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Note

Assay of prostaglandins in the epithelial cells and fibroblasts of the rat mammary gland

CHARLES E. MORREAL*, DILIP K. SINHA, CHRISTOPHER J. WHITE and DAVID T. NEMOTO

Department of Breast Surgery and Breast Cancer Research Unit, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, NY 14263 (U.S.A.)

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The actual role which prostaglandins (PG) play in mammary cancer is not clear, but high prostaglandin production and poor prognosis go hand-in-hand [1—3]. Experimental evidence has also revealed that high levels of PGE_2 may serve as a marker of the metastatic potential of human breast tumors [4, 5]. The synthesis of prostaglandins in biological samples has been demonstrated in rat chloroleukemia cells in culture [6] and has been studied in patients with severe atherosclerosis [7]. The formation of prostaglandins has been studied in mammary tissue and its homogenates [2] and in epithelial cells of tumors [8]. The assay of prostaglandins by the measurement of their fluorescent derivatives has been demonstrated previously [9], but its application to the mammary gland has not been reported. The present work reveals the independent assessment of the presence of PGE_2 and $PGF_{2\alpha}$ in the epithelial cells and the fibroblasts of the rat mammary gland by high-performance liquid chromatography (HPLC) of their panacyl esters.

EXPERIMENTAL

Chemical studies

Prostaglandins were purchased from Sigma (St. Louis, MO, U.S.A.). Cesium fluoride and 18-crown-6 were obtained from Aldrich (Milwaukee, WI, U.S.A.). Tritium-labeled PGE_2 was obtained from New England Nuclear (Boston, MA, U.S.A.). Panacyl bromide, *p*-(9-anthroyloxy)phenacyl bromide, was a complimentary sample generously supplied by Dr. W. Morozowich of Upjohn

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(Kalamazoo, MI, U.S.A.), and additional material was synthesized in this laboratory from anisole and chloroacetyl chloride. Panacyl bromide is available commercially from Molecular Probes, (Junction City, OR, U.S.A.). Silica gel was purchased from Davison (Baltimore, MD, U.S.A.).

Biological studies

The cells were prepared by the methods currently in use in these laboratories [10]. These are summarized as follows: mammary glands were surgically dissected out and minced with razor blades. The mince was then transferred to a 150-ml fleaker containing 0.45% (w/v) collagenase (Type III, Worthington) solution (10 ml/g of tissue). The fleaker with the mammary gland mince was incubated in a Dubnoff metabolic shaker at 37°C under constant agitation (60 cycles/min) for 90 min. At the end of this time, the digested tissue was filtered through 340 Nylon mesh. The filtrate was then passed through 100-μm Nylon mesh. The cell suspension (filtrate) was rinsed with Eagles' balanced salt solution (EBSS) (Ca^{2+}- and MG^{2+}-free) and centrifuged at 325 *g* for 15 min (Beckman J6; JS rotor). The supernatant was removed, the pellet was resuspended in EBSS and centrifuged again as above. The final pellet was resuspended in 10 ml Eagles' medium containing 10% porcine serum. The final cell suspension was then layered over a preformed Ficoll gradient (2—8% continuous, cushion 20%). The gradient with the cells was spun at 60 *g* for 10 min. The epithelial cells and fibroblasts were thus separated. Epithelial cells accumulate near the bottom of the gradient above the 20% cushion. Cells were collected from this section of the gradient and verified in a phase contrast microscope. The fibroblasts were similarly isolated from the upper layers of the gradient.

Analytical procedures

Mammary gland epithelial cells or fibroblasts ($15 \cdot 10^6$—$150 \cdot 10^6$) in 2 ml RPMI 1640 medium at pH 7.8 were treated with approximately 30 000 cpm tritium-labeled PGE_2 in 20 μl acetonitrile to serve as an internal standard. To determine the endogenous levels of the prostaglandins, this solution was neutralized to pH 4.0 with 1 ml of 0.1 *M* citric acid and extracted with 3 ml ethyl acetate, twice. The combined extracts were dried with sodium sulphate and evaporated. A separate, identical aliquot of cells was treated with a solution of 20 μg arachidonic acid in 20 μl acetonitrile and incubated at 37°C for 15 min, followed by neutralization with citric acid and extraction as above.

The biological samples and the representative standards (1 μg in 20 μl each of PGD_2, PGE_2, and $PGF_{2\alpha}$) were diluted with 50 μl tetrahydrofuran and treated with 200 μg panacyl bromide in 100 μl of 20% tetrahydrofuran in acetonitrile, 5 μg triethylamine in 20 μl acetonitrile, 4 μg 18-crown-6 in 20 μl acetonitrile, and 13.5 μg cesium fluoride in 10 μl tetrahydrofuran (prepared by diluting 10 μl of a water solution of cesium fluoride to 10 ml with tetrahydrofuran). The samples were left at room temperature (21°C) for 18 h. Columns of 7 $\times$ 0.5 cm were prepared with silica gel (100—200 mesh) which had been washed with methanol and activated at 100°C for 1 h. The columns were then pretreated with 5 ml of 95% tetrahydrofuran, 5 ml acetonitrile, and 5 ml methylene chloride, as described previously [9]. After the samples were

applied, the columns were eluted with 4 ml of 10% acetonitrile in methylene chloride, which was discarded, followed by 4 ml of 5% methanol in acetonitrile. The final solution, which contains the prostaglandin esters, was evaporated; the samples containing the standards were diluted to 3 ml, the samples representing arachidonic acid metabolism were diluted to 2 ml, and the samples containing endogenous levels of prostaglandins were diluted to 1 ml.

The prostaglandins were then separated and quantitated by modifications of the HPLC method of Morozowich [11] and Watkins and Peterson [9]. A DuPont Model 848 liquid chromatograph was fitted with a Zorbax-Sil column (25 cm × 4.6 mm I.D., particle size 7 μm). The column was eluted at ambient temperature with 2% (v/v) methanol in methylene chloride at 68.9 bar. Fluorescence spectra of the metabolites and standards were measured with a DuPont Model 836 fluorescence detector. The magnitude of the prostaglandin levels was obtained by measuring the peak height and comparing this value to those obtained by concurrently running appropriate standards [9].

RESULTS

Original plans included PGD_2 in the analytical scheme, until it was realized that an unknown cellular component has a retention time almost identical to the PGD_2 ester in the systems used in this work (see Figs. 1 and 2). Fig. 1 represents a typical chromatogram illustrating the separation of 5.74 ng each of the 9-anthroyloxyphenacyl esters of PGD_2 (retention time 3 min), PGE_2 (retention time 4.1 min) and $PGF_{2\alpha}$ (retention time 8 min). Fig. 2 represents a biological sample containing 7.65 ng PGE_2 and 1.86 ng $PGF_{2\alpha}$.

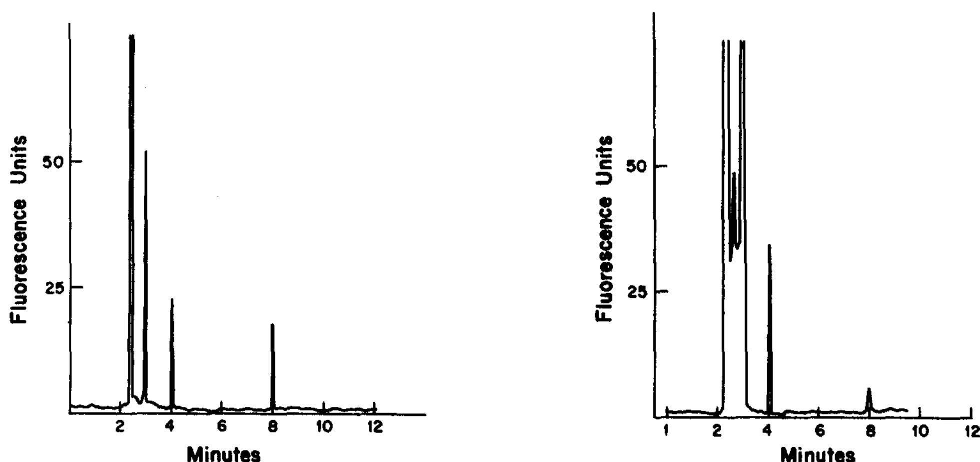

Fig. 1. Chromatogram of 5.74 ng each of standard prostaglandins. Order of elution is: PGD_2, 3 min; PGE_2, 4.1 min; $PGF_{2\alpha}$, 8 min. Zorbax-Sil column, 25 cm × 4.6 mm, eluted with 5% methanol in methylene chloride at 68.9 bar.

Fig. 2. Chromatogram of extract from rat epithelial cells incubated with arachidonic acid. Order of elution is: PGE_2 (7.65 ng), 4.1 min; $PGF_{2\alpha}$ (1.86 ng), 8 min. Zorbax-Sil column, 25 cm × 4.6 mm, eluted with 5% methanol in methylene chloride at 68.9 bar.

TABLE I

ENDOGENOUS LEVELS OF PGE_2 IN MAMMARY CELLS FROM SPRAGUE–DAWLEY RATS

Type of animal	PGE_2 level (ng per 10^6 cells)	
	Epithelial cells	Fibroblasts
58-Day-old female	2.03	1.80
Adenocarcinoma from 141-day-old female	0.62	0.56
111-Day-old male	0.69	0.65

Table I illustrates single measurements of the endogenous levels of PGE_2 and $PGF_{2\alpha}$ obtained from the epithelial cells and the fibroblasts of Sprague–Dawley rats, expressed as ng per 10^6 cells. The total number of cells in the experiments ranged from $15 \cdot 10^6$ to $150 \cdot 10^6$. In the study of the endogenous levels of prostaglandins, it was observed that as much as 50 μl of a 1000-μl biological sample derived from $15 \cdot 10^6$ cells could be injected into the instrument and provide acceptable peaks. This is the lowest number of cells used thus far in this study and was obtained from two animals. Thus, using a single animal as the source of cells and operating the instrument at its highest level of performance, less than one tenth of this amount of cells would be sufficient for the measurement of PGE_2.

Table II shows single measurements representing the capacity of Sprague–Dawley rats to synthesize prostaglandins from added arachidonic acid.

TABLE II

SYNTHESIS OF PGE_2 AND $PGF_{2\alpha}$ FROM ARACHIDONIC ACID BY MAMMARY CELLS FROM SPRAGUE–DAWLEY RATS

Type of animal	PGE_2 level (ng per 10^6 cells)		$PGF_{2\alpha}$ level (ng per 10^6 cells)	
	Epithelial cells	Fibroblasts	Epithelial cells	Fibroblasts
58-Day-old female	6.69	2.45	2.85	0.78
Adenocarcinoma from 141-day-old female	2.23	1.29	1.75	0.97
111-Day-old male	2.27	N.D.*	1.87	N.D.

*N.D. = Not detectable.

DISCUSSION

Original procedures using different grades of silica gel employed methylene chloride to remove unreacted reagent from the esters. Since it was found in this work that excessive amounts of panacyl bromide were present in the fraction containing the prostaglandin esters, elution was conducted with 10% acetonitrile in methylene chloride. This modification, although it effectively removes virtually all of the unreacted reagent, results in a slight loss of the product.

Nevertheless, consistent recovery of the 9-anthroyloxyphenacyl esters in the range of 65—85% with 5% methanol acetonitrile was achieved.

Formation of the fluorescent derivatives was accomplished as described [9], except that cesium fluoride and crown ether were added to the derivative-forming system. Either catalyst system — a tertiary amine or an alkali metal fluoride in crown ether — has been found effective in the formation of prostaglandin esters. Although the amine is a more effective catalyst, it cannot tolerate small amounts of protic solvents. Even though the biological extracts are dried with sodium sulfate, on some occasions it was found that derivative formation was inhibited when only triethylamine or diisopropylethylamine were used and it was presumed that small amounts of water may have been responsible for this effect. Following the introduction of cesium fluoride and crown ether into the protocol, not one case of inhibition has been observed.

Potassium fluoride has been used as a catalyst in the formation of esters [12], a procedure known to be effective in the presence of small amounts of water and alcohols, but it is difficult to maintain potassium fluoride in solution in organic solvents. Cesium fluoride was selected since it tended not to precipitate from solution when aqueous samples were diluted with tetrahydrofuran. Radioimmunoassay procedures have been used to measure the levels of prostaglandins in biological systems. The present HPLC methodology supplements this approach and further provides a medium by which subsequent metabolism or intermediate metabolic products can be studied in detail in a sensitive system.

The primary focus of interest of this group is the possible link between prostaglandins and breast cancer. The role of prostaglandins in carcinogenesis has been implicated by studies which demonstrated that polyunsaturated fats, which are known to be rich in linoleic acid, promote mammary carcinogenesis to a much greater extent than saturated fats, which do not contain this precursor of arachidonic acid. A direct link between dietary linoleate and prostaglandin levels has in fact been established [13, 14].

The prostaglandins themselves have been directly implicated in mammary cancer, but conflicting results have been reported, some indicating stimulation and others inhibition of tumor growth [15]. Prostaglandin formation is elevated most at the early stages of cancer development [5, 16, 17].

Dietary fat has been implicated epidemiologically as a factor in the genesis of breast cancer in humans [18]. The evidence that a high-fat diet in experimental animals serves to promote mammary carcinogenesis induced by polycyclic hydrocarbons is overwhelming [19—21], but the mechanism by which this event occurs has still not been identified.

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