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Developmental onset of mixed-function oxidase activity in preimplantation mouse embryos

(carcinogen activation/embryonic development/teratogenesis)

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ABSTRACT **Two-cell embryos, obtained from the C57BL/6N and DBA/2N strains, were cultured in media that supported *in vitro* differentiation and that contained [^{3}H]benzo[*a*]pyrene. High-pressure liquid chromatography of the activated intermediates formed during *in vitro* early embryonic development indicated that the onset of polynuclear aromatic hydrocarbon activation coincided with blastocyst formation. Comparison of individual oxygenated intermediates metabolically formed from embryos genetically "responsive" or "nonresponsive" to aromatic hydrocarbons revealed significant quantitative differences in the production of dihydrodiol, quinone, and phenolic derivatives. In addition to exhibiting basal mixed-function oxidase activity, blastocysts were also responsive to enzymatic induction when exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. The presence of operative metabolite-detoxifying pathways was also assayed. Enzymatic treatment of water-soluble metabolites with β-glucuronidase or arylsulfatase revealed that neither glucuronic acid conjugates nor sulfate ester derivatives were present. These data, therefore, provide direct evidence that late preimplantation mouse embryos (day 3½ of gestation) are similar to later developmental stages in having the enzymatic capability for xenobiotic activation and enzyme induction but are dissimilar with respect to their detoxification mechanism(s). Moreover, the ability of preimplantation embryos to activate directly polynuclear aromatic hydrocarbon to bioreactive intermediates may be of importance in assessing the ontological susceptibility of the developing embryo to carcinogenic or teratogenic chemicals.**

The polycyclic aromatic hydrocarbon benzo[*a*]pyrene (B*a*P) is representative of a class of environmental agents that require biotransformation by the microsomal mixed-function oxidase system (MFO) to produce reactive intermediates (1). In various strains of mice, there is a genetic difference in the inducibility of aryl hydrocarbon hydroxylase (2) and of several other enzyme components of the MFO system (3). This genetic difference, as observed between C57BL/6N (responsive) and DBA/2N (nonresponsive) mice, has been correlated with a differential susceptibility to B*a*P-induced developmental effects (4–6).

The influence of environmental chemicals on mammalian embryonic development may result in selective embryotoxic effects, in modifying cellular division rates, or in altering the differentiational potential of primordial cell types (reviewed in refs. 7 and 8). The extent of embryonic response in part may be influenced by the mother's or developing embryo's metabolic activities in producing bioreactive species (9). Previous investigations have concentrated on determining metabolic activities in maternal and in middle to late gestational fetal systems (10–12). A recent report on B*a*P-induced sister chromatid exchange in postimplantation mouse embryos suggests that the activating enzymes in biotransformation pathways may be functioning at gestational day 7½ (13).

The role of activation and deactivation reaction pathways during the preimplantation period of embryonic development has not been investigated because of the requirement for sensitive analytical techniques to detect subtle levels of reactive intermediates produced by limited amounts of early mammalian embryos. The purpose of this investigation was to determine the *in vitro* metabolic activity of preimplantation mouse embryos in order to define better the enzymatic capability of the overall gestational period to activate and detoxify xenobiotics. To accomplish this goal we used B*a*P as a probe for detecting the developmental onset of early embryonic metabolism and high-pressure liquid chromatography (HPLC) as a sensitive analytical technique.

MATERIALS AND METHODS

Chemicals. [G-^{3}H]B*a*P (37 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels), obtained from Amersham, was purified before use by HPLC. Nonradioactive derivatives of B*a*P and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) were provided by the Cancer Research Program, Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, MD.

Embryos. Female C57BL/6N and DBA/2N mice between 10 and 16 weeks of age were superovulated with hormone injections as described (14), mated with adult males of the same strain, and checked for vaginal plugs the next day. Approximately 300 embryos were flushed from the oviducts at the 2-cell stage and cultured to the 8-cell, morula, and late blastocyst stages in Brinster's medium (15) supplemented with 6% calf serum and 50 μCi of [^{3}H]B*a*P. Induction experiments were performed by incubating early preimplantation mouse embryos to the late blastocyst stage in the presence of TCDD at 0.2 ng/ml and radioactive B*a*P. Preliminary experiments with TCDD indicated that there was no embryotoxic effect at this concentration of inducer.

Metabolite Analysis. Incubations were terminated at the 8-cell, morula, and late blastocyst stages. Embryo cultures were extracted with ethyl acetate and the organic solvent extract was prepared for HPLC as described (16). Metabolites were separated for identification by using a DuPont 850 instrument fitted with a Zorbax C_8 column. Gradient elution was performed with a reverse-phase from an initial solvent ratio of 58% methanol/42% water to a final composition of 100% methanol. Individual

Abbreviations: B*a*P, benzo[*a*]pyrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; HPLC, high-pressure liquid chromatography; MFO, mixed-function oxidase; 9,10-diol, 9,10-dihydro-9,10-dihydroxybenzo[*a*]pyrene; 4,5-diol, 4,5-dihydro-4,5-dihydroxybenzo[*a*]pyrene; 7,8-diol, 7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene; 1,6-quinone, benzo[*a*]pyrene-1,6-quinone; 3,6-quinone, benzo[*a*]pyrene-3,6-quinone; 6,12-quinone, benzo[*a*]pyrene-6,12-quinone; 9-OH-B*a*P, 9-dihydroxybenzo[*a*]pyrene; 3-OH-B*a*P, 3-hydroxybenzo[*a*]pyrene.

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metabolites of B*a*P were identified by cochromatography of nonradioactive derivatives.

Determination of Water-Soluble Conjugates. Glucuronide and sulfate esters were assayed independently on media derived from blastocyst incubations containing [^{3}H]B*a*P. For the determination of glucuronide derivatives, media were extracted four times with 2 vol of ethyl acetate. The reaction mixture consisted of medium, 0.1 M phosphate buffer (pH 7.1), and 5000 Fishman units of β-glucuronidase (EC 3.2.1.31; from *Escherichia coli*) per ml. After incubating for 2 hr at 37°C, the reaction mixture was extracted twice with 5 vol of ethyl acetate. Enzymatically released metabolites were identified as a decrease in water-soluble radioactivity. The effectiveness of β-glucuronidase on the hydrolysis of glucuronide conjugates was determined initially by subjecting radiolabeled medium, obtained from incubating hamster embryo cells with [^{3}H]B*a*P, to β-glucuronidase treatment. Under our incubation conditions, there was a 47% release of water-soluble radioactivity (unpublished data). To assay for the presence of sulfate esters culture medium was incubated for 2 hr at 37°C in 0.2 M sodium acetate (pH 5.0) containing 100 units of arylsulfatase (EC 3.1.6.1; from *Patella vulgata*). The entire reaction mixture was extracted with ethyl acetate and the sample was prepared for HPLC analysis by the procedures described in the previous section. Release of sulfate esters was determined directly as an increase in the quantity of individual metabolites in the organic phase after enzyme treatment.

RESULTS

Developmental Onset of MFO Activity in Preimplantation Embryos. The metabolic capabilities of responsive and nonresponsive preimplantation mouse embryos were determined during the 2-cell to blastocyst stages of development. HPLC of the organic solvent-extractable metabolites obtained at the 8-cell and morula developmental end points indicated that the majority of the radioactivity was unmetabolized parent hydrocarbon (Fig. 1). However, the HPLC elution profiles of the metabolites formed by responsive and nonresponsive blastocysts revealed a marked biotransformation of B*a*P to several activated intermediates, with the dihydrodiols being the most abundant class. The overall metabolic activity of C57BL/6N blastocysts was 1.5 times greater than that of DBA/2N blastocysts (Table 1). Of the vicinal glycols, C57BL/6N blastocysts formed 77% more 9,10-diol and 53% more 7,8-diol than did DBA/2N blastocysts. Both genetic strain embryos, however, formed similar quantities of the K-region diol. Quinones were also present in incubations from C57BL/6N and DBA/2N blastocysts and represented 29% and 38%, respectively, of the total metabolites. The phenolic derivatives of B*a*P were also major oxygenated intermediates formed by the blastocysts. Responsive strain blastocysts formed 2.5 times more of the monohydroxylated species than did nonresponsive strain blastocysts, and the ratio of 3-OH- to 9-OH-B*a*P was 1.5 for C57BL/6N embryos; the production of these phenols was approximately equivalent for DBA/2N embryos.

Induction of MFO System in Responsive and Nonresponsive Blastocysts. The presence of MFO activity in early mouse embryos suggested the possibility that this enzyme system might be sensitive to induction. Fig. 2 shows the HPLC separation of B*a*P-activated intermediates obtained from the organic solvent extraction of media from TCDD-treated C57BL/6N and DBA/2N blastocysts. The overall metabolic activation of parent hydrocarbon by responsive and nonresponsive embryos increased 4.3 and 2.2-fold, respectively (Table 1). Furthermore, all oxygenated intermediates were increased significantly by blastocysts from both genetic strains for each of the primary

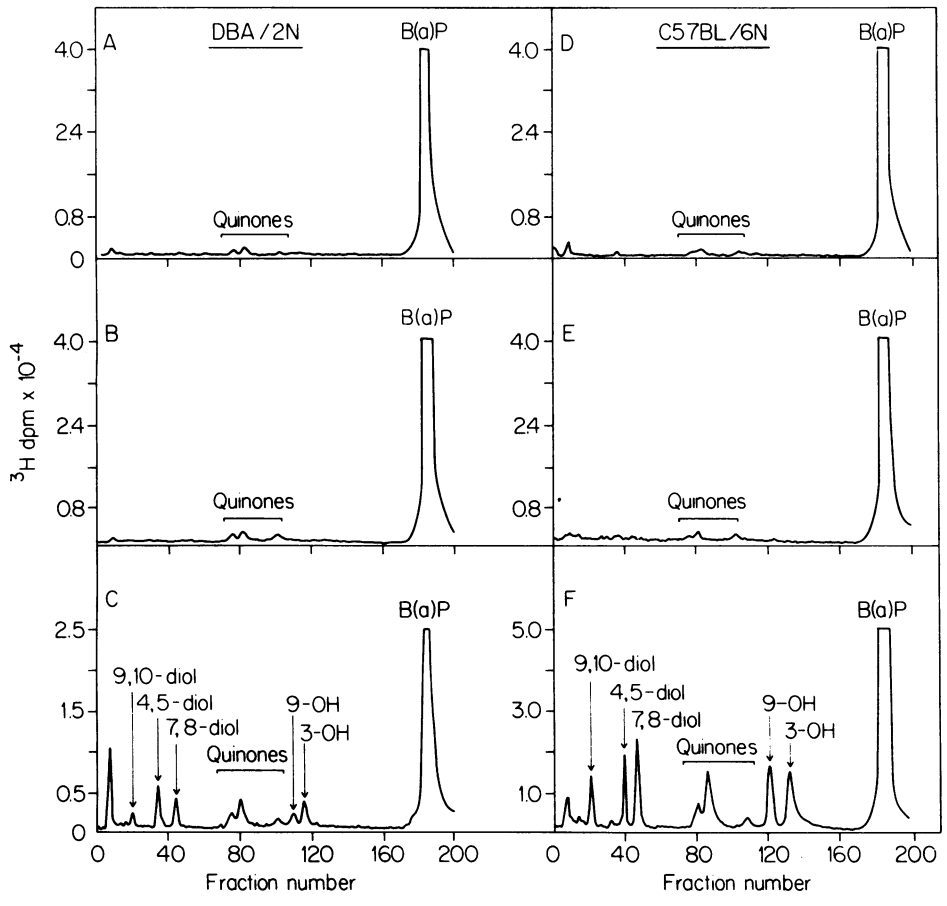

FIG. 1. Developmental onset of metabolic activation capability in murine preimplantation mouse embryos. DBA/2N and C57BL/6N 2-cell embryos were cultured to the 8-cell (*A* and *D*), morula (*B* and *E*), or late-blastocyst (*C* and *F*) stage of development in the presence of [^{3}H]B*a*P. Culture media were extracted with organic solvent, and equivalent amounts of radioactivity were analyzed by HPLC. Overall metabolism of parent hydrocarbon by DBA/2N blastocysts (*C*) was 3.1%, of which 1.1% was water-soluble products (50 fmol per embryo) and 2% was organic solvent-soluble products (90 fmol per embryo). C57BL/6N blastocysts (*F*) metabolized 5.4% of the input, of which 2.5% was water-soluble products (113 fmol per embryo) and 2.9% was organic solvent-soluble metabolites (131 fmol per embryo). Values are the mean of three independent determinations.

Table 1. Genetic differences in metabolism of BaP by preimplantation mouse blastocysts

Metabolite	DBA/2N fmol/embryo No TCDD	DBA/2N fmol/embryo With TCDD	DBA/2N Inducibility ratio	C57BL/6N fmol/embryo No TCDD	C57BL/6N fmol/embryo With TCDD	C57BL/6N Inducibility ratio
9,10-diol	10.8	29.3	2.7	19.1	64.7	3.4
4,5-diol	24.6	55.4	2.3	20.7	44.8	2.2
7,8-diol	20.1	30.1	1.5	30.8	116.1	3.8
1,6-quinone	11.8	28.5	2.5	16.6	49.8	3.0
3,6-quinone	17.7	39.6	2.2	32.5	285.4	8.8
6,12-quinone	25.2	41.6	1.7	10.1	33.2	3.3
9-OH-BaP	11.8	44.6	3.8	24.9	145.2	5.8
3-OH-BaP	12.9	23.2	1.8	37.0	75.5	2.0

DBA/2N and C57BL/6N embryos were incubated with [^{3}H]BaP in the presence or absence of TCDD. The culture media were extracted with ethyl acetate and then an aliquot was subjected to HPLC. The amount of each metabolite was determined from the HPLC chromatograms and then corrected for the total fmol found in the original organic solvent-soluble fraction. Values are expressed as the mean of three independent determinations.

metabolite classes. C57BL/6N blastocysts biotransformed BaP more effectively than did DBA/2N blastocysts.

Of particular interest is the difference between genetically responsive and nonresponsive embryos in the formation of dihydrodiols and phenols after treatment with TCDD. In C57BL/6N embryo incubations, the 7,8-diol represented more than half of the total extractable diols, and genetically responsive blastocysts produced 3.9-fold more of this activated intermediate than did genetically nonresponsive blastocysts. In contrast to the amount of 7,8-diol extractable from C57BL/6N embryo media, DBA/2N embryos accumulated the 4,5-diol intermediate as an equivalently proportional amount of its total diols. Genetically responsive and nonresponsive embryos produced quantitatively similar amounts of the K-region diol. With regard to phenol formation, C57BL/6N and DBA/2N blastocysts produced an increase of 3.6- and 2.8-fold, respectively, in the total phenolic products. Concomitant with this induction in monohydroxylated derivatives, responsive and nonresponsive blastocysts had a significant increase in accumulation of 9-OH-BaP relative to 3-OH-BaP. The ratio of 9-OH derivative to the 3-OH derivative, consequently, varied 10-fold for C57BL/6N embryos and 2-fold for DBA/2N embryos. In parallel with these TCDD-mediated increases in reactive metabolites, there was a 3.4-fold increase in quinone formation from responsive blastocysts relative to that from nonresponsive blastocysts.

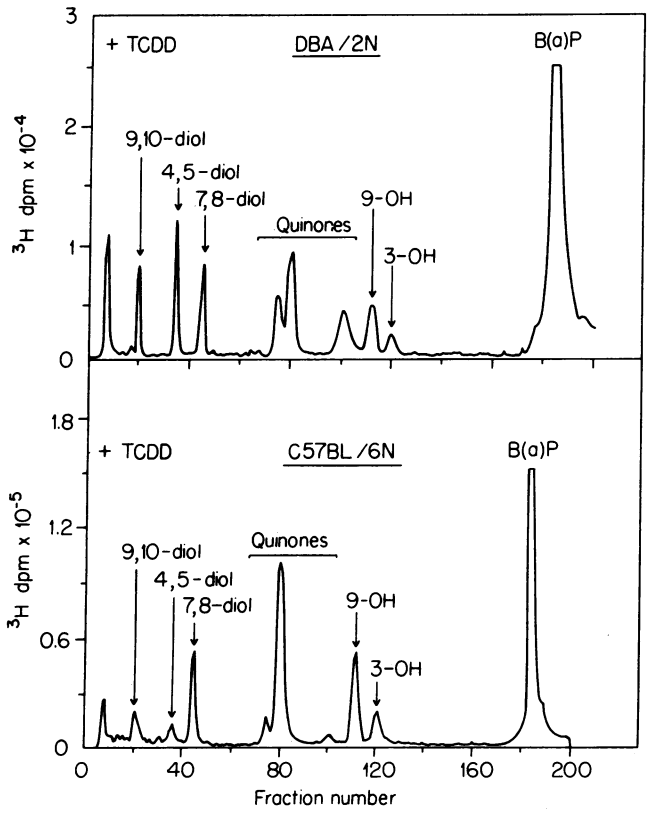

FIG. 2. Induction of MFO activity in murine blastocysts. Responsive and nonresponsive embryos were incubated in the presence of 0.2 ng of TCDD and 50 μCi of [^{3}H]BaP. Overall metabolism of [^{3}H]BaP by TCDD-treated DBA/2N blastocysts was 5% of the input, of which the amount of water-soluble products formed was similar to that in uninduced incubations, but there was a 2-fold increase in formation or organic solvent-soluble metabolites (180 fmol per embryo). TCDD-treated C57BL/6N blastocysts metabolized approximately 15% of the input. Although the amount of water-soluble products formed was similar to that in Fig. 1, there was a 4-fold increase in formation of ethyl acetate-soluble metabolites (689 fmol per embryo).

Determination of Early Embryonic Detoxification Pathways. In order to define more fully the overall characteristics of xenobiotic biotransformation by preimplantation embryos, we assayed for the ability of blastocysts to detoxify hydrocarbon oxidation products by means of conjugation with glucuronic acid or with sulfate. Media from C57BL/6N and DBA/2N blastocyst incubations were treated either with β-glucuronidase to hydrolyze glucuronide conjugates or with arylsulfatase to hydrolyze sulfate ester derivatives. The data in Tables 2 and 3 clearly demonstrate that neigher β-glucuronidase nor arylsulfatase had any effect on releasing water-soluble metabolites from the media. Although we have demonstrated that TCDD is an effective inducer in blastocysts for the bioactivation of BaP, no analogous inductive effect was observed for either of these two conjugation pathways (unpublished data).

DISCUSSION

The assessment of embryonic and fetal xenobiotic activation and deactivation reactions is crucial in the determination of potential developmental effects that may arise from exposure of pregnant mammals to environmental agents. Although previous investigations (10) have attempted to elucidate the biotransformational capabilities associated with maternal and fetal systems, these studies have been ambigous because of the possibility of transplacental transfer of maternally produced reactive intermediates. The *in vitro* culturing of early pre- and postimplantation embryos offers an experimental approach for the une-

Table 2. Effect of β-glucuronidase treatment on conjugated metabolites of BaP formed by blastocysts

Genetic strain	Water-soluble radioactivity remaining after enzyme treatment	
	Control	β-Glucuronidase
C57BL/6N	1.30	1.33
DBA/2N	0.57	0.56

Media obtained from embryo cultures that had been incubated in the presence of [^{3}H]BaP were extracted with ethyl acetate, and the aqueous phase was incubated for 2 hr with β-glucuronidase at 5000 units/ml. Values are expressed as dpm × 10^{-6}.

Table 3. Effect of arylsulfatase treatment on conjugated metabolites of BaP formed by blastocysts

Genetic strain	% of total ethyl acetate extractable radioactivity					
	9,10-diol	4,5-diol	7,8-diol	Quinones	9-OH-BaP	3-OH-BaP
C57BL/6N						
Control	9.2	10.0	14.9	28.6	12.1	17.9
Arylsulfatase	9.0	9.8	15.0	29.1	11.9	18.1
DBA/2N						
Control	8.0	18.2	14.9	40.6	8.8	9.6
Arylsulfatase	7.8	17.9	15.0	41.0	9.0	9.8

The ability of arylsulfatase to release sulfate conjugates was determined directly by incubating media from embryos that had been cultured in the presence of [^{3}H]BaP with enzyme (100 units/ml) for 2 hr. The media were then extracted with ethyl acetate and equivalent amounts of radioactivity were prepared for HPLC. The percentage recovery of total metabolites was similar for the organic solvent extracts from control and enzyme-treated media. Values are expressed as the mean of three independent determinations.

quivocal identification of an enzyme system(s) that potentiates the detrimental effects of a biologically inert parent compound by activating it to various electrophiles. The data presented here clearly demonstrate that the onset of measurable MFO activity occurs at the blastocyst stage of embryonic development, which is equivalent to gestational day 3½. We have shown directly, by HPLC that late preimplantation embryos activate BaP to diols, quinones, and phenolic derivatives. In addition, the genetic difference associated with the *Ah* locus that is observed in fetuses and adults (2, 17) is expressed in blastocysts derived from responsive and nonresponsive strains of mice. Futhermore, the inducibility of the early embryonic MFO suggests that cellular regulatory mechanisms present at later developmental stages (18, 19) are also operative in blastocysts.

In mammalian development, the earliest indication of morphological differentiation of embryonic cells occurs at the blastocyst stage, at which time blastomeres segregate into two distinct populations, a peripheral single-cell layer (trophectoderm), and a group of cells, the inner cell mass, positioned to one side of the blastocoele cavity (20). The developmental fate of the progeny of these two cell populations is also distinct. Totipotency is restricted to the cells of the inner cell mass; these give rise primarily to the adult organism. The cells of the trophectoderm form extraembryonic membranes exclusively (21). This divergence of developmental fate may also mark the onset of expression of new genetic information such that those cells committed to the formation of the embryo proper develop specific metabolic capabilities. Trophectoderm and inner cell mass are distinguishable by their rates of cellular division, enzyme activities, cell surface properties, and susceptibilities to antimetabolites and radiation (22). Recent observation in our laboratory have demonstrated that pluripotent embryonal carcinoma cells isolated from murine teratocarcinomas experimentally induced in responsive and nonresponsive mice (16) express their genetic difference in the metabolic activation of BaP, as do the blastocysts. Because embryonal carcinoma cells have been shown to be developmentally analogous to inner cell mass cells (23), this suggests that MFO activity in blastocysts is probably associated with the inner cell mass. The inability of blastocysts and embryonal carcinoma cells to detoxify reactive intermediates by glucuronide or sulfate ester formation is additional supportive evidence for biochemical analogy between the inner cell mass and embryonal carcinoma cells. Therefore, our results demonstrating the presence of MFO activity in blastocysts is consistent with previous data on early embryonic macromolecular differentiational processes.

Various exogenous chemical agents can penetrate and accumulate in the developing preimplantation embryo (24). These chemicals have the potential to affect adversely the development of the embryo (25) or to interfere with implantation (26). In this regard, *in vivo* experiments with rabbit blastocysts (27) have demonstrated that the preimplantation embryo appears to have specialized regulatory system(s) for the accumulation of chemical agents. Although a relatively small fraction of the initially administered maternal dose is accumulated by blastocysts, there is a significant delay in the rate of clearance from the embryo in comparison to that from the maternal system (27). This delay could allow for more extensive biotransformation of unreactive parent compound. The persistence of reactive electrophiles within the developing embryo affords the opportunity for subsequent interactions with critical embryonic target cells during the developmentally sensitive period of organogenesis.

Therefore, our present observations suggest that in addition to its potential vulnerability to maternally derived metabolites, the early developing embryo may also be susceptible to embryo-derived reactive intermediates that are in close proximity to active regions of embryonic cell proliferation.

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