Metabolism of N-[4-(5-Nitro-2-furyl)-2-thiazolyl]formamide by Prostaglandin Endoperoxide Synthetase

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ABSTRACT

Cooxidative metabolism of the urinary bladder carcinogen N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) was examined using solubilized and particulate microsomal preparations from the rabbit renal inner medulla and the ram seminal vesicle. Metabolism was measured by the rate of decrease in absorbance at 400 nm. In these soluble and particulate preparations, FANFT metabolism was observed only in the presence of specific fatty acids. These fatty acids are substrates for prostaglandin endoperoxide synthetase. Structurally dissimilar inhibitors of prostaglandin endoperoxide synthetase such as indomethacin, aspirin, 5,8,11,14-eicosatetraynoic acid, ethoxyquin, and meclofenamic acid specifically inhibited FANFT metabolism. Other inhibitor and substrate specificity studies suggest that FANFT was not metabolized by nitroreductase, xanthine oxidase, lipoxygenase, lipid peroxidation, or mixed-function oxidases. In addition, the lack of detectable 2-amino-4-(5-nitro-2-furyl)thiazole formation suggests that aryiformamidase was not participating in FANFT metabolism measured in these experiments. The data indicate that prostaglandin endoperoxide synthetase can mediate FANFT metabolism by a coaxidative process.

INTRODUCTION

Carcinoma of the urinary bladder is induced by a number of chemical carcinogens (6). The 5-nitrofuran FANFT(3) is a potent urinary bladder carcinogen (9). When fed to experimental animals, FANFT is associated with a high incidence of carcinoma of the urinary bladder. Virtually 100% of weaning Fischer rats develop bladder cancer when fed a diet containing 0.2% FANFT for 26 weeks. It has been proposed that FANFT requires metabolic activation to induce carcinoma of the urinary bladder (25). Several enzyme systems have been shown to metabolize FANFT. These include nitroreductase (3, 27), xanthine oxidase (27), and aryiformamidase (28). Metabolism by these systems, however, has been difficult to relate to the formation of carcinoma of the bladder. For example, when the xanthine oxidase inhibitor allopurinol is administered to rats in conjunction with FANFT, there appears to be enhancement of the carcinogenic potential of FANFT to induce carcinoma of the bladder (29). ANFT is the product of FANFT deamination in the kidney and is observed in the urine of animals fed FANFT (30). However, ANFT is not a urinary bladder carcinogen (7). The purpose of these experiments was to evaluate the possibility that FANFT could be metabolized by a different enzyme system, prostaglandin endoperoxide synthetase. The results indicate cooxidation of FANFT by prostaglandin endoperoxide synthetase prepared from the rabbit inner medulla and the ram seminal vesicle.

MATERIALS AND METHODS

Material. Metyparone, indomethacin, allopurinol, hemoglobin (type I, bovine), butylated hydroxytoluene, sodium benzoate, Tween 20, aspirin (acetylsalicylic acid), 11,14,17-eicosatetraynoic acid, and 9,12,15-octadecatrienoic acid were purchased from Sigma Chemical Co., St. Louis, Mo.; 5,8,11,14-Eicosatetraynoic acid and 8,11,14-eicosatrienoic acid were purchased from Nu-Chek Prep, Inc., Elysian, Minn.; FANFT was purchased from Saber Laboratories, Morton Grove, Ill. The purity of FANFT was greater than 99.5% as judged by previously published criteria (8). 5,8,11,14-Eicosatetraynoic acid, ethoxyquin, and prostaglandin E2 were the generous gifts of Dr. W. E. Scott, Hoffmann-La Roche Inc., Nutley, N. J.; Dr. G. L. Romoser, Monsanto Chemical Co., St. Louis, Mo.; and Dr. John Pike, The Upjohn Co., Kalamazoo, Mich., respectively. All other chemicals were purchased in the highest possible grade from standard sources. Male New Zealand rabbits weighing 1.5 to 2.0 kg were obtained from Eldridge Laboratory Animals, Barnhart, Mo.

Preparation of Microsomes. Rabbits were anesthetized with sodium thiopental (20 mg/kg i.v.). The kidneys were quickly removed and placed in ice-cold 0.9% NaCl solution (33). Renal inner medullary tissue was isolated by careful dissection and washed free of hemoglobin. Ram seminal vesicles were obtained fresh from a local packing plant and dissected free of adjacent tissue. Microsomes from either tissue were prepared as follows. Minced tissue was homogenized for 15 sec with a Polytron homogenizer in 3 volumes of 0.1 m phosphate buffer, pH 7.8, containing 20% glycerol and 0.1 m dithiothreitol. The homogenate was centrifuged at 10,000 × g for 15 min and the subsequent supernatant at 100,000 × g for 60 min. This pellet was layered with 1.15% KCl and stored at −40° C. Before use, the microsomal pellets were suspended in 1.15% KCl by hand homogenization and centrifuged for 60 min at 105,000 × g. Pellets were then resuspended in 0.1 m phosphate buffer, pH 7.8, with a Potter-Elvehjem Teflon-glass homogenizer to give a final concentration of about 10 mg microsomal protein per ml. Prostaglandin endoperoxide synthetase was solubilized with Tween 20 using previously published procedures (21). The final concentration of Tween 20 was 1%. Aliquots were either used immediately or stored frozen at −40° C. Protein content was determined in microsomes by the method of Lowry et al. (18), and in the solubilized preparations by the method of

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3 The abbreviations used are: FANFT, N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide; ANFT, 2-amino-4-(5-nitro-2-furyl)thiazole.
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Bensadoun and Weinstein (4). In both methods, bovine serum albumin was used as the standard.

Incubation Conditions and Analysis of FANFT Metabolism. The rate of oxygenation of FANFT was determined by the rate of decrease in absorbance at 400 nm at 25°. An extinction coefficient of 8.1 mM⁻¹ cm⁻¹ for FANFT was used. Kinetic measurements were made using a Beckman Acta VI recording spectrophotometer, with a scale expander and a temperature control device. The reaction mixture contained microsomal protein, 0.1 M phosphate buffer (pH 7.8), 0.05 mM FANFT, and various test substances in a final volume of 0.2 ml. Reactions were started by the addition of fatty acid. Following addition of arachidonic acid, the reaction was linear with respect to protein concentration and, as shown in Chart 1, with respect to time. Inhibitors were added to both the sample and reference cuvets.

Inhibitors were preincubated with microsomes for 2 min at room temperature prior to addition of fatty acid. All inhibitors and initiators of cooxidation were found to elicit their respective effects in a dose-dependent manner. In most cases, only results with maximally effective concentrations of inhibitors are provided for brevity. To investigate the effects of oxygen on arachidonic acid-dependent metabolism of FANFT, a Thunberg cuvet was used to achieve anaerobic conditions. The cuvet contained the microsomal preparation with FANFT, and the side arm contained the fatty acids. The cuvet was evacuated with a water aspirator for 2 to 3 min to remove air. The enzyme reaction was started by mixing together the solutions in the side arm and the cuvet.

Thin-Layer Chromatographic Analysis. The reaction mixture was extracted with an equal volume of methylene chloride. The organic phase was removed following centrifugation, washed with aqueous NaHCO₃ and water, dried with Na₂SO₄, evaporated under N₂ gas, dissolved in dimethylformamide, and subjected to thin-layer chromatography on 0.1-mm cellulose plates (EM Laboratories, Inc., Elmsford, N. Y.). Plates were developed with an aqueous solvent of 2% formic acid and visualized under UV light. The Rₜ's for the FANFT and ANFT standards were 0.20 and 0.35, respectively. These values are similar to those previously reported (28). ANFT was synthesized from FANFT using a modification of a previously published procedure (7).

High-Pressure Liquid Chromatographic Analysis. Metabolism of FANFT was analyzed on a 4.6-mm (inside diameter) × 25-cm Whatman Partisil-10 ODS (Whatman Inc., Clifton, N. J.). An Instrumentation Specialities Company Model 1440 high-pressure liquid chromatograph was used. The column was eluted at room temperature with a 20-min linear gradient from 80% methanol in water to 100% methanol at a flow rate of 0.8 ml/min. Eluent fractions (0.4 ml) were collected and used for UV-visible spectral analysis (Beckman Acta VI). Samples were prepared as described above.

Results represent the mean ± S.E. of at least 3 separate experiments. Statistical differences were evaluated by Student’s t test for unpaired values.

RESULTS

Metabolism of FANFT was not observed prior to addition of arachidonic acid (Chart 1). Arachidonic acid-dependent metabolism of FANFT by inner medullary microsomes is illustrated in Table 1. A maximum rate of FANFT metabolism was achieved between 0.12 and 0.18 mM arachidonic acid. With 0.18 mM arachidonic acid, greater than 30% of the FANFT is metabolized. Metabolism of FANFT was completely inhibited by 0.025 mM indomethacin, and there was no measurable metabolism in the absence of oxygen. Reaction mixtures from samples in Table 1 were subjected to thin-layer chromatographic analysis. In samples containing 0.12 mM arachidonic acid, 2 spots were observed, one at the origin and the other at an Rₜ of 0.20. The spot at Rₜ 0.20 was identical to the FANFT standard and was the only spot observed with reaction mixtures that did not contain arachidonic acid (no addition). ANFT, the deformylated

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**Chart 1.** Arachidonic acid-dependent oxidation of FANFT (0.05 mM) by rabbit inner medullary microsomes. Oxidation was measured by a decreased absorbance at 400 nm. Arachidonic acid (0.12 mM) was added as indicated, followed by 0.05 mM ethoxyquin.

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product of FANFT metabolism, has an Rf of 0.35, and a corresponding spot was not observed at this position. However, previous studies have shown a nonidentified urinary metabolite of FANFT (Y1) at the origin, using this same chromatographic solvent system (13).

Samples were also analyzed by high-pressure liquid chromatography. Shown in Chart 2A is the elution profile of authentic FANFT. A similar elution profile was observed in samples incubated without arachidonic acid or with both 0.18 mM arachidonic acid and 0.025 mM indomethacin. However, in the presence of 0.18 mM arachidonic acid (Chart 2B), 2 peaks were observed. Peak II corresponds to authentic FANFT, while Peak I represents the product of FANFT metabolism. Using this same solvent system, ANFT had an elution time of 4.7 min.

High-pressure liquid chromatographic fractions corresponding to Peak I were collected, and the UV absorbance spectra were determined (Chart 3). The UV absorbance spectra for FANFT and the material in Peak I were different. The material in Peak I only exhibited an absorbance peak in the 280 nm region. However, FANFT exhibited absorbance peaks in both the 280 nm and 390 to 400 nm regions.

The fatty acid substrate requirement for FANFT metabolism is illustrated in Table 2, using solubilized inner medullary microsomal preparations. In addition to arachidonic acid, 8,11,14-eicosatetraenoic acid, the substrate for monoenoic prostaglandins, was also effective in eliciting FANFT metabolism. Prostaglandin E2, the major product of arachidonic acid metabolism by inner medullary microsomes (32), was not effective. 5,8,11,14-Eicosatetraynoic acid (the acetylene analog of arachidonic acid), 11,14,17-eicosatetraenoic acid, and 9,12,15-octadecatrienoic acid were not effective in initiating FANFT metabolism. The specific activity of FANFT metabolism with 0.12 mM arachidonic acid was higher in the renal solubilized preparations (17.5 ± 0.4 nmol per mg protein per min) than in the nonsolubilized microsomal preparations (11.8 ± 0.4 nmol per mg protein per min). The metabolism of FANFT using solubilized ram seminal vesicle preparations was 23 ± 0.5 nmol per mg protein per min with 0.12 mM arachidonic acid.

Effect of various compounds on arachidonic acid-dependent FANFT metabolism is illustrated in Table 3. All compounds which inhibited FANFT metabolism have been shown previously to inhibit prostaglandin endoperoxide synthetase (10). These compounds include indomethacin, aspirin, 5,8,11,14-eicosatetraynoic acid, ethoxyquin, meclofenamic acid, and butylated hydroxytoluene. Ethoxyquin inhibition of FANFT metabolism is also illustrated in Chart 1. Allopurinol, benzoate, and me tyrapone did not inhibit FANFT metabolism. FANFT metabolism was not observed when incubated with arachidonic acid and either heated microsomes (3 min at 100°C) or in the absence of microsomes.

The solubilized prostaglandin endoperoxide synthetase from ram seminal vesicles demonstrated qualitatively the same substrate and inhibitor specificity as did the enzyme isolated from the renal inner medulla.

### DISCUSSION

The data demonstrate metabolism of FANFT by renal inner
FANFT metabolism by prostaglandin endoperoxide synthetase

medulla. The product of FANFT metabolism has been characterized and shown to be different from FANFT in its thin-layer chromatographic, high-pressure liquid chromatographic and UV spectral properties. FANFT was metabolized by a process of cooxidation by prostaglandin endoperoxide synthetase. This conclusion is supported by the substrate specificity of the reaction and inhibitor data. Arachidonic acid and 8,11,14-eicosatrienoic acid, both substrates for prostaglandin endoperoxide synthetase, initiated inner medullary metabolism of FANFT. Other unsaturated fatty acids which are substrates for lipooxygenase enzymes (11) and susceptible to lipid peroxidation but are not substrates for synthetase (1) did not initiate metabolism of FANFT. Benzoate, an inhibitor of hepatic microsomal lipid peroxidation (16), had no effect on FANFT metabolism. FANFT metabolism was inhibited by aspirin, meclofenamic acid, and indomethacin, all known inhibitors of prostaglandin endoperoxide synthetase (10). Conversely, allopurinol, a xanthine oxidase inhibitor (29), and metyrapone, an inhibitor of the microsomal mixed-function oxidase system (14), had no effect upon FANFT metabolism. In addition, NADPH, a necessary cofactor for mixed-function oxidases, was not required for arachidonic acid-dependent FANFT metabolism. These results are consistent with the lack of renal inner medullary cytochrome P-450 content and mixed-function oxidase activity (2, 33). Although rodent kidneys have been shown to enzymatically deformylate FANFT to ANFT, ANFT was not identified as a reaction product. In previous studies examining kidney de-

formation of FANFT, the dialyzed 900 x g supernatant from whole kidney was used (28). Our studies used an entirely different kidney preparation. Therefore, our studies are not inconsistent with whole kidney deformation of FANFT. The proposed mechanism of metabolism of FANFT in inner medulla, cooxidation by prostaglandin endoperoxide synthetase, is consistent with our previous findings with another furan, 1,3-diphenylisobenzofuran (34), and with another known urinary bladder carcinogen, benzidine (35). Other studies have also implicated prostaglandins in carcinogenesis. Such studies have demonstrated increased prostaglandin synthesis with tumor-producing agents (12, 17) and malignant tumors (15, 26) and decreased prostaglandin synthesis with agents, such as ethoxyquin and butylated hydroxytoluene, which inhibit the development of chemically induced tumors (23, 31).

FANFT metabolism by nitroreductase and cooxidation by prostaglandin endoperoxide synthetase are both monitored by following the decrease in absorbance at 400 nm. However, the metabolism of FANFT reported in this paper is distinctly different from that observed with nitroreductase (27). Oxygen is a necessary requirement for both prostaglandin synthesis and cooxidation of FANFT. By contrast, nitroreductase-mediated metabolism of FANFT is inhibited by oxygen. In addition, nitroreductase has not been shown to require arachidonic acid for metabolism.
activation, nor has it been shown to be inhibited by aspirin and indomethacin.

Prostaglandin endoperoxide synthetase-mediated cooxidation appears due to the peroxidative activity of this enzyme. Purified prostaglandin endoperoxide synthetase has both cyclooxygenase and hydroperoxidase activities (22). The cyclooxygenase activity is responsible for the oxidative conversion of arachidonic acid to prostaglandin G2 (a hydroperoxy prostaglandin endoperoxide), while the hydroperoxidase activity conver erts prostaglandin G2 to prostaglandin H2 (a prostaglandin endoperoxide). In addition to arachidonic acid, prostaglandin G2 and other organic hydroperoxides but not prostaglandin H2 can elicit cooxidation (19, 20). Therefore, the hydroperoxidase and not the cyclooxygenase activity is necessary for cooxidation. Recently, the cooxidative metabolism of 1,3-diphenylisobenzofuran was reported to occur by a radical chain pathway (19). The similarity in the furan structure between 1,3-diphenylisobenzofuran and FANFT suggests a common mechanism of metabolism of both furans by prostaglandin endoperoxide synthetase.

These data are consistent with the hypothesis that the renal inner medulla is a site for the metabolism of chemicals which induce bladder cancer. Prostaglandin endoperoxide synthetase mediates cooxidative metabolism of the bladder carcinogens FANFT and benzidine (35). The renal inner medulla has the highest content of kidney prostaglandin endoperoxide synthetase (32), with the epithelial cells of the collecting duct containing the highest level of synthetase (24). In addition, these epithelial cells are the last cells in contact with urine prior to its entry into the urinary space (5). The renal inner medulla is the site of drug and xenobiotic concentration and excretion. Therefore, the unique anatomical, physiological, and biochemical characteristics of the inner medulla, along with the close proximity of the renal inner medulla to the bladder, make the inner medulla ideally suited for playing a key role in the pathogenetic mechanisms involved in the induction of bladder cancer.

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REFERENCES


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