TOXICOLOGY AND APPLIED PHARMACOLOGY **83,** 590–595 (1986)

Carbon Monoxide Inhibits Monooxygenation by the Conceptus and Embryotoxic Effects of Proteratogens *in Vitro*[1]

Carbon Monoxide Inhibits Monooxygenation by the Conceptus and Embryotoxic Effects of Proteratogens *in Vitro*. FAUSTMAN-WATTS, E. M., GIACHELLI, C. M., AND JUCHAU, M. R. (1986). *Toxicol. Appl. Pharmacol.* **83,** 590–595. Evidence that Day 10 to 11 rat conceptuses can contain sufficient quantities of xenobiotic-oxidizing *P*-450(s) to catalyze the conversion of proteratogens to reactive teratogenic intermediates is presented. Quantities of reactive intermediates generated by conceptuses from 2-acetylaminofluorene (AAF) were sufficient to result in the appearance of gross morphologic abnormalities with a high incidence of neural tube anomalies. Carbon monoxide effectively inhibited the conversion of AAF to hydroxylated metabolites by rat conceptuses, and also inhibited the capacity of AAF to elicit neural tube anomalies in cultured embryos. Inhibition by CO of either parameter was observed only with conceptuses exposed *in utero* to the potent *P*-450 inducer, 3-methylcholanthrene.

Recent investigations have provided evidence that conceptuses of common experimental animals possess benzo[*a*]pyrene (BaP)-oxidizing enzyme systems during very early stages of their development. For example, Filler and Lew (1981) demonstrated that preimplantation blastocysts could convert BaP to various oxidized metabolites. Lambert and Nebert (1977) and Shum *et al.* (1979) published data indicating that midgestational mouse conceptuses could oxidatively biotransform the same substrate. Cultured conceptuses from strains of mice that respond to the *P*-450-inducing effects of 3-methylcholanthrene (MC) converted BaP to products that caused sister chromatid exchange (Galloway *et al.*, 1980). Pedersen *et al.* (1985) have presented results suggesting that cytochrome *P*-450 is active in BaP metabolism in most tissues of mouse conceptuses at several stages of development.

In the above studies, oxidizing activities were extremely low as compared to those commonly measured in adult rat livers. Recently, we presented data showing that rat conceptuses (treated with MC *in utero* prior to culturing) could convert sufficient quantities of another substrate, 2-acetylaminofluorene (AAF), to reactive intermediates to produce readily observable gross morphologic abnormalities in the same embryos (Juchau *et al.*, 1985a,b). We postulated that the abnormalities resulted from *P*-450-dependent bioactivation of AAF by the embryos' own enzymes. These observations were somewhat surprising in view of the very low BaP-oxidizing and AAF-oxidizing activities observed in each of the various studies cited above. An alternate interpretation of the morphologic data is that treatment of embryos *in utero* with MC may have predisposed the embryos to potential dysmorphogenic effects of parent AAF via biologic effects independent of *P*-450 induction. Furthermore, in common with other investigators who have explored xenobiotic biotransformation/bioactivation during the early stages of rodent gestation, we did not provide definitive evidence that the generation of reactive intermediary metabolites by the conceptus was actually dependent on a *P*-450 hemoprotein(s). It could therefore be argued that peroxidative or other enzymic mechanisms may have been responsible for generation of reactive AAF metabolites in our earlier studies. The data presented in this publication provide definitive evidence that neither alternative is viable and that *P*-450-dependent bioactivation of AAF (a model substrate for *P*-450-dependent bioactivation) to dysmorphogenic intermediates can be catalyzed by the conceptus' own enzymes.

[1] Supported by USPHS Grants OH-01281 and ES-03157 and Training Grant ES-07032.

0041-008X/86 $3.00

Importantly, the bioactivation by the conceptus is sufficiently extensive to elicit readily observable gross abnormalities in the target embryos. The data have strong implications for the importance of regulation of embryonic *P*-450(s) as an important determinant for chemical teratogenesis and other forms of chemically elicited embryonic damage.

METHODS

Sources and purities of all chemicals and reagents utilized have been reported earlier (Juchau *et al.*, 1985a). The postimplantation culture system developed by New (1978) was used as an experimental model in these investigations. The system as utilized in our laboratories has been described in detail (Greenaway *et al.*, 1982; Faustman-Watts *et al.*, 1983). Conceptuses were treated *in utero* on Day 8 by injecting either MC (40 mg/kg, ip) dissolved in corn oil or an equal volume of the corn oil vehicle into the dams. MC-pretreated conceptuses (MC conceptuses) and corn oil-pretreated conceptuses (control conceptuses) were explanted on Day 10 (10 ± 2 somites) and exposed to AAF in culture. AAF was dissolved in dimethyl sulfoxide (DMSO) and final concentrations were 282 μM AAF and 35 mM DMSO. In the absence of an active bioactivating system, this combination produces no detectable embryotoxic effects (Faustman-Watts *et al.*, 1983). Cultures were gassed with either O_2:N_2 (5:95) or O_2:CO:N_2 (5:5:90). It has been shown that CO produces no detectable embryotoxic effect on cultured embryos (Greenaway *et al.*, 1982). After the 24-hr culture period, embryos were removed from the culture flasks and scored, without knowledge of treatment, for viability, malformations, and somite numbers. Embryos were then sonicated and protein content was measured by the method of Bradford (1976).

For measurements of the capacity of conceptuses to biotransform AAF, control and MC-pretreated conceptuses (10 ± 2 somites) were homogenized in 0.1 M potassium phosphate buffer, pH 7.4, on Day 10 at 0900 hr. These homogenates (approximately 2 mg protein, 15 conceptuses) were added to reaction tubes containing 1.92 mM NADPH, 3.42 mM glucose 6-phosphate, and 2 units of glucose 6-phosphate dehydrogenase in phosphate buffer. Homogenates heated for 10 min at 100°C served as background controls. Reactions were initiated by addition of 1.01 μCi 9-^{14}C-AAF (52 Ci/mol) such that the final concentration in the reaction was 40 μM. Tubes were incubated for 2 hr at 37°C under embryo culture gassing conditions described above. Reactions were terminated by addition of ice-cold 1.0 M sodium acetate, pH 6.0. Nonpolar metabolites were extracted into diethyl ether and organic layers were reextracted into 0.5 N NaOH. Samples were neutralized and radioactivity quantified by liquid scintillation counting. Enzyme activities were expressed as picomoles of alkaline-extractable radioactivity produced per milligram protein per 2 hours of incubation. Preliminary experiments indicated that reactions were linear for at least 2 hr. Data were also expressed as "fold induction" by calculating ratios of extracted radioactivity from incubations containing MC conceptus homogenates to that from incubations containing control conceptus homogenates.

Specific alkaline-extractable AAF metabolites were also analyzed with high-performance liquid chromatography (HPLC). Metabolites were first separated from parent AAF by neutralizing the alkaline extraction layer with HCl and then back extracting four times with 10 volumes of diethyl ether. Under these conditions, over 99% of the radioactivity associated with the parent compound was eliminated. The organic layers were dried under N_2, redissolved in methanol, and injected into a Beckman HPLC system.

A modification of the HPLC system used by Smith and Thorgeirsson (1982) was used to separate AAF and eight standard metabolites, and has been described in detail previously (Juchau *et al.*, 1985a).

Specific activities for individual AAF metabolites were determined by measuring radioactivity which coeluted with known AAF standards; radioactivity associated with incubations containing heat-inactivated embryo homogenates was subtracted. The following recovery data were used to account for differential alkaline extraction recovery rates for the various AAF metabolites: N-OH-AAF, 80%; 3-OH-AAF, 70%; 5-OH-AAF, 90%; 7-OH-AAF, 63%; 9-keto, 9-OH, and 1-OH-AAF, 75%.

Student's *t* test was used to evaluate treatment differences in somite numbers, metabolic activities, and protein content. The χ^2 statistic was used for parameters measured on a quantal scale (viability, malformations). Statistical methods are described by Steele and Torrie (1960).

RESULTS AND DISCUSSION

The effects of CO (O_2:CO = 1:1) on the capacity of AAF to produce embryotoxic effects in embryos preexposed *in utero* to MC are presented in Table 1. The percentage of malformations as well as other measures of embryotoxicity were markedly reduced when CO was utilized in the culture system. (The effect on protein content was somewhat less than expected and is currently unexplained.) In agreement with previous results (Greenaway *et al.*, 1982), CO itself produced no measurable deleterious effects on the cultured embryos. Likewise, AAF elicited no embryotoxic effects unless conceptuses were preexposed to a *P*-450-inducing agent *in utero* (Juchau *et al.*, 1985a,b). The incidence, severity, and nature

TABLE 1

EFFECT OF CARBON MONOXIDE (CO) ON THE CAPACITY OF 2-ACETYLAMINOFLUORENE (AAF) TO ELICIT EMBRYOTOXICITY *IN VITRO* AFTER TREATMENT OF EMBRYOS *IN UTERO* WITH 3-METHYLCHOLANTHRENE (MC)

CO[a]	AAF[b]	MC[c]	Viable/ cultured	Malformed (%)	Somite number[d]	Protein (μg/embryo)[d]
−	+	+	34/35	60.0[e]	17.5 ± 2.4[e]	128 ± 30[e]
−	+	−	11/11	9.0	20.0 ± 2.1	193 ± 76
+	+	+	8/8	12.5	19.5 ± 1.7	148 ± 17
+	−	+	6/6	0.0	19.5 ± 1.5	180 ± 43
+	−	−	16/17	6.3	20.1 ± 1.4	189 ± 32
−	−	−	42/43	4.8	20.0 ± 1.6	191 ± 37

[a] $CO:O_2:N_2 = 5:5:90$.
[b] Initial concentration in the culture medium was 282 μM.
[c] Dams treated on Day 8 with a single injection of MC (40 mg/kg, ip).
[d] Somite numbers and protein values are given as $\bar{x} \pm$ SD.
[e] Values differ statistically from those of all other groups ($p < 0.05$).

of the malformations produced by AAF on MC-pretreated embryos in the absence of CO were similar to those observed previously (Faustman-Watts *et al.*, 1983; Juchau *et al.*, 1985a,b). Abnormal neurulation was the most characteristic malformation; morphologic details have been described previously (Faustman-Watts *et al.*, 1983). CO dramatically reduced the incidence but not the nature of the malformations produced.

Effects of CO on the capacity of conceptuses to generate base-extractable AAF metabolites are indicated in Table 2. The effect of CO was significant statistically ($p < 0.05$) but was not profound. Analyses of the effects of CO on individual hydroxylated metabolites (Fig. 1), however, revealed that CO markedly inhibited the generation of 7-hydroxy and 3-hydroxy metabolites, had little effect on the generation of certain others (5-hydroxy and 1-hydroxy), and actually produced increases in quantities of metabolite(s) cochromatographing with 9-hydroxy-AAF. Since 9-hydroxy-AAF can be generated via *P*-450-independent pathways, inclusion of CO probably resulted in the preferential generation of this metabolite. The reduction in generation of the 7-hydroxy metabolite paralleled the reduction in embryopathic effect. This was of considerable interest because of the recent finding (Faustman-Watts *et al.*, 1985) that authentic, chemically synthesized 7-hydroxy-AAF produced neural tube

TABLE 2

TOTAL RADIOACTIVITY EXTRACTABLE INTO 0.1 N NaOH FROM INCUBATION FLASKS CONTAINING WHOLE CONCEPTUS HOMOGENATES AND ^{14}C-AAF[a]

Embryo pretreatment	Carbon monoxide	Specific activity (pmol/mg/2 hr)	Ratio (treated/control)
Corn oil	−	4.2 ± 0.9	1.0
3-Methylcholanthrene	−	31.4 ± 2.6[b]	7.5 ± 1.1[b]
3-Methylcholanthrene	+	26.4 ± 3.2	6.3 ± 0.7

[a] $\bar{x} \pm$ SD of three experiments.
[b] Values differ statistically from each of the other two groups ($p < 0.05$).

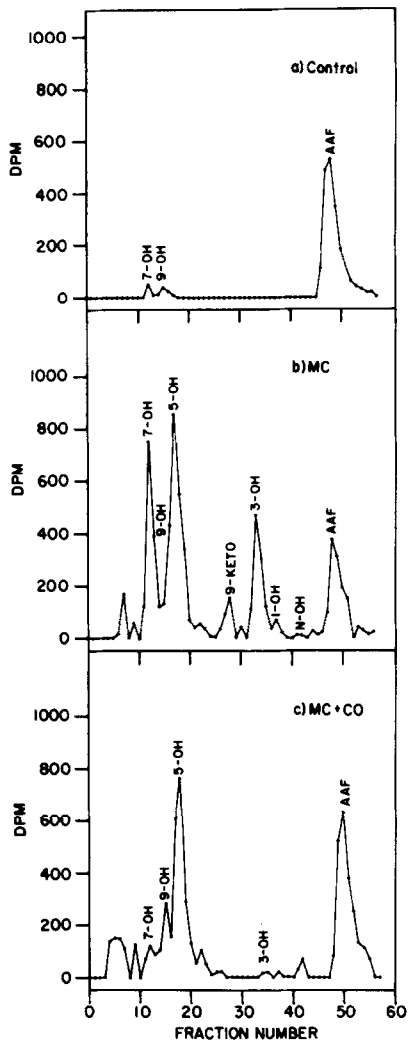

FIG. 1. HPLC metabolite profiles of ^{14}C-AAF generated by Day 10 rat conceptuses. Representative profiles are shown for reactions using (a) control conceptus homogenates, (b) MC-treated conceptus homogenates, and (c) MC-treated embryo homogenates incubated with carbon monoxide. Elution patterns for authentic AAF metabolites are indicated on the profiles. Homogenates were derived from 15 pooled conceptuses for each experiment.

defects when added to embryo culture systems in the absence of an exogenous biotransformation system.

The effects of MC-treatment of embryos *in utero* on the capacity of the conceptus to catalyze generation of specific AAF metabolites are depicted in Table 3. It is of interest that very profound effects on the conversion to 7-, 5-, 3-, and 1-hydroxy metabolites occurred with lesser effects on the 9- and N-hydroxy and 9-keto metabolites.

In these experiments, we took advantage of the fact that CO produces no detectable deleterious effects on Day 10 rat embryos cultured for 24 hr (Greenaway *et al.*, 1982). CO exhibited the capacity to inhibit the generation of hydroxylated AAF metabolites by the conceptus as well as the AAF-elicited embryotoxic and dysmorphogenic effects observed in the cultured embryos. We previously demonstrated that CO prevented exogenous bioactivation of cyclophosphamide to embryotoxic intermediates in an embryo culture system (Greenaway *et al.*, 1982). CO is a time-honored and specific inhibitor of *P*-450-mediated hydroxylation reactions and is an especially valuable tool in these kinds of studies not only because of its lack of embryotoxicity but also because other inhibitors would likely be much less useful. *P*-450 antibodies, for example, would probably not transfer across the visceral yolk sac and other membranes in sufficient quantities to produce effective inhibition. Other known inhibitors of *P*-450 (e.g., 7,8-benzoflavone) would likely be embryotoxic themselves at the concentrations required to effectively inhibit the monooxygenation reactions.

The observed capacity of CO to prevent AAF from eliciting malformations (as well as inhibiting biotransformation in MC-induced conceptuses) is readily explicable only in terms of *P*-450-dependent generation of dysmorphogenic intermediates in tissues of the conceptus. That the early conceptus can bioactivate sufficient quantities of AAF to produce gross structural abnormalities is of great importance in considerations of chemical teratogenesis. It will be of considerable interest to determine whether *P*-450s of the conceptus may be capable of catalyzing the conversion of other chemicals to embryotoxic intermediates. Additionally of importance is the question as to whether the embryo proper or other membranes (e.g., yolk sac) are primarily responsible for the bioactivation.

TABLE 3

EFFECTS OF 3-METHYLCHOLANTHRENE (MC) PRETREATMENT OF SPECIFIC METABOLITES OF 2-ACETYLAMINOFLUORENE BY HOMOGENATES OF DAY 10 RAT CONCEPTUSES[a]

Cochromatographing metabolite(s)	Specific activities (pmol/mg/2 hr)		Ratio (induced/control)
	Control	MC pretreated	
7-Hydroxy	0.28 ± 0.11	8.08 ± 1.31	28.9
9-Hydroxy	0.21 ± 0.18	1.04 ± 0.53	5.0
5-Hydroxy	ND[b]	11.0 ± 2.94	NC[c]
9-Keto	0.41 ± 0.20	1.33 ± 0.48	3.2
3-Hydroxy	ND	6.60 ± 1.24	NC
1-Hydroxy	ND	0.40 ± 0.29	NC
N-Hydroxy	ND	0.15 ± 0.07	NC

[a] $\bar{x}$ ± SD of three experiments.

[b] ND indicates not detectable or not significantly different from background values. Detection limit was approximately 0.05 pmol/mg/2 hr.

[c] NC indicates not calculated because controls were not detectable.

Previous studies have shown that AAF can produce teratogenic effects in rats (Alexandrov, 1973), chicks (Jelinek, 1982), and mice (Izumi, 1962a,b) following administration *in vivo*. Skeletal malformations were observed in mice and chicks and hydrocephalus was elicited in rats. Relatively large doses were required to produce these effects and, in view of the potent mutagenicity and carcinogenicity of AAF, a rationale for its apparently weak teratogenic effect *in vivo* remains to be elucidated.

REFERENCES

ALEXANDROV, V. A. (1973). Embryotoxic and teratogenic effects of chemical carcinogens. *IARC Sci. Publ.* **4,** 112–126.

BRADFORD, M. M. (1976). A rapid, sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* **72,** 248–254.

FAUSTMAN-WATTS, E. M., GREENAWAY, J. C., NAMKUNG, M. J., FANTEL, A. G., AND JUCHAU, M. R. (1983). Teratogenicity *in vitro* of 2-acetylaminofluorene. *Teratology* **27,** 19–28.

FAUSTMAN-WATTS, E. M., NAMKUNG, M. J., GREENAWAY, J. C., AND JUCHAU, M. R. (1985). Analysis of metabolites of 2-acetylaminofluorene generated in an embryo culture system: Relationship of biotransformation to teratogenicity *in vitro*. *Biochem. Pharmacol.* **34,** 2953–2959.

FILLER, R., AND LEW, K. J. (1981). Developmental onset of mixed-function oxidase activity in preimplantation mouse embryos. *Proc. Natl. Acad. Sci. USA* **78,** 6991–6995.

GALLOWAY, S. M., PERRY, P. E., MENESES, J., NEBERT, D. W., AND PEDERSEN, R. A. (1980). Cultured mouse embryos metabolize benzo[*a*]pyrene during early gestation: Genetic differences detectably by sister chromatid exchange. *Proc. Natl. Acad. Sci. USA* **77,** 3524–3528.

GREENAWAY, J. C., FANTEL, A. G., SHEPARD, T. H., AND JUCHAU, M. R. (1982). The *in vitro* teratogenicity of cyclophosphamide in rat embryos. *Teratology* **25,** 335–343.

IZUMI, T. (1962a). Developmental anomalies in offspring of mice induced by administration of 2-acetylaminofluorene during pregnancy. *Acta Anat. Nippon* **37,** 239–249.

IZUMI, T. (1962b). Congenital skeletal abnormalities in offspring of mice induced by administration of 2-acetylaminofluorene during pregnancy. *Acta Anat. Nippon* **37,** 250–259.

JELINEK, R. (1982). Use of chick embryos in screening for embryotoxicity. *Teratog. Carcinog. Mutagen.* **2,** 255–261.

JUCHAU, M. R., GIACHELLI, C. M., FANTEL, A. G., GREENAWAY, J. C., SHEPARD, T. H., AND FAUSTMAN-WATTS, E. M. (1985). Effects of 3-methylcholanthrene and phenobarbital on the capacity of embryos to bioactivate teratogens during organogenesis. *Toxicol. Appl. Pharmacol.* **80,** 137–146.

JUCHAU, M. R., BARK, D. H., SHEWEY, L. M., AND GREENAWAY, J. C. (1985b). Generation of reactive

dysmorphogenic intermediates by rat embryos in culture: Effects of cytochrome *P*-450 inducers. *Toxicol. Appl. Pharmacol.* **81,** 533–544.

LAMBERT, G. H., AND NEBERT, D. W. (1977). Genetically mediated induction of drug metabolizing enzymes associated with congenital defects in the mouse. *Teratology* **16,** 147–153.

NEW, D. A. T. (1978). Whole embryo culture and the study of mammalian embryos during organogenesis. *Biol. Rev.* **53,** 81–122.

PEDERSEN, R. A., MENESES, J., SPINDLE, A., WU, K., AND GALLOWAY, S. M. (1985). Cytochrome P-450 metabolic activity in embryonic and extraembryonic tissue lineages of mouse embryos. *Proc. Natl. Acad. Sci. USA* **82,** 3311–3315.

SHUM, S., JENSEN, N. M., AND NEBERT, D. W. (1979). The Ah locus: *In utero* toxicity and teratogenesis associated with genetic differences in benzo[*a*]pyrene metabolism. *Teratology* **20,** 365–376.

SMITH, C. L., AND THORGEIRSSON, S. S. (1982). An improved high-pressure liquid chromatographic assay for 2-acetylaminofluorene and eight of its metabolites. *Anal. Biochem.* **113,** 62–66.

STEELE, R. G. D., AND TORRIE, J. H. (1960). *Principles and Procedures of Statistics.* McGraw–Hill, New York.

ELAINE M. FAUSTMAN-WATTS

Department of Environmental Health
School of Public Health

CECELIA M. GIACHELLI
MONT R. JUCHAU[2]

Department of Pharmacology
School of Medicine
University of Washington
Seattle, Washington 98195
Received August 20, 1985

[2] To whom all correspondence should be addressed.