRIFICED 24 HOURS AFTER INDUCER INJECTION
N.S. : NON SIGNIFICANT.

TABLE 2

Action of methylcholanthrene as a function of delay between injection and sacrifice

Injection	Delay (h)	Estrogen Receptor fmoles / mg prot. m ± S.D. (n = 4)	Variation %	p
Vehicle (oil)	24	102.8 ± 10.8	-----	-----
	48	132.0 ± 43.8	-----	N.S.
	72	137.4 ± 22.1	+ 33.7*	$p < 0.02$
Methylcholanthrene	0	112.0 ± 16.9	-----	-----
	5	99.0 ± 18.4	-----	N.S.
	24	57.7 ± 8.1	- 43.9	$p < 0.001$
	72	25.9 ± 10.9	- 81.1	$p < 0.001$

* - : Decrease

+ : Increase

N.S.: Non-significant

TABLE 3

ACTION OF INDUCERS *IN VITRO*

INDUCERS	CONC. (μM)	DECREASE %
β-Naphtoflavone	200	6.6
Phenobarbital	100	0
Benzopyrene	200	10.2
Methylcholanthrene	100 50 10	48.0 29.0 12.2
Cyanopregnenolone	100	0

A comparison of the estrogen receptor levels observed after MC treatment and the activities of aryl hydrocarbon hydroxylase (AHH) in liver microsomes measured in the same rats exhibited a high negative correlation ($n = 17$, $r = -0.93$, $p < 0.001$) (figure 1). This was observed regardless of whether the decrease in the ER levels was obtained as a function of the dose of the inducer administered, or as a function of the time elapsed. On the other hand, the same correlation was not obtained when PB or PCN was used as the inducer.

This observation indicated that the receptor levels and AHH activities were linked to a common biochemical event. This may have been an increase in the metabolism of the tracer [^{3}H]-moxestrol by the induced enzymes. This hypothesis could be discarded because, after incubation, the chromatography of the labelled ligand exhibited no difference between the control and treated rats. Moreover, MC induced the enzymes essentially bound to endoplasmic reticulum, a subcellular fraction absent from the incubation medium used for the receptor assay.

It is also interesting to note that the phenomenon was restricted to the liver, as we did not observe a similar modification in other target tissues (i.e. the uterus) (date not shown).

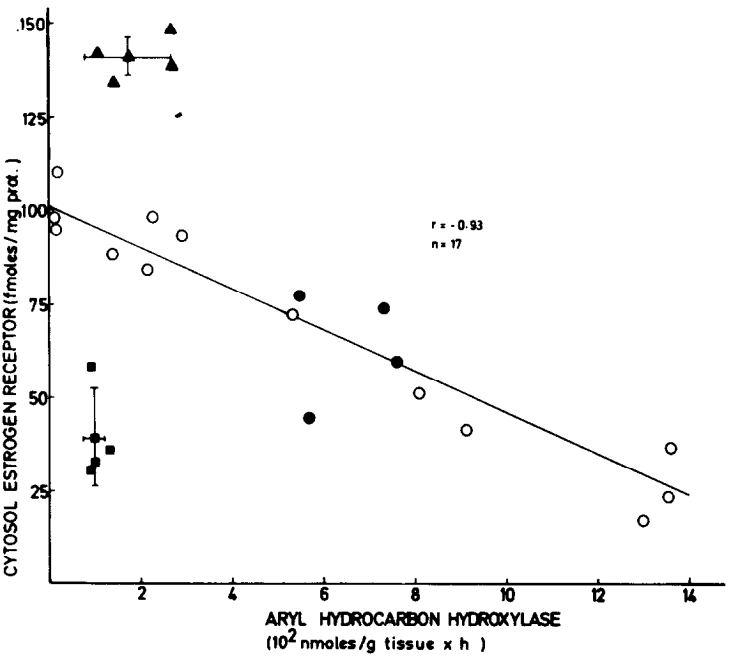

FIGURE 1 Correlation between specific binding of estradiol in liver cytosol and AHH activities measured in microsomes of the same adult female rats after treatment by various doses of MC (○) or BP (●). Animals were sacrificed 1 to 3 days after injection of the inducers. Results (n = 4) obtained for PB (▲) and PCN (■) are also plotted with the mean ± standard deviation for both groups.

In the case of MC and BP, it was possible to assume that the binding sites of the estrogen receptor were modified *in vivo* by the interaction with their electrophilic metabolites. However, lactic dehydrogenase assays performed in the cytosol showed no decrease in this enzymatic activity, but on the contrary, exhibited a slight and significant increase (5.1 ± 0.3 I.U./mg prot. in the control rats, in contrase to 6.6 ± 0.6 I.U./mg prot. in the MC-treated rats after 72 hours, $p < 0.001$). Therefore, if there was a direct alteration, it did not randomly change the binding and enzymatic activity of any cytosol protein. Moreover, it was not impossible that the nuclear mechanisms which elicited an increase in AHH activity were also responsible for the simultaneous decrease in the cytosol ER levels. Another explanation might be a direct but selective action of electrophilic hydrocarbon metabolites on a

few cytosol proteins, among which would be ER. The answer to this question is the object of our present research.

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