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Prostaglandin Concentrations and Prostaglandin Synthetase Activity in *N*-Nitrosomethylurea-induced Rat Mammary Adenocarcinoma*

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Abstract—*A comparison of tissue concentrations and biosynthesis of prostaglandin (PG)E_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ (degradation production of PGI_2) and thromboxane (TX)B_2 (degradation product of TXA_2) was made in normal mammary glands obtained from virgin female Sprague–Dawley rats and in N-nitrosomethylurea (NMU)-induced mammary adenocarcinomas. The tissue concentrations* (ng/g *wet weight) of all 4 compounds were significantly higher in the NMU-induced tumor than in normal mammary tissue:* PGE_2, 210 ± 37 *vs* 25 ± 6; $PGF_{2\alpha}$, 287 ± 48 *vs* 23 ± 8; 6-keto-$PGF_{1\alpha}$, 294 ± 42 *vs* 31 ± 8; *and* TXB_2, 260 ± 49 *vs* 27 ± 5 *(mean ± S.E.M.). Microsomal prostaglandin synthetase activity in NMU-induced tumors was also significantly higher than in normal tissue for all but* 6-keto-$PGF_{1\alpha}$: PGE_2, 226 ± 16 *vs* 50 ± 9; $PGF_{2\alpha}$, 28 ± 3 *vs* 4 ± 1; 6-keto-$PGF_{1\alpha}$, 14 ± 2 *vs* 11 ± 2; *and* TXB_2, 17 ± 1 *vs* 10 ± 1 ng/mg *protein (mean ± S.E.M.). There was no apparent relationship between either tumor size or age and the ability of microsomal enzyme to synthesize prostaglandins, although the content of prostaglandins extracted from tumor tissue was inversely related to the tumor size.*

INTRODUCTION

EXCESSIVE prostaglandin (PG) production, particularly of the E series, has been reported in several types of malignant tissues—including mammary tumors—in both humans and experimental animals (reviewed in [1–3]). Moreover, PGs have been implicated in certain symptoms commonly associated with human breast cancer such as metastatic spread to bone and hypercalcemia [4–7], as well as survival after surgery [8]. Prostaglandins apparently play a role in mediating the physiologic responses of normal mammary gland to hormonal stimulation (e.g. prolactin) [9]; and since many mammary tumors are hormone-responsive [10], it is logical to assume that PGs may also serve a regulatory function in mammary tumor growth. The precise nature of this function, however, is unclear at present. Published reports suggest both a stimulatory and an inhibitory role for PGs in cultures of tumor cell lines and in experimental tumor systems (reviewed in [1–3]).

One reason for this state of uncertainty may be found in recent studies suggesting that the many physiological and pathological effects formerly attributed to the classical PGs, namely PGE_2 and $PGF_{2\alpha}$, may, in fact, be attributable to the action of other oxygenated metabolites of arachidonic acid, including the biologically potent cyclic endoperoxides, prostacyclin and thromboxane A_2. At present little is known about the ability of mammary tumors to metabolize precursor essential fatty acids to these potent but unstable derivatives. In the light of these considerations it is apparent that further progress in this area will require assessment of other prostaglandin moieties together with the basic parameters which govern prostaglandin metabolism in established mammary tumors.

Due to the greater control of experimental variables afforded by animal models a reasonable

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Abbreviations: PGs, prostaglandins; DMBA, 7,12-dimethylbenz(a)anthracene; RIA, radioimmunoassay; NMU, *N*-nitrosomethylurea.

point of departure for such an endeavor is a well-characterized experimental mammary tumor model. In this regard there is considerable current interest in the use of the nitrosomethylurea (NMU)-induced mammary adenocarcinoma as a model for human breast cancer, primarily because NMU-induced tumors exhibit characteristics typical of human breast cancer such as the ability to (a) metastasize to bone marrow, spleen and lung, (b) respond to hormones and (c) induce hypercalcemia [11].

Accordingly, in order to gain further insight into the role of PGs in human breast cancer we report here a comparison of tissue concentrations and biosynthesis of PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ (a stable degradation product of PGI_2) and TXB_2 (a stable degradation product of thromboxane A_2) in normal mammary glands obtained from virgin female rats and in NMU-induced adenocarcinomas.

MATERIALS AND METHODS

Mammary adenocarcinoma

Mammary tumors were induced in female Sprague–Dawley rats (Camm Research, Wayne, NJ) by a modification of the procedure of Gullino *et al.* [11]. *N*-Nitrosomethylurea (NMU) was obtained from Ash Williams Inc., Detroit, MI. It was first mixed with 3% acetic acid and then dissolved in distilled H_2O at 10 mg/ml. The NMU solution was then injected once via the dorsal tail vein at a concentration of 25 mg/kg body weight into 50-day-old rats which were anesthetized with ether. Induction of tumors was assessed by palpation and tumor size was measured as the largest and smallest diameters which were perpendicular to each other (r_1 and r_2). Tumor volume was determined according to the formula for an ellipsoid ($4/3\pi r_1^2 r_2$). The position and time of appearance of all tumors were recorded weekly.

Animals were housed 3 to a cage in a temperature (74°F), light (14-hr cycle) and humidity (50%) controlled room and fed the recommended NIH-07 diet [12]. Both food and water were provided *ad libitum*.

Approximately 5 months after NMU injection animals were killed by exposure to CO_2. Mammary tumors were surgically excised, cleared of surrounding tissue and 2-mm square segments cut, weighed and immediately frozen in liquid nitrogen until used for prostaglandin analysis. The remainder of the tumor was fixed in buffered formalin, sectioned and stained with hematoxylin –eosin for histopathological examination.

Mammary fat pads were removed from control, age-matched, untreated female Sprague–Dawley rats and prepared similarly for prostaglandin analysis and histopathological examination, with the exception that frozen sections were used to preserve adipocyte integrity.

Prostaglandin radioimmunoassay procedures

Materials. Prostaglandin standards, PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and TXB_2 were kindly supplied by Dr. J. Pike (Upjohn Company, Kalamazoo, MI). Tritium-labelled compounds were purchased from New England Nuclear (Boston, MA). Rabbit antiserum to PGE_2 was obtained from the Pasteur Institute (Paris, France) and was prepared by the method of Dray *et al.* [13]. Antibodies to $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and TXB_2 were raised in our laboratory (R.K.) and were prepared as described earlier [14]. The specificities and cross-reactivities of these antibodies are listed in Table 1. Unlabelled arachidonic acid (sodium salt) was obtained from Sigma (St. Louis, MO).

Table 1. Cross-reactivity patterns for various antisera (%)

	PGE_2	$PGF_{2\alpha}$	6-Keto-$PGF_{1\alpha}$	TXB_2
PGE_1	10.7		<0.1	
PGE_2	100	0.2	1.2	0.3
$PGF_{1\alpha}$	0.01		1.9	
$PGF_{2\alpha}$	0.11	100	2.6	0.07
6-Keto-$PGF_{1\alpha}$	<0.01	1.2	100	0.05
TXB_2	<0.01	<0.1		100
PGA_1	0.04		<0.1	
PGA_2	0.3	<0.1	<0.1	<0.05
PGB_1	<0.01		<0.1	
PGB_1	<0.01			
PGD_1	<0.01			
PGD_2	<0.01	0.4		2.5
13,14Dihydro-15-keto-PGE_1	0.03			
13,14Dihydro-15-keto-PGE_2	0.6			
13,14Dihydro-15-keto-$PGF_{1\alpha}$	<0.01		0.1	
13,14Dihydro-15-keto-$PGF_{2\alpha}$		1.1		

Analytical methods. Extraction: the procedure for extracting the prostaglandins was described earlier [14, 15]. Briefly, a trace of [^{3}H]-PG (1200 counts/min) was added to aliquots of standards and samples before being extracted once with 3.5 ml petroleum ether. After acidification to pH 3.5 the samples were extracted twice with diethyl ether, dried under nitrogen and reconstituted in assay buffer. We have previously compared the extraction efficiency of various PGs using the different solvent systems currently employed [14]. We found the petroleum ether/ether (1/1) system to be the most efficient. The efficiency of the extraction procedure to this point was 85–95%. Samples with an extraction efficiency outside this range were extracted again.

Radioimmunoassay (RIA): standard quantities of each prostaglandin (0–1000 pg) or the unknown extracted sample to be measured were prepared in 0.1-ml aliquots of assay buffer. Antiserum and label were added successively in 0.1-ml aliquots and incubated at 4°C for 8–12 hr. Bound and free [^{3}H]-PG were separated by 0.5 ml dextran-coated charcoal (0.5–1.0% by weight) to estimate the amount of each compound in the unknown samples.

The sensitivity of the assays has routinely been found to be approximately 10 pg. The intra-assay coefficient of variation for each of the compounds was: $PGE_{2\alpha}$, 6.1%; $PGF_{2\alpha}$, 5.3%; 6-keto-$PGF_{1\alpha}$, 9.0%; and TXB_2, 8.2%. When each compound was measured in 5 aliquots (100 μl) of the same control plasma pool the mean ± S.D. was: PGE_2, 460 ± 28; $PGF_{2\alpha}$, 330 ± 17.3; 6-keto-$PGF_{1\alpha}$, 335 ± 30 pg; and TXB_2, 436 ± 35.8.

Assay of prostaglandins in mammary tissue. Solid tumor fragments, weighing between 0.5 and 1 g, were ground using a pestle and mortar at 2°C in a 1:5 (g:ml) tissue:buffer volume of MES buffer (1 M 2*n*-morpholinoethane sulphonic acid, pH 7.4, containing 2 mM $CaCl_2$, 2% glycerol and 1 mM monothioglycerol). Homogenates were centrifuged at 800 ***g*** for 15 min in a refrigerated centrifuge (4°C). The supernatant thus obtained was further spun at 150,000 ***g*** for 1 hr at 4°C. The subsequent supernatant was collected and stored in duplicate at −20°C. [^{3}H]-PG was added as a tracer and prostaglandins were extracted from this sample and measured by RIA as described. Such measurements represented the concentrations of the 5 compounds in normal and tumor tissues. The remaining microsomal pellet obtained after centrifugation at 150,000 ***g*** was saved to carry out studies of cyclooxygenase activity *in vitro*.

Prostaglandin synthetase assay. The microsomal pellet was suspended in MES buffer and its protein content was measured by the method of Lowry *et al.* [16]. The final concentration of the microsomal fraction was adjusted to 0.5 mg protein/ml in MES buffer. Biosynthesis of prostaglandins by microsomal preparations of normal and neoplastic mammary tissue was assayed by a modification of the procedure described by Rolland *et al.* [7].

The microsomal fraction (0.2 ml) was incubated at 37°C with 0.8 ml MES buffer containing 1.25 mM reduced gluthathione, 1.25 mM epinephrine and 1.25 μM arachidonic acid (Na salt). After an incubation of 10 min tritiated PG (1200 counts/min) was added as a tracer to the above mixture to evaluate procedural losses. Prostaglandins were extracted with a petroleum ether/diethyl ether mixture as described earlier [14]. The organic extracts were then dried under nitrogen and taken up in buffer for subsequent measurement by RIA of PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and TXB_2.

RESULTS

Histological observations

Histologically the mammary tumors were adenocarcinomas. There was considerable variation in tissue architecture from section to section in the same tumor and between different tumors. The tumors were encapsulated in a thin layer of connective tissue. Varying amounts of stromal tissue arranged in bands ran between nests of parenchymal cells. The stromal bands contained infiltrating mast cells and histiocytes. The major cell types were neoplastic parenchymal cells and mesenchymal cells; inflammatory cell infiltrates represented a minor tissue component.

The tumors ranged in age from 7 to 132 days, with a mean age (± S.E.M.) of 61 ± 8.7 days. Tumor volumes ranged from 0.006 to 7.74 cm^3, with a mean volume (± S.E.M.) of 1.6 ± 0.45 cm^3. No metastases to spleen, lung, bone or liver were seen in any of the NMU-treated animals.

Mammary fat pads were typical of non-pregnant female rats at 6 months of age. In whole mounts the ductal tree was readily apparent, with numerous ductal ramifications extending into the surrounding adipose tissue matrix. Thin sections revealed small nests of epithelial cells 1–3 cells thick, with a basal layer of myoepithelial cells bounded by adipocytes. Parenchymal cells comprised approximately 10–20% of the glandular mass.

Concentrations of prostaglandins and thromboxane in normal and neoplastic tissues

The tissue concentrations (ng/g wet weight) of all 4 compounds, PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and TXB_2, were significantly higher ($P < 0.001$ by Student's *t* test) in the NMU tumor than in normal mammary tissue (Table 2).

Effect of tumor size and age

When tumor prostaglandin concentrations (ng/g tissue) were analyzed with respect to tumor size an inverse correlation was observed. The correlation coefficients between prostaglandin levels and log of tumor volume were between −0.45 and −0.39. No correlation was found between prostaglandin concentrations and tumor age (defined as the time of appearance to the time of termination).

Characterization of microsomal prostaglandin synthetase activity in normal and neoplastic tissues

The microsomal enzyme obtained from tumor homogenates generated on average 226, 28, 14 and 17 ng of PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and TXB_2/mg microsomal protein/10 min respectively, while that obtained from normal mammary tissue generated much reduced amounts, being on average 50, 4, 11 and 10 ng of PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and TXB_2/mg microsomal protein/10 min respectively.

The activity of microsomal prostaglandin synthetase from tumor and normal mammary gland was studied under optimal conditions, i.e. at pH 7.4 using identical concentrations of substrate (sodium salt of arachidonic acid) and enzyme. Under these conditions synthesis of PGE_2 and $PGF_{2\alpha}$ was significantly higher in tumor tissue than in normal tissue, whereas the synthesis of PGI_2 (measured as 6-keto-$PGF_{1\alpha}$) was similar in normal and neoplastic tissue. The major product of tumor microsomal prostaglandin synthetase was PGE_2: biosynthesis of this compound was 10-fold that of the other 3 metabolites.

Effect of tumor size and tumor age

There was no apparent relationship between either tumor size or age and the ability to synthesize prostaglandins—with the exception of PGE_2 synthesis, which was related to the tumor volume. However, this correlation was only marginally significant ($r = 0.35$; $P = 0.05$).

Correlation between microsomal prostaglandin synthetase activity and prostaglandin levels in NMU-tumors

No significant correlation was found between enzyme activity and tissue prostaglandin levels for any of the four compounds.

DISCUSSION

The present results show clearly that the tissue levels of all 4 compounds examined were significantly elevated in the NMU-tumor compared to normal mammary tissue. While previous studies in both human and experimental mammary tumors [1] reported elevated PGE_2

Table 2. Tissue levels of PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and TXB_2 in NMU-induced mammary tumors: ng/g *tissue (mean ± S.E.M.)*

	Normal mammary tissue ($n = 6$)	NMU-induced mammary tissue ($n = 16$)
PGE_2	24.9 ± 6.43	210.3 ± 37.02
$PGF_{2\alpha}$	22.6 ± 7.61	287.0 ± 48.35
6-Keto-$PGF_{1\alpha}$	30.9 ± 8.24	294.2 ± 41.9
TXB_2	26.6 ± 5.15	259.5 ± 48.7

Normal mammary tissue or NMU-tumors were ground using a mortar and pestle at 2°C in MES buffer and centrifuged at 800 *g* for 15 min at 4°C. The supernatant thus obtained was further centrifuged at 150,000 *g* for 1 hr at 4°C. The subsequent supernatant was analyzed for the 4 compounds examined.
Student's *t* test with separate variance estimates: $P < 0.001$ for all 4 compounds.

Table 3. Prostaglandin synthetase activity in normal mammary tissue and NMU-induced mammary tumors: ng/mg *protein*/10 min *(mean ± S.E.M.)*

	Normal mammary tissue ($n = 6$)	NMU-induced mammary tissue ($n = 16$)
PGE_2	49.9 ± 8.63	226.3 ± 16.36
$PGF_{2\alpha}$	4.4 ± 0.77	28.1 ± 2.86
6-Keto-$PGF_{1\alpha}$	10.8 ± 1.96	13.5 ± 1.76
TXB_2	10.1 ± 0.95	16.5 ± 1.29

Nanograms of each compound generated by microsomes from 1 mg microsomal protein incubated for 10 min in the presence of 1.25 μM arachidonic acid, 1.25 mM reduced glutathione and 1.25 mM epinephrine at 37°C and pH 7.4.
Student's *t* test: $P < 0.001$ for all but 6-keto-$PGF_{1\alpha}$ (N.S.).

levels, this study additionally demonstrates elevated levels of the stable degradation products of PGI_2 and TXA_2.

The present results also show that microsomal fractions obtained from NMU-induced tumors contain the prostaglandin synthetase that is capable of transforming arachidonic acid (C20:4) to various prostaglandins and to the non-prostanoate derivative thromboxane. Tumor prostaglandin synthetase activity was significantly greater than that of normal tissue with regard to PGE_2 and $PGF_{2\alpha}$ but not with regard to 6-keto-$PGF_{1\alpha}$ and TXB_2.

The reason for the observed elevation in tissue prostaglandin levels in mammary tumors is uncertain. Several possibilities have been proposed: (a) increased activity of prostaglandin synthetase; (b) decreased activity of enzymes metabolizing the prostaglandins; (c) increased availability of precursor fatty acids; or (d) a breakdown in the negative feedback controls which normally regulate the prostaglandin levels [17]. Regarding (a), Kibbey *et al.* [18] recently reported that PGE_2 levels correlated closely with PGE_2 synthetic capacity in a series of human breast tumors and concluded that elevated PGE_2 levels in these tumors could be attributed to the increased biosynthetic capacity of the tumor rather than to enhanced precursor availability. In the present study increased tumor microsomal prostaglandin synthetase activity could account for the higher tissue levels of PGE_2 but not for that of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and TXB_2. The discrepancy between the low microsomal biosynthesis of the latter 3 compounds and their high tissue concentrations could also be due to a methodological artefact. As discussed by Moncada and Vane [19], the use of reduced glutathione in the prostaglandin synthetase assay mixture may favor the production of the stable, classical prostaglandins, PGE_2 and $PGF_{2\alpha}$, at the expense of the other, less stable derivatives. Furthermore, contrary to findings of PGE_2 in human breast cancer, no significant correlations were found between tissue levels of the 4 compounds examined and the corresponding microsomal prostaglandin synthetase activity. Hence the prostaglandin profile generated by microsomal preparations *in vitro* may not accurately reflect the actual prostaglandin biosynthetic capacity of NMU-induced tumors *in situ*. Alternatively, this discrepancy could be real and due instead to differences in the activities of the enzymes involved in metabolizing the prostaglandins (b). However, little is known about the activity of such enzymes (15-hydroxyprostaglandin dehydrogenase and 13,14,prostaglandin reductase) in mammary tumors and this possibility cannot be commented upon at present. Regarding (c), the possible role of enhanced precursor availability, Tan *et al.* [20] have demonstrated that the phospholipid fraction of the dimethylbenzanthracene (DMBA)-induced tumor contains higher levels of arachidonic acid than normal mammary tissue; and in the same vein, Kitada and co-workers [21] have demonstrated the existence of a 'lipid-mobilizing factor' in tumor-bearing mice which mobilizes polyenoic fatty acids from fat stores and transfers them to tumors. Together these studies suggest that tumors may have greater stores of prostaglandin precursor fatty acids available than normal tissues. Related studies by Rillema [22] indicate that the DMBA-induced tumor exhibits increased phospholipase A_2 activity in comparison to either normal mammary tissue or tissue obtained from pregnant animals. Since phospholipase A_2 cleaves fatty acids from the glycerol moiety of membrane phospholipids for utilization by prostaglandin synthetase, one reason for the elevated levels of prostaglandins in mammary tumors may be the increased availability of precursor fatty acids for prostaglandin synthesis. However, since the fatty acid content and phospholipase A_2 activity of the NMU-induced tumors are unknown at present, the relevance of the studies to the NMU-induced mammary tumor remains to be determined. With regard to (d), there is evidence to suggest that under physiological conditions prostaglandins regulate one another's biosynthesis by a complex negative feedback system and that this feedback system breaks down in transformed cells [17].

It is important to note that several intervening variables complicate interpretation of prostaglandin studies in solid tumors. For example, it is well established that simple mechanical disruption—a necessity in analyzing prostaglandins in solid tumors—can stimulate prostaglandin production. In order to control for this variable indomethacin was included in some preparations and the results compared with samples from the same tumor prepared in the absence of indomethacin. Differences between the two samples were insignificant, indicating that the method of disruption was only minimally stimulating prostaglandin synthesis. Moreover, because the same procedures were used for preparation of both normal and tumor tissue, the results obtained can be considered comparable. A second intervening variable is the question of tissue heterogeneity. Tumor tissue, for example, consists of 60–80% epithelial cells, with the remaining cells consisting of mesenchymal tissue, plus some lymphocytes and histiocytes. Normal mammary gland, on the other hand, consists of 60–80% adipose tissue and 10–20% parenchymal

cells. Hence homogenates of the two tissue types differ not only by virtue of their normal and neoplastic character but also in terms of their cellular composition. For this reason the present results can be meaningfully evaluated at the tissue but not the cellular level.

Because lymphocyte and monocyte reactions are present to varying extents in human breast cancers, it has been suggested that elevated prostaglandins in mammary tumors may actually be due to the inflammatory cell infiltrate rather than the neoplastic epithelial component of the tumor. This appears unlikely, however, in view of the fact that the proportion of lymphocytes and monocytes in the NMU-induced tumor is very small. Moreover, Rolland *et al.* [7] recently reported that the degree of lymphocyte and monocyte reaction in a series of human mammary tumors was unrelated to prostaglandin production. Nonetheless, we are currently testing this possibility in a more definitive manner using cultured NMU-tumor cells *in vitro*, where host immune responses are entirely absent.

In summary, the present results suggest that (a) prostaglandin synthesis is 'turned on' in the NMU-induced mammary tumor in comparison to the normal mammary gland; (b) the NMU-induced tumor contains high concentrations of two biologically potent but unstable derivatives of arachidonic acid, namely PGI_2 and TXA_2; (c) the concentration of prostaglandins in a given tumor is inversely related to the size of the tumor but unrelated to its age; and (d) the capacity of NMU-tumor microsomes to synthesize prostaglandins is unrelated to either tumor size or age. Further studies will be necessary to ascertain what enzymatic defects or alterations may account for the excess prostaglandin production and tissue content characteristic of mammary tumors and, more important, to elucidate the role played by prostaglandins in the development and maintenance of mammary tumors.

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