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Metabolism of 7,12-Dimethylbenz[a]anthracene by Mouse Mammary Cells in Serum-Free Organ Culture Medium

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Abstract—*The murine mammary gland is a prime target organ for* 7,12-*dimethylbenz[a]anthracene,* (*DMBA*)*-induced carcinogenesis. We analyzed the metabolism of* ^{3}H-*DMBA in a cell-free microsomal activation system derived from mouse mammary microsomes and in a whole mammary organs in culture. The* in vitro *microsomal activation system failed to show the more polar diol derivative of DMBA afer HPLC. The metabolites obtained directly from the* ^{3}H-*DMBA-treated whole mammary organs, however, revealed the presence of both the diol as well as the phenolic derivatives of DMBA. Analysis of the glands and the culture medium further showed that nearly* 95% *of the radioactivity added to the culture medium was associated with the adipose tissue and complete solubilization of the fat pad released substantial amounts of DMBA and its metabolites. It appears that a large portion of DMBA and its metabolites remain entrapped in the adipose tissue surrounding the parenchyma. Formic acid digestion of the gland releases the DMBA and the metabolites allowing their ethylacetate extraction, and HPLC characterization.*

INTRODUCTION

7,12-DIMETHYLBENZ[a]ANTHRACENE (DMBA), a potent oncogenic hydrocarbon, can induce tumors in the mammary gland and in a variety of other organs of experimental animals [1–3]. Moreover, DMBA can induce preneoplastic and neoplastic transformation of mouse mammary epithelial cells also in an isolated whole organ in a hormonally defined serum-free culture medium [4]. Also the mammary cell transformation model in organ culture provides a suitable *in vitro* system for studies on the chemoprevention of DMBA-induced transformation of the mammary cells [5–7]. The procarcinogen DMBA is known to be metabolized by the cellular mixed function mono-oxygenase system yielding the reactive electrophilic derivatives, diol epoxides which alkylate cellular DNA forming covalent adducts, consequent mutagenesis, and initiation of transformation [8, 9].

However, despite the fact that DMBA is a potent mammary carcinogen [1] there has been no previous study on the fate of DMBA in the mammary gland *in vivo* or *in vitro*, although binding of polycyclic aromatic hydrocarbon (PAH) carcinogens to mammary cell DNA has been observed [10–12]. A few recent studies have measured the metabolism of carcinogenic PAH including DMBA in monolayer cultures of mammary cells from rat and human [13–14]. However, a determination of the endogenous metabolites obtained directly from the intact mammary gland is desirable and the results then are likely to reflect the metabolic events that occur within the mammary organ. The organ culture model of the mouse mammary gland provides the conditions for these determinations and in the present paper we report the results of the studies on DMBA metabolism in the isolated whole mammary organ in a hormonally define serum-free culture medium.

MATERIALS AND METHODS

Chemicals

DMBA was purchased from Sigma Chemical Co., St. Louis, Missouri (95% carcinogenic). ^{3}H-DMBA (specific activity 50 μCi/mmole) was sup-

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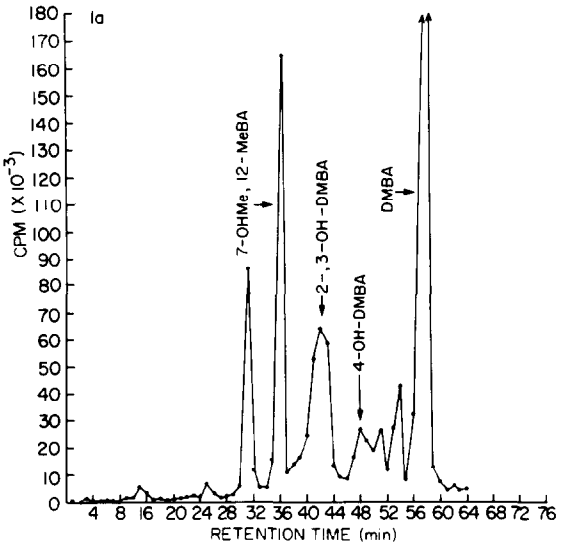

Fig. 1. HPLC profiles of the ethylacetate : acetone (2 : 1) extractable metabolites of ^{3}H-DMBA activated by cell-free mammary microsomal systems was derived from mouse microsomes. 2 × 10^6 cpm was injected for HPLC analysis. The metabolites were analyzed on an ODS column (4.6 × 150 mm) using a methanol : water gradient described by Chou and Yang [16]. Fractions were collected at 1 min intervals. Arrows indicate the elution time of the reference standards. The minced mammary tissue was homogenized in a sucrose–Tris buffer (0.5 M Tris, sucrose 0.05 M, Tris–HCl, pH 7.5). Microsomes were obtained by centrifugation of the post-mitochondrial supernatant at 105,000 g for 60 min at 4° C. Metabolites were obtained by in vitro incubation of ^{3}H-DMBA (2.5 μmol; 10 μCi/reaction mixture) in the dark at 37° C in a 3 ml reaction mixture containing 0.15 mmol Tris–HCl pH 7.5, 0.009 mmol $MgCl_2$, 0.3 units of glucose-6-phosphate dehydrogenase (type X, Sigma, St. Louis, Missouri, U.S.A.), 3 mg $NADP^+$, 1.95 mg glucose-6-phosphate and 1.5 mg protein equivalent of mammary microsomes from methylcholanthrene-induced mice. The metabolites were extracted 3 times with 2 vol. of ethyl-acetate : acetone mixture (2 : 1, v/v). The extract was then dried under a stream of nitrogen and the residue was dissolved in 300 μl of HPLC grade methanol. For HPLC 100 μl of this preparation was used.

plied by Amersham/Searle Corporation, Arlington Heights, Illinois. ^{3}H-DMBA was purified as described by DiGiovanni *et al.* [15], purity 98%. DMBA metabolites used as markers were obtained from the National Cancer Institute (NCI) Chemical Repository, National Institutes of Health, Bethesda, Maryland, U.S.A. DMBA-*cis*-5,6-diol, also obtained from NCI repository, was a gift from Dr. S. Nandi, University of California, Berkeley.

Mammary microsomal assay

Female BALB/c mice weighing 20–22 g were induced by intraperitoneal injections of 3-methylcholanthrene (25 mg/kg body wt), the animals were killed by cervical dislocation and the mammary or the liver microsomes were prepared and the cell-free monoxygenase assay was done according to the standard procedure [16]. Additional details of the procedures are in the legend of Fig. 1.

Organ culture

The procedures for organ culture of the whole mammary gland have been described [17, 18]. Briefly, three-week-old female BALB/c mice were primed daily by subcutaneous injections of 1 μg estradiol-17β and 1 mg progesterone for 9 days as a prerequisite for the whole mammary gland organ culture procedure. On the 10th day the entire second thoracic mammary gland was excised on a dacron raft and incubated in a 95% O_2 and 5% CO_2 atmosphere at 37° C in Waymouth's medium MB752/1, supplemented with the hormones insulin, prolactin, hydrocortisone (5 μg/ml each) and aldosterone (1 μg/ml) to obtain full lobulo-alveolar development. In accordance with the protocol for the neoplastic transformation studies described [19, 20] the glands were exposed to 7.8 μM ^{3}H-DMBA (20 μCi/ml) for 24 hr between the 3rd and 4th day of culture (initiation stage). The cultures were terminated at 16 and 24 hr after exposure to DMBA. DMSO was used as a vehicle for DMBA and its concentration in medium was 0.1%. A concentration of 7.8 μM DMBA is optimal for neoplastic transformation of the mammary cells in organ culture [19].

DMBA metabolites from the mammary glands and the culture medium

After termination of the cultures following exposure of the glands to ^{3}H-DMBA, the tissue was weighed and frozen in liquid nitrogen. The tissue and the frozen culture medium were then stored separately at −70° C. For each HPLC analysis metabolites from 4 mammary glands (≃ 80 mg) were used. The tissue samples were suspended in 2 ml of formic acid (88% v/v) containing 2 mM tocopheral as an antioxidant. The mixture was then vigorously agitated on a vortex for 10–15 min. The turbid suspension then was extracted 3 times with 2 vol. of ethylaceate : acetone mixture (2 : 1 v/v). The ethylacetate extracts were then dried under a stream of nitrogen and the residue was dissolved in 400 μl of HPLC grade methanol. In order to prevent photolysis of the metabolites all extraction procedures were done under a Kodak safety light (type GBX). For analysis of the medium, 4 ml of the culture medium was extracted twice with 2 vol. of ethylacetate : acetone mixture (2 : 1 v/v).

DMBA metabolites in collagenase dissociated cells

Mammary glands in organ culture were treated with ^{3}H-DMBA for 16 and 24 hr. Epithelial cells were then isolated by the collagenase dissociation procedure [21]. The single cell suspension was pelleted by centrifugation at 700 rpm for 10 min and the cell pellet was lysed in a hypotonic buffer (0.015 M NaCl; 0.01 M Tris, pH 7.4, 1 mM $MgCl_2$). The metabolites from the lysate were

extracted by the ethyl-acetate extraction procedure described above.

Reversed phase HPLC

Metabolites of DMBA were separated on HPLC using a Beckman system equipped with an ultrasphere octadecyl silane column (4.6 × 150 mm). The columns were maintained at ambient temperature with a contant flow rate of 0.8 ml/min, and the metabolites were eluted with a methanol : water gradient system as described by DiGiovanni *et al.* [15] and Chou *et al.* [16]. The eluates were monitored at 254 nm.

RESULTS

DMBA metabolites in the mammary microsomal system in vitro

Figure 1 shows the metabolites of DMBA following the *in vitro* incubation of 80 μM ^{3}H-DMBA with mouse mammary microsomes in a cell-free NADPH-regenerating system. While significant amounts of the phenols were present in the HPLC eluate, only a trace amount of the diols, which elute early in the polar region, were detectable in the microsomal reaction product. A similar *in vitro* assay derived from 3-MCA-induced rat mammary microsomes also showed virtually identical results (data not shown). The reason for the absence of the diol metabolites in the cell-free mammary microsomal reaction product is unclear at this time, although there have been reports indicating that the cell-free system may not always faithfully show the endogenous metabolites [22, 23]. The reaction products from the cell-free microsomal systems derived from the liver of the same animals, however, showed abundant phenols and the diols (data not shown). Consistent with the virtual absence of the diols in the mammary microsomal system, incubation of exogenous calf thymus DNA (2 mg/ml incubation mixture) in the microsomal activation system showed only a trace amount of binding of the metabolite to DNA. As expected the level of carcinogen binding to exogenous DNA added to the mouse and rat liver microsomal activation system was as high as 166 and 133.09 pmol/mg DNA respectively (Table 1).

Metabolites of DMBA in the mammary glands in the organ culture medium

Of the total organosoluble material extracted from the glands exposed to DMBA in organ culture for 16 or 24 hr, 54% was DMBA and the remaining 46% constituted the metabolites of DMBA. Figure 2 shows the HPLC elution profile of the ethyl-acetate : acetone extract of the formic acid digest of cultured mammary glands after being exposed to ^{3}H-DMBA for 24 hr *in vitro*. A prominent peak at about 22 min, eluted just ahead of the DMBA-*cis*-5,6-diol marker. Based on the close relationship with DMBA-*cis*-5,6-diol this peak likely represents DMBA-*trans*-5,6-diol. DMBA-*trans*-8,9-diol and DMBA-*trans*-3,4-diol were also present, eluting with their respective markers. Thus the present studies show that DMBA metabolism in the mouse mammary gland in culture yields the 5,6-diol, 8,9-diol and 3,4-diol derivatives. The HPLC profile in Fig. 2 also shows the 2-, 3-, and 4-phenols, and the 12-hydroxymethyl and 7-hydroxymethyl derivatives were also identified using the appropriate markers. Thus the results demonstrate that the carcinogenic metabolites of DMBA are formed in the mammary gland *in vitro*, and these are extractable directly from the gland. The mammary metabolic derivatives of

Table 1. *Binding of* ^{3}H-*DMBA metabolites to calf thymus DNA*

Microsomal system	DNA binding activity (pmol/mgDNA)
Mouse	
Mammary	24.00
Liver	166.00
Rat	
Mammary	6.60
Liver	133.09

The extent of binding of ^{3}H-DMBA metabolites to exogenous DNA was assessed by including calf thymus DNA in the microsomal activation system derived from the mammary gland and liver of the mouse and rat. The reaction was terminated after 2 hr of incubation; DNA was isolated, purified and the radioactivity associated with the DNA was estimated.

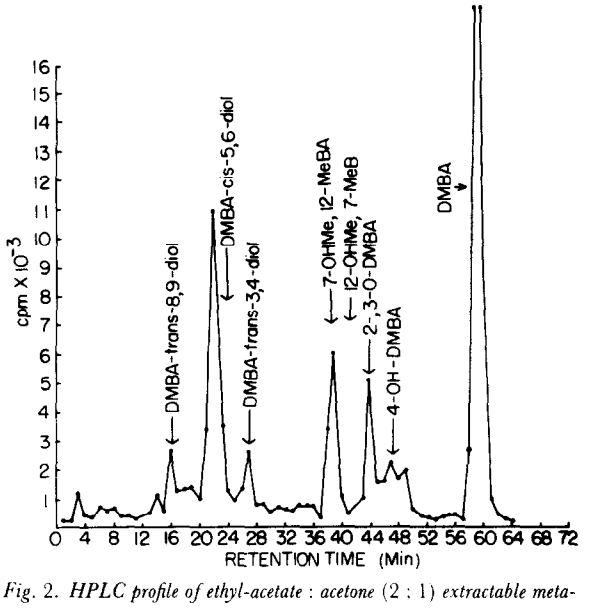

Fig. 2. *HPLC profile of ethyl-acetate : acetone* (2 : 1) *extractable metabolites from formic acid digests of whole mammary glands exposed to* ^{3}H-*DMBA for* 16 hr *in organ culture.* 2 × 10^5 cpm *was injected for HPLC analysis. The metabolites were analyzed on an ODS column* (4.6 × 150 mm) *using a methanol : water gradient* [15]. *Other details are as described in Fig.* 1.

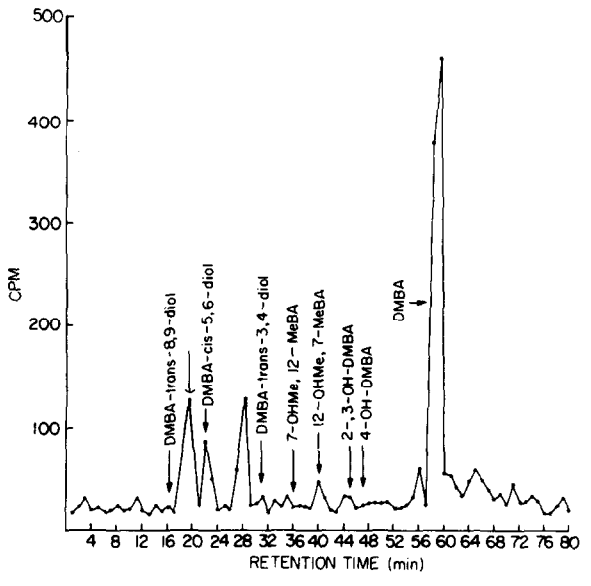

Fig. 3 HPLC profile of ethylacetate : acetone (2 : 1) extractable material from mammary epithelial cells isolated by collagenase dissociation of whole organs exposed to ^{3}H-DMBA for 16 hr in culture. 5000 cpm was injected for HPLC analysis. The metabolites were analyzed on an ODS column (4.6 × 150 mm) using a methanol : water gradient described by DiGiovanni et al. [15]. Arrows indicate the elution time of the metabolites. As stated in the text, radioactivity eluting was very low and in some instances little radioactivity was measurable. Average background in these experiments was 9 cpm, other details are as in Fig. 1.

DMBA, however, did not contain any water-soluble material. The metabolites of the glands treated with ^{3}H-DMBA for 24 hr after exposure to DMBA was similar to that obtained at 16 hr (data not shown). Analysis of ^{3}H-DMBA radioactivity in the culture medium showed that 95% of the DMBA added to the medium remained associated with the mammary glands and 99% of the remaining radioactivity in the medium was organosoluble material. HPLC analysis of the organosoluble material in the medium showed it to be exclusively DMBA. The remaining 1% of the radioactivity (3.9 pmol) in the medium was taken to be as water-soluble conjugates of DMBA, but analysis of the water-soluble fraction according to the method of Autrup *et al.* [24] showed virtually no conjugates, presumably due to an extremely low concentration of the residues. Thus the material in the 1% fraction resistant to extraction remained unidentified.

DMBA metabolites in the collagenase-dissociated mammary epithelial cells

Approximately 61% of the organosoluble material isolated from the dissociated mammary cells was DMBA. Figure 3 shows the HPLC profile of DMBA and its metabolites in the dissociated epithelial cells isolated from 20 cultured mammary glands, exposed to ^{3}H-DMBA for 16 hr. It is evident that the characteristic DMBA diols including the *trans*-5,6-diol, the 12-hydroxymethyl and the phenolic derivatives of DMBA were resolved in the HPLC profile. However, the concentration of the DMBA metabolites in the collagenase-dissociated epithelial cells was much lower that that obtained from the whole mammary glands, even though almost all (97–98%) of the added radioactivity could be accounted for after extraction in both cases. Quantitative analysis of the data further revealed that the metabolites recovered from 4 whole glands was 13.63 nmol, whereas only 613 pmol were recovered from the dissociated epithelial cells pooled from 20 glands. Thus the concentration of the metabolites in the whole mammary gland was 110-fold higher than that obtained from the dissociated cells. The concentration of the metabolites in the epithelial cells from collagenase-digested glands treated with DMBA for 16 hr or 24 hr was essentially similar. Thus the yield of DMBA metabolites from the intact glands is markedly greater than the dissociated glands.

DISCUSSION

Although the ability of DMBA to induce mammary carcinomas in rats and mice is well documented, there has been no study on DMBA metabolism in the mammary gland. Studies on DMBA metabolism in a variety of other organs have employed cell-free microsomal activation systems or monolayer cultures of cells, mostly fibroblasts derived from the organ of interest [25]. Thus, the present report, although belated, constitutes the first study of DMBA metabolism as it occurs within the mouse mammary gland in organ culture. The cell-free microsomal assay system, however, has made it possible to identify the major pathways of DMBA activation, but evidence indicates that the *in vitro* system may not always reflect the endogenous events occurring within the mammary gland [23, 24]. Isolated mammary cells in monolayer culture are capable of generating the polar and non-polar derivatives of DMBA [14]. The present observations in organ culture are thus consistent with the concept that intact cells with a full complement of oxidative and conjugative enzymes would be more appropriate activators of PAH carcinogens [8, 26]. Thus the ability of the isolated mammary organ to metabolize DMBA yielding the expected products is in agreement with the above contention.

Present results thus demonstrate that the mammary glands in culture are capable of activating DMBA yielding both polar and non-polar derivatives and this finding also corroborates our earlier observation on DMBA transformation of the mammary cell in organ culture. Although about 95% of the DMBA added to the culture medium partitioned into the mammary fat pad, dispersion of the mammary fat pad with formic acid released the trapped DMBA and its metabolites, which were mainly

phenols and diols. A small portion of the radioactivity that remained associated with the residue after extraction probably constitutes DMBA metabolites covalently bound to cellular macro-molecules.

In contrast to the observations in mammary epithelial cells in monolayer culture [13, 14], virtually no organosoluble material was released into the organ culture medium. Also no conjugates were formed. These results again may reflect a consequence of trapping of DMBA and its derivatives in the stromal compartment of the mammary organ which is mostly (70–80%) adipocytes in the quiescent gland. The importance of the stromal component in trapping the DMBA metabolites is also demonstrated by the finding that the whole glands contained 110-fold more metabolites than the collagenase-treated cells. A substantial portion of the metabolites is lost apparently during the collagenase-digestion procedure.

It is also important to mention that because of the entrapment of material(s) in the adipose compartment distribution of PAH substrate and the metabolites is likely to be different in the mammary epithelium in organ culture compared to that in the mammary cells in monolayer culture medium. Thus the levels of the individual metabolites obtained from the mammary cells in organ culture may not be comparable with those present in the mammary cells in monolayer culture. Therefore the metabolic product obtained from the mammary cells in organ culture is more likely to resemble the events occurring in the mammary gland *in vivo.*

The mammary fat pad may also serve as an efficient reservoir for sustained release of the procarcinogen into the parenchymal tissue with little chance for detoxification by conjugation. The process thus may account for the greater susceptibility of the mammary epithelial cells to DMBA carcinogenesis. The phenomenon of procarcinogen trapping in the fat pad enhancing the carcinogenic susceptibility of the mammary cells in the organ culture is conceivable also in the mammary glands *in vivo.* In the animal, the procarcinogen carried in the humoral system reaches the parenchymal tissue through the capillary system embedded in the mass of adipose tissue. Thus the eventual transit of the lipid-soluble procarcinogen into the mammary cells by diffusion is likely to encounter some entrapment also in the mammary fat pad *in vivo.* Therefore, the accumulation of the procarcinogen in the mammary fat pad *in vivo* also is a distinct possibility.

Finally, the results of the present study demonstrate that the measurement of DMBA metabolism in the whole mammary organ *in vitro* is feasible and this important finding provides the means for studying the possible site of action of the chemopreventive agents during the multistage initiation of transformation of the mammary gland *in vivo.* For example, it will now be possible to analyze whether selective inhibition of adduct formation caused by selenium [27] may prevent DMBA-induced tumorigenesis.

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