Estradiol Metabolism: An Endocrine Biomarker for Modulation of Human Mammary Carcinogenesis

Nitin T. Telang, Meena Katdare, H. Leon Bradlow, and Michael P. Osborne

Strang Cancer Research Laboratory, The Rockefeller University, New York, New York

The natural estrogen 17β-estradiol (E_2) has a profound influence on proliferation and neoplastic transformation of mammary epithelium. The role of cellular metabolism of E_2 in mammary carcinogenesis, however, remains to be elucidated. Explant culture and cell culture models developed from noncancerous human mammary tissue were used to examine modulation of E_2 metabolism in response to treatment with prototype rodent mammary carcinogens and the ability of the naturally occurring phytochemical indole-3-carbinol (I3C) to influence E_2 metabolism and regulate aberrant proliferation. In the two models, treatment with the chemical carcinogens 7,12-dimethylbenz[*a*]anthracene and benzo[*a*]pyrene altered the metabolism of E_2 as determined from the radiometric (tritium release) and gas chromatography–mass spectrometry (GC–MS) assays. This alteration in E_2 metabolism was accompanied by aberrant proliferation and abrogation of apoptosis as determined by the extent of replicative DNA synthesis, *S*-phase fraction and Sub G_0 (apoptotic) peak. Exposure of carcinogen-initiated cultures to I3C resulted in induction of C2-hydroxylation of E_2 and of apoptosis and downregulation of hyperproliferation. Determination of altered cellular metabolism of E_2 in response to initiators and modulators of carcinogenesis and evaluation of cell cycle related markers for proliferation and apoptosis may provide a mechanism-oriented approach to validate E_2 metabolism as an endocrine biomarker for induction and prevention of human mammary carcinogenesis. — *Environ Health Perspect* 105(Suppl 3):559–564 (1997)

Key words: estrogen metabolites, mammary carcinogenesis, chemoprevention, *in vitro* models

Introduction

Breast cancer is one of the prevalent causes of death in women in the United States. The American Cancer Society has estimated a 31% incidence of breast cancer (184,300 new breast cancer cases) and about 17% mortality (44,300 cancer related deaths) in 1996 (*1*). These estimates emphasize a need to identify markers for risk, early detection, and effective prevention.

Laboratory investigations on animal models have provided compelling but circumstantial evidence that human mammary carcinogenesis may be a multifactorial and multistep process involving early-occurring molecular, biochemical, and cellular events that represent preneoplastic transformation and late-occurring epigenetic events that represent promotion and progression of the preneoplastic phenotype to tumorigenic phenotype with metastatic potential (*2,3*). Identification and validation of biomarkers for preneoplastic transformation, therefore, may provide important leads not only for identifying markers of risk for developing breast cancer but also for evaluating effective primary or secondary prevention (*4–9*).

In the estrogen-responsive mammary tissue, the natural estrogen 17β-estradiol (E_2), in concert with other steroid and polypeptide hormones that have mammotropic or lactogenic effects, supports epithelial cell proliferation and neoplastic transformation (*6–8*). The mitogenic stimulus mediated via E_2 may predispose nontransformed cells for initiation of carcinogenesis, while in preinitiated cells, this stimulus may promote the expression of the transformed cell phenotype (*2,3,5–8,10–12*).

The cellular biotransformation of E_2 represents a complex enzymatic process by which metabolically competent cells convert the mitogenic estrogen E_2 to its less active metabolites. Cytochrome P450 (cyp450)-dependent steroid hydroxylases are critical for E_2 metabolism, while estrogen receptor, a nuclear transcription factor, is indispensable for transcriptional activation, expression of early response genes c-*fos*, c-*jun*, c-*myc*, and resultant E_2-mediated positive regulation of growth (*12–16*).

Our studies on murine mammary explant cultures and on immortalized nontumorigenic mammary epithelial cell cultures have demonstrated that treatment with chemical carcinogens and transfection with oncogenes results in altered cellular metabolism of E_2 and aberrant hyperproliferation *in vitro* prior to tumorigenicity *in vivo* (*17–19*). In these studies alteration in E_2 metabolism was detected by specific and significant increase in C16α-hydroxylation, with a concomitant decrease in C2-hydroxylation pathways, while aberrant hyperproliferation was quantified by the relative extent of cell proliferation in anchorage-dependent and anchorage-independent conditions of growth. Altered cellular metabolism of E_2 and aberrant hyperproliferation, therefore, represent biochemical and cellular surrogate end point biomarkers for mammary carcinogenesis (*4,9,17–21*). The clinical relevance of these biochemical and cellular perturbations, however, depends on extrapolation and therefore is largely equivocal. Clinical investigations on breast cancer patients, disease-free subjects,

This paper was presented in part at the Workshop on Hormones, Hormone Metabolism, Environment, and Breast Cancer held 28–29 September 1995 in New Orleans, Louisiana. Manuscript received at *EHP* 6 June 1996; manuscript accepted 15 August 1996.

Supported in part by Department of Defense grant DAMD-17-94-J-4208, National Institute of Health grant P01 CA 29502, Indo-US Fulbright fellowship#17267, and philanthropic funds to the Strang Cancer Prevention Center.

The authors acknowledge the expert technical assistance of M. Zvanovec and skillful editorial assistance of K.J. Brady.

Address correspondence to Dr. N.T. Telang, Division of Carcinogenesis and Prevention, Strang Cancer Research Laboratory, The Rockefeller University, Box 231, 1230 York Avenue, New York, NY 10021. Telephone: (212) 734-0567, ext. 213. Fax: (212) 472-9471.

Abbreviations used: AP, apoptosis; B[*a*]P, benzo[*a*]pyrene; BSTFA, bis(trimethylsilyl)trifluroacetamide; cyp450, cytochrome P450; DMBA, 7,12-dimethylbenz[*a*]anthracene; DMSO, dimethyl sulfoxide; E_2, 17-β estradiol; FACS, fluorescene assisted cell sorting; G_0, resting phase; G_1, first growth phase; G_2, second growth phase; GC–MS, gas chromatography–mass spectrometry; I3C, indole-3-carbinol; M, mitosis; NS, not significant; *S*, synthesis phase; 2-OHE_1, 2-hydroxyestrone; 16α-OHE, 16α-hydroxyestrone; TDLU, terminal duct lobular units.

and subjects at risk have demonstrated a correlation between elevated C16α-hydroxylation of E_2, presence of atypical hyperplasia or proliferative breast disease and increased risk for developing breast cancer (*2,5,6–8,13,14,20,21*). Thus, altered E_2 metabolism may represent a biochemical or endocrine marker for breast cancer development. A systematic investigation of this aspect using appropriate human tissue-derived models should provide important leads that will help researchers evaluate the clinical relevance of specific molecular, biochemical, endocrine, and cellular biomarkers for human mammary carcinogenesis and its prevention (*4,9,22*).

The present report provides an overview of the experiments designed on explant and cell culture models developed from noncancerous human mammary tissue to establish potential clinical relevance of E_2 metabolism as a biochemical or an endocrine biomarker for effective chemoprevention of human mammary carcinogenesis.

Experimental Systems and Biomarker Assays

Human Mammary Explant Culture and Cell Culture Models. The mammary explant culture system and mammary epithelial cell culture system provide useful *in vitro* models to examine the responsiveness of noncancerous mammary tissue to agents that affect cell proliferation, cytodifferentiation, and neoplastic transformation at the molecular, biochemical, and cellular levels (*2,4,17–19*). The tissue culture technology and biomarker assays established for the murine models have been optimized for human mammary tissue (*4,22,23*).

The explant cultures were prepared from human mammary terminal duct lobular unit (TDLU) obtained from surgical samples. The TDLU are the endocrine responsive and proliferatively active intact organoids that represent target tissue for carcinogenesis (*2,4,8*). These organoids were maintained in a chemically defined, serum-free Waymouth's MB 752/1 medium (GIBCO/BRL, Grand Island, NY) supplemented with 5 μg/ml insulin, 1 ng/ml E_2, 2 mM L-glutamine, and antibiotics. The medium was routinely changed every 48 hr and the cultures were maintained in a humidified atmosphere of 95% air: 5% CO_2 at 37°C.

The human mammary epithelial 184-B5 cell line was maintained in chemically defined, serum-free KBM–MEM medium (Clonetics Corp., San Diego, CA, and GIBCO/BRL) supplemented with 10 μg/ml insulin, 10 ng/ml epidermal growth factor, 10 μg/ml transferrin, 0.5 μg/ml hydrocortisone, and 5 μg/ml gentamycin (*24,25*). The medium was routinely changed every 48 hr and the cells were subcultured by a 1:4 split when approximately 70% confluent.

Chemical Carcinogens and Chemopreventive Agent. The stock solutions (1000×) of the chemical carcinogens 7,12-dimethylbenz[*a*]anthracene (DMBA) and benzo[*a*]pyrene (B[*a*]P) were made up in dimethyl sulfoxide (DMSO). The stock solution of the naturally occurring phytochemical indole-3-carbinol (I3C), to be used as the chemopreventive test compound, was made up in 100% ethanol. These stock solutions were appropriately diluted with the culture medium to obtain the effective nontoxic concentrations. The selection of chemical carcinogens and of the naturally occurring phytochemical was based on their documented tumorigenic or tumor modulating effects on the rodent models (*3,10,26–28*).

Cellular Metabolism of 17β-estradiol. The metabolism of E_2 by TDLU and 184-B5 cultures was determined by the radiometric assay that measures the tritium exchange from specifically labeled E_2 to form ^3H_2O (*4,17,19*) and by the gas chromatography–mass spectrometry (GC–MS) assay that involves product isolation and identification of the metabolites (*29*).

For the radiometric assay, cultures were incubated with 8.0×10^{-10} M [C2-^{3}H] E_2 or [C16α-^{3}H] E_2 for 48 hr at 37°C. Aliquots of 500 μl of the incubation medium were diluted to 3.0 ml with distilled water and lyophilized to separate ^3H_2O from the residual radioactive E_2. The relative extent of ^3H_2O formed provided an indirect measure of 2-hydroxyestrone (2-OHE_1) or 16α-hydroxyestrone (16α-OHE_1) formed via the C2-hydroxylation and C16α-hydroxylation pathways of E_2 metabolism, respectively. Based on the stoichiometric conversion and the specific activity of labeled E_2, the amounts of the metabolites formed were calculated. The data were expressed as pmol metabolite/48 hr/mg tissue.

For the GC–MS assay, cultures were incubated for 48 hr at 37°C with 10^{-8} M nonradioactive E_2. Fifteen milliliters of incubation medium was added with deuterated E_2 as an internal standard and processed for solid phase extraction. The extracted sample was derivatized in the presence of 50 μl dry pyridine and 10 μl bis(trimethylsilyl)trifluoroacetamide (BSTFA). The derivatized samples were analyzed under select ion mode and mass ion, and GC elution times of the metabolites were determined (*29*). The data were expressed as relative abundance of E_2 metabolites per 10^7 cells.

Cell Cycle Analysis and Cellular Apoptosis. The effect of initiators and modulator of carcinogenesis on alteration in cell cycle progression and on the relative extent of cellular apoptosis was examined on 184-B5 cells using the fluorescence-assisted cell sorting (FACS) flow cytometric assay. For this assay, trypsinized cell suspensions fixed in 2% formaldehyde and subsequently in ice-cold 70% ethanol were stained with propidium iodide according to the published procedure (*30*). The extent of proliferation was expressed as percent synthesis (*S*)-phase fraction, while the extent of apoptosis was expressed as the intensity of Sub G_0 (apoptotic) peak obtained from FACS analysis.

Results

Effect of Chemical Carcinogens on Terminal Duct Lobular Units. The experiment presented in Table 1 was performed on explant cultures of human mammary TDLU to examine the effects of well known rodent carcinogens DMBA and B[*a*]P on the cellular metabolism of E_2. The relative extent of E_2 metabolism via C2-hydroxylation and C16α-hydroxylation pathways was determined using the radiometric assay. The results obtained from

Table 1. Effect of chemical carcinogens on the metabolism of 17β-estradiol (E_2) in explant cultures of human mammary terminal duct lobular units.

Treatment[a]	E_2 Metabolite,[b] pmol/mg tissue		
	2-OHE1	16α-OHE_1	C2/C16α ratio
DMSO	2.32 ± 0.24[c]	0.48 ± 0.08[f]	4.83 ± 0.30
DMBA	1.36 ± 0.24[d]	2.24 ± 0.72[g]	0.61 ± 0.20
B[*a*]P	1.12 ± 0.08[e]	3.12 ± 0.24[h]	0.36 ± 0.10

[a]Explant cultures incubated with 0.1% DMSO, 39 μM DMBA, or 39 μM B[*a*]P for 24 hr and with 10^{-10}M [C2-^{3}H]E_2 or [C16α-^{3}H]E_2 for the subsequent 48 hr. The culture medium was processed for the radiometric assay. [b]Calculated from the stoichiometric convertibility and the specific activity of [^{3}H]-E_2. Values are mean ± SD; $n = 12$. [c–d, c–e] $p = 0.005$. [f–g, f–h] $p = 0.001$.

this experiment clearly demonstrate that both the carcinogens DMBA and B[*a*]P inhibit 2-OHE_1 formation and increase 16α-OHE_1 formation. This alteration in metabolism leads to an 87.4 and a 92.5% decrease by DMBA and B[*a*]P, respectively, in C2/C16α-hydroxylation ratio relative to that observed in the solvent-treated control cultures. The preliminary experiments on the TDLU explant culture system were designed to examine whether the solvents DMSO or ethanol used to solubilize DMBA, B[*a*]P, and I3C, respectively, influence E_2 metabolism. The extent of C2/C16α-hydroxylation ratios obtained from untreated cultures (master controls) and those treated with 0.1% DMSO or 0.1% ethanol (solvent controls) was 5.7 ± 1.1, 4.9 ± 0.2, and 5.1 ± 0.1, respectively. The lack of substantial difference in the ratios from the three groups indicates that the two solvents at 0.1% concentrations did not influence the constitutive level of E_2 metabolism.

In the explant cultures of TDLU the epithelial component is surrounded by intralobular and extraductular stroma. It is therefore not possible to demonstrate whether the epithelial component is directly responsible for E_2 metabolism. In addition, the radiometric assay measures the reaction kinetics of C2- and C16α-hydroxylation pathways and therefore represents an indirect assay for the formation of 2-OHE_1 or 16α-OHE_1. In an attempt to eliminate the above-mentioned limitations, experiments were conducted on human mammary epithelial 184-B5 cells, and E_2 metabolism was evaluated by the GC–MS assay that measures the specific metabolites formed.

Table 2. Effect of chemical carcinogens on the metabolism of 17β-estradiol (E_2) in human mammary epithelial 184-B5 cells.

Treatment[a]	E_2 Metabolite,[b] relative abundance /10^7 cells		
	2-OHE_1	16α-OHE_1	C2/C16α ratio
DMSO	342 ± 73[c]	53 ± 13[f]	6.45 ± 0.20
DMBA	52 ± 3[d]	65 ± 5[g]	0.80 ± 0.20
B[*a*]P	45 ± 5[e]	78 ± 8[h]	0.58 ± 0.10

[a]Cell cultures incubated with 0.1% DMSO, 39 μM DMBA or 39 μM B[*a*]P for 24 hr and with 10^{-8}M E_2 for the subsequent 48 hr. The culture medium was processed for the product isolation and identification by the GC–MS assay. [b]Values are mean ± SD; n = 4. [c–d, c–e] $p = 0.001$. [f–g] NS [f–h] $p = 0.01$.

Effect of Chemical Carcinogens on 184-B5 Cells. The data presented in Table 2 demonstrate that treatment of 184-B5 cells with DMBA and with B[*a*]P results in a decreased abundance of 2-OHE_1 relative to that observed in the solvent-treated control cultures. Furthermore, B[*a*]P was more effective in upregulating 16α-OHE_1 formation than was DMBA. Because of the observed distinct effects of DMBA and B[*a*]P on the metabolic pathways, the carcinogen-mediated alteration in the 184-B5 system also resulted in an 87.6 and a 91% inhibition in the C2/C16α-hydroxylation ratio, respectively, relative to that observed in the solvent-treated controls. The effect of DMBA and B[*a*]P on cellular apoptosis in confluent cultures of 184-B5 cells is presented in Figures 1A–C. The differential effects of DMBA and B[*a*]P were also evident on the cell cycle progression. Treatment of 184-B5 cells with DMBA exhibited 63.3 ± 10.5% *S*-phase fraction and 5.8 ± 1.3% apoptosis. In contrast, treatment with B[*a*]P resulted in 34.5 ± 0.3% *S*-phase fraction and 1.6 ± 0.9% apoptosis, in comparison with 26.1 ± 3.6% *S*-phase fraction and 15.1 ± 3.1% apoptosis that was observed in 0.1% DMSO-treated solvent controls. It is possible that the two carcinogens enhance aberrant hyperproliferation and inhibit cellular apoptosis via distinct mechanisms. Additional experiments focused on the specific cell cycle regulatory gene expression may elucidate the possible mechanisms responsible for effects of DMBA and B[*a*]P on 184-B5 cells.

Effect of I3C on Chemical Carcinogenesis in Terminal Duct Lobular Units. Having demonstrated that treatment of TDLU explant cultures or 184-B5 cell cultures with the chemical carcinogen B[*a*]P results in decreased

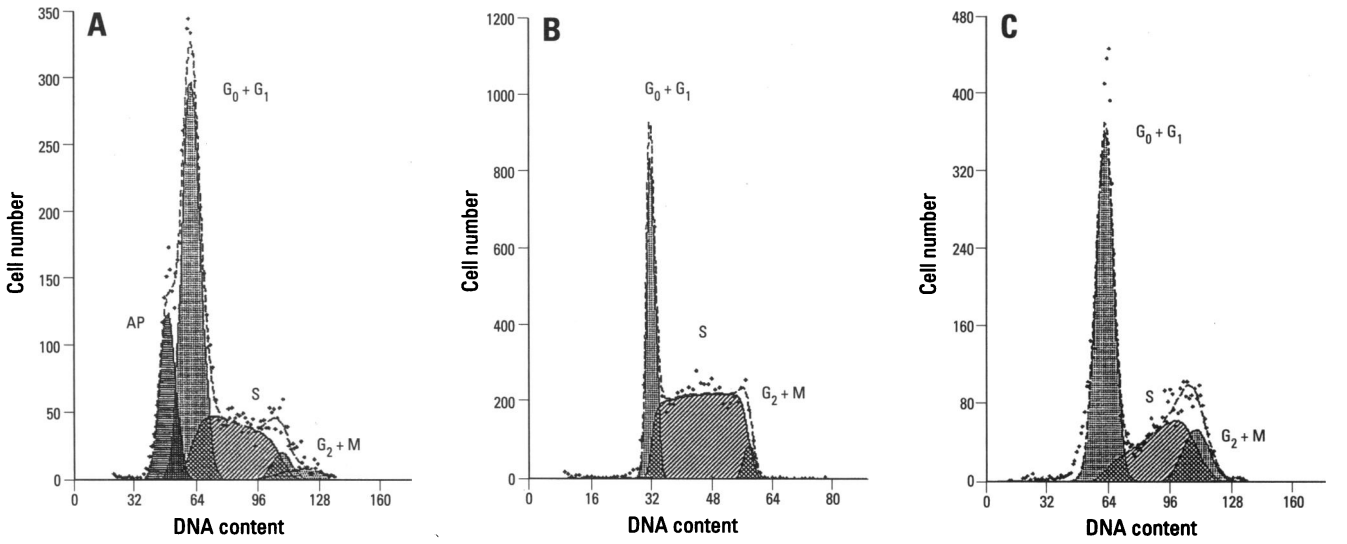

Figure 1. Cell cycle analysis of 184-B5 cells. (*A*) A 24-hr treatment with 0.1% DMSO (solvent control); (*B*) A 24-hr treatment with 39 μM DMBA; (*C*) A 24-hr treatment with 39 μM B[*a*]P. Note the inhibition of Sub G_0 (apoptotic) peak in cells treated with DMBA or B[*a*]P.

Table 3. Effect of I3C on chemical carcinogen-treated human mammary explant cultures.

Treatment[a]	Replicative DNA synthesis,[b] cpm × 10^6/mg DNA	E_2 Metabolism,[c] C2/C16α ratio
DMSO	3.0 ± 0.4[d]	4.3 ± 0.7[g]
B[*a*]P	6.3 ± 0.3[e]	0.5 ± 0.1[h]
B[*a*]P + I3C	3.6 ± 0.9[f]	6.5 ± 1.6[i]

[a]Explant cultures incubated with 0.1% DMSO or 39 μM B[*a*]P for 24 hr and were maintained with or without 50 μM I3C for the subsequent 10 days. [b]Pulse labeled with 5 μci/ml of ^{3}H-thymidine between days 9 and 10 of culture. Radioactivity was determined as trichloroacetic acid precipitable counts. [c]Determined by the radiometric assay as in Table 1. [d–f]Mean ± SD, n = 6 per treatment group. [d–e, d–f]p = 0.001. [g–i]Mean ± SD, n = 18 per treatment group. [g–h]p = 0.0001. [h–i]p = 0.005.

Table 4. Effect of I3C on chemical carcinogen-treated human mammary epithelial 184-B5 cells.

Treatment[a]	*S*-Phase fraction[b], %	Apoptosis,[b] %	E_2 Metabolism[c] (C2/C16α ratio)
DMSO	29.9 ± 0.9[d]	14.9 ± 1.2[g]	3.7 ± 0.5[j]
B[*a*]P	34.5 ± 0.3[e]	1.6 ± 0.9[h]	0.6 ±0.1[k]
B[*a*]P + I3C	17.0 ± 1.7[f]	5.1 ± 0.5[i]	2.6 ± 0.3[l]

[a]Cell cultures were incubated with 0.1% DMSO or 39 μM B[*a*]P for 24 hr and were maintained with or without 50 μM I3C for the subsequent 48 hr. [b]Determined from propidium iodide-stained cell suspensions using FACS. Mean ± SD, n = 8/treatment group. [c]Determined by the radiometric assay as in Table 1. Mean ± SD, n = 4/treatment group. [d–e]p = 0.01. [e–f]p = 0.001. [g–h]p = 0.001. [h–i]p = 0.005. [j–k, k–l]p = 0.001.

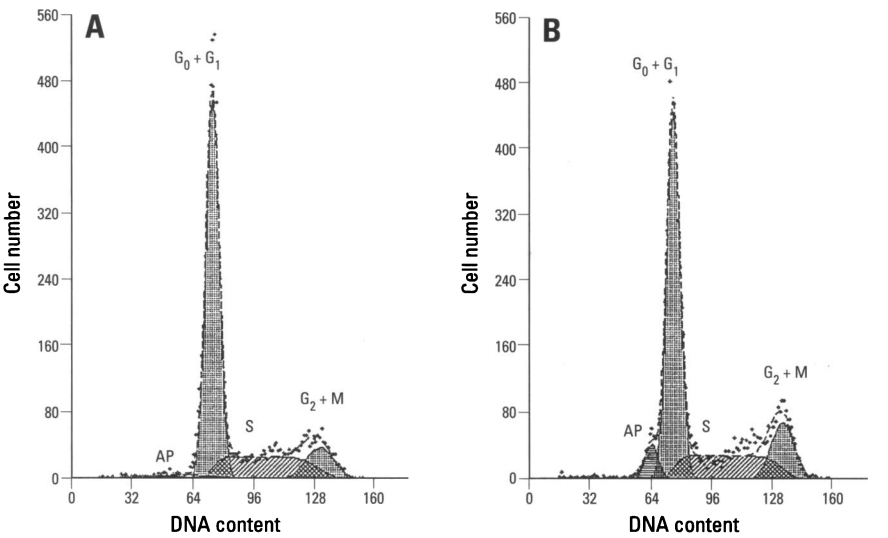

Figure 2. Effect of I3C on cellular apoptosis in B[*a*]P-initiated 184-B5 cells. (*A*) A 24-hr treatment with 39 μM B[*a*]P; (*B*) A 24-hr treatment with 39 μM B[*a*]P + 50 μM I3C. Note the presence of Sub G_0 (apoptotic) peak in cells treated with B[*a*]P + I3C, but not in cells treated with B[*a*]P alone.

C2/C16α-hydroxylation ratio, it was important to provide evidence for validation of E_2 metabolism as an endocrine marker for preventive efficacy of agents known to inhibit rodent mammary carcinogenesis. In the experiment presented in Table 3, the naturally occurring plant product I3C represented the chemopreventive test compound, while replicative DNA synthesis and E_2 metabolism provided two independent markers for quantitation. Treatment of TDLU explant cultures with 50 μM I3C resulted in a ^{3}H-thymidine uptake of 3.6 ± 0.9 cpm × 10^6/mg DNA and a C2/C16α-hydroxylation ratio of 5.0 ± 0.6. These data were comparable to those observed in the DMSO-treated or ethanol-treated solvent controls (data not shown). Exposure of TDLU explants to B[*a*]P resulted in increased DNA synthetic activity and in decreased C2/C16α-hydroxylation ratio. The B[*a*]P-initiated explant cultures, upon treatment with I3C, exhibited a 42.8% decrease in replicative DNA synthesis and a 12-fold increase in C2/C16α-hydroxylation ratio. These results indicate that I3C by itself does not influence the constitutive status of the biomarkers; however, this agent is able to reverse the hyperproliferative effects of B[*a*]P, in part, by upregulating the C2/C16α-hydroxylation ratio.

Effect of I3C on Chemical Carcinogenesis in 184-B5 Cells. The experiment presented in Table 4 was conducted on the 184-B5 cells to examine the effect of I3C on B[*a*]P-induced cellular and biochemical alterations. Treatment of 184-B5 cells with B[*a*]P resulted in increased *S*-phase fraction, inhibited Sub G_0 (apoptotic) peak and decreased C2/C16α-hydroxylation ratio. In the 184-B5 cell culture system, treatment with 50 μM I3C exhibited 29.7 ± 0.7% *S*-phase fraction, 15.5 ± 0.6% apoptosis, and a C2/C16α-hydroxylation ratio of 4.1 ± 0.3. These data were comparable to those observed in untreated master controls and in 0.1% ethanol-treated solvent controls (data not shown). Thus, I3C, at the dose level tested, does not appear to influence the constitutive status of the biochemical and cellular quantitative end points.

Treatment of B[*a*]P-initiated cells with I3C exhibited inhibition of the *S*-phase fraction, increase in cellular apoptosis, and increase in C2/C16α-hydroxylation ratio of E_2 metabolism. The induction of cellular apoptosis by I3C in B[*a*]P-initiated 184-B5 cells was also evidenced by a substantial increase in Sub G_0 (apoptotic) peak in cells treated with B[*a*]P+I3C relative to those treated with B[*a*]P alone (Figure 2A,B). Thus, the ability of I3C to inhibit the effect of B[*a*]P may, in part, be due to growth regulation by decreased proliferation, increased apoptosis, and increased C2-hydroxylation of E_2.

The effect of DMBA appears to be distinct from that of B[*a*]P in the present experimental system. It will therefore be of considerable interest to examine whether I3C in DMBA initiated cells downregulates the status of *S*-phase-specific gene expression.

Discussion

The experiments in this study were conducted on *in vitro* models for human mammary carcinogenesis to examine the role of E_2 metabolism in chemical carcinogen-induced initiation of tumorigenic transformation and to validate E_2 metabolism as a surrogate end point biomarker for efficacy of chemopreventive agents. Human mammary TDLU explant cultures and 184-B5 cell cultures represented the experimental systems derived from noncancerous human mammary tissue. Biochemical determination of E_2

metabolism by radiometric and GC–MS assays, replicative DNA synthesis by [^{3}H]thymidine uptake assay, and cell cycle analysis by FACS flow cytometry assay represented the quantitative parameters.

The observed increase in replicative DNA synthesis or *S*-phase fraction in response to the chemical carcinogens may be due to a combination of replicative DNA synthesis and DNA repair synthesis. Mammary tissue has been reported to effectively metabolize the procarcinogens and generate DNA-damaging oxidative metabolites that may induce DNA repair synthesis (*2,18,31–35*).

In the multistep process of mammary carcinogenesis, the natural estrogen E_2 functions as a potent tumor promoter, acting prevalently during the late-occurring, postinitial (promotional) stage of tumorigenesis (*3,6,7,10*). The possible mechanisms responsible for the positive growth regulation by E_2 include mitogenic signal transduction via upregulation of early response genes c-*jun*, c-*fos*, and c-*myc*; induction of DNA synthesis in quiescent cells; and activation of nuclear transcriptional factors estrogen receptor and nuclear factor (*12,15,16,36*). In contrast, the biological activity of various oxidative metabolites of E_2 is pleiotropic depending upon the experimental systems (*37–41*), and therefore the role of E_2 metabolites in mammary carcinogenesis remains equivocal.

The experiments on TDLU explant culture system clearly demonstrated that prototypic rodent carcinogens DMBA and B[*a*]P decrease the C2/C16α-hydroxylation ratio of E_2 metabolism. This observation is consistent with our previous studies that demonstrated a similar perturbation in *in vitro* models of rodent mammary carcinogenesis in response to DMBA or c-*myc* (*17,18,20,21,31,32*). In addition, clinical investigations on patients with breast cancer, as well as laboratory investigations on noncancerous breast tissue, or on human mammary carcinoma-derived cell lines, have provided convincing evidence for a role of E_2 metabolism in breast cancer development (*4,13,14,20,22,23,40–42*). Taken together, these observations suggest that altered metabolism of E_2 may represent an endocrine biomarker for carcinogenic insult to the mammary tissue.

The presence of interlobular and interductular stroma in the TDLU explant culture system represents the nontarget component for mammary carcinogenesis. This cellular heterogeneity therefore compromises the specificity of E_2 metabolism as a biomarker assay. In addition, the radiometric assay measuring the reaction kinetics of the two pathways provides an indirect measurement for the formation of E_2 metabolites. The two technical limitations were eliminated by experiments that utilized the mammary epithelial 184-B5 cells to determine E_2 metabolites by the GC–MS assay. The experiments on the 184-B5 cell culture system demonstrated that while both DMBA and B[*a*]P decrease 2-OHE_1 formation, upregulation of 16α-OHE_1 is observed only in response to B[*a*]P. Decrease in C2/C16α-hydroxylation ratio by the two carcinogens in the two experimental systems is comparable, and the human mammary tissue is more susceptible to B[*a*]P than it is to DMBA. The observed preferential susceptibility of human tissue to the polycyclic aromatic hydrocarbons is consistent with that reported previously (*33–35*). The results obtained from the TDLU explant culture and 184-B5 cell culture systems, taken together, suggest that the target epithelial component responds directly to the carcinogenic insult.

The naturally occurring plant product I3C represents a major phytochemical in such cruciferous vegetables as cabbage, broccoli, and brussels sprouts. Our previous *in vitro* and *in vivo* studies on rodent models of mammary carcinogenesis have shown that exposure to I3C protects the target tissue from chemical carcinogen-induced transformation or from mammary tumor virus-induced mammary tumorigenesis (*27,31*). Furthermore, I3C has been reported to induce C2-hydroxylation of E_2 in human mammary carcinoma cells as well as in human subjects (*42–45*). We therefore sought to examine whether I3C is also an effective inhibitor of carcinogenesis in human mammary TDLU explant culture and cell culture models. In the experiments utilizing the TDLU explant culture system, treatment of cultures with B[*a*]P resulted in induction of aberrant proliferation as evidenced by increased replicative DNA synthesis, and in altered E_2 metabolism, as seen by inhibition of C2/C16α-hydroxylation ratio. B[*a*]P-initiated cultures in the presence of I3C exhibited inhibition of aberrant proliferation and enhancement of C2/C16α-hydroxylation ratio. In the experiments on 184-B5 cell culture system, exposure to B[*a*]P exhibited an increase in *S*-phase fraction, and a decrease in cellular apoptosis and in C2/C16α-hydroxylation ratio. The B[*a*]P-initiated cultures in the presence of I3C exhibited downregulation of cell proliferative activity and upregulation of apoptosis and of C2/C16α-hydroxylation ratio. The possible mechanisms responsible for the protective effect of I3C against B[*a*]P-induced human mammary carcinogenesis, however, remain to be identified. In this context, it is noteworthy that I3C functions as an inducer of cyp4501A1-dependent C2-hydroxylation of estradiol (*42*) and 2-OHE_1 has been reported to antagonize the genotoxic and transforming effects of DMBA (*31*). These observations, taken together, raise the possibility that increased production of antiproliferative E_2 metabolite 2-OHE_1 by I3C in carcinogen-initiated human mammary tissue may negatively regulate aberrant hyperproliferation, in part by induction of cellular apoptosis.

In conclusion, the present study on human mammary tissue-derived *in vitro* models for carcinogenesis has provided evidence that E_2 metabolism and cell cycle-related markers for proliferation and apoptosis may represent valuable surrogate end point biomarkers to evaluate efficacy of chemopreventive agents for human mammary carcinogenesis.

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