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A stable epoxide of estrone: Evidence for formation of a 'new' estrogen metabolite

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ABSTRACT

Oxidative metabolism of estrogens is an important feature in liver and some non-hepatic tissues. In initial studies on estrogen metabolism in tissues from the reproductive tract of the stallion, where testicular estrogen secretion is remarkably high, a prominent radiolabeled product from [^{3}H]-estrone (E1) was noted on chromatography; it had a retention time (Rt) between 17β-estradiol (E2) and E1. Unexpectedly, when non-radiolabeled E1 was the substrate no UV absorption at 280 nm was seen at the Rt for the [^{3}H]-labeled product—suggesting a non-aromatic ring A. The following efforts were made to reveal more about the nature of the "unknown" compound. Reduction and acetylation showed, separately, the presence of a single keto and hydroxyl group. Exposure to acid gave a single radiolabeled peak with Rt of 6α-hydroxy-E1—suggesting the presence of a third molecule of oxygen. Mass spectrometry with limited material was inconclusive but supportive for a formula of $C_{18}H_{22}O_3$. Thus, an epoxide involving the aromatic ring of E1 is suggested as a labile intermediate in the formation of the "unknown" metabolite. Estrogen epoxides as labile, reactive intermediates have been considered as potential precursors of the 2- and 4-hydroxy catechol estrogens with implications in breast cancer [Soloway, 2007]. Because of the association of the "unknown" metabolite with 6α-hydroxy-E1, the structural form proposed for the stable epoxide is that for 5α,6α-epoxy-estrone. This represents an alternative to the production of the 2- and 4-hydroxy-catechol estrogens. The broad range in normal tissues where the "unknown" compound was shown to be a persistent metabolite (e.g. mouse mammary glands, ovary, uterus, brain, muscle, equine conceptus, stallion and domestic boar reproductive tracts) suggests more general biological implications.

1. Introduction

Steroid epoxides in biological systems were the subject of an early review [1]. They have been postulated as being participants in, or products of, selective biological oxidations. Attention was first drawn to them as possible intermediates in the biosynthesis of steroids when squalene epoxide was shown to undergo cyclization leading to the ultimate formation of cholesterol. Their biosynthesis and hydrolysis can occur by enzymatic or non-enzymatic processes—for example, cholesterol-5α,6α-epoxide is formed by ultraviolet light irradiation of skin. Although this epoxide of cholesterol was shown to be a stable compound it is generally conceded that steroid epoxides as oxidative metabolites in nature are quite labile. As a consequence, few have been detected and isolated under relatively physiological conditions. The formation of stable 5,6-epoxides of pregnenolone by liver microsomal preparations is at least one other exception [2].

The position of epoxides in the metabolism of estrogens has generated interest largely in relation to cancer. In analogy with the need for metabolic activation of some polycyclic aromatic hydrocarbons (PAHs) to become carcinogenic [3], a cytochrome P-450-mediated oxidative metabolism of the primary estrogens, 17β-estradiol (E2) and estrone (E1), seems necessary for carcinogenicity [4]. Some uncertainty remains as to the actual nature of the oxidized estrogen products that account for the initiation of tumor formation and support. It was proposed that epoxidation of E2 and E1 may be the underlying mechanism for inducing the carcinogenicity of these estrogens [5]. In addition, it has been shown that after reacting with dimethyldioxirane (DMDO), a versatile epoxide-forming oxidant, E2 was able to bind DNA forming DNA adducts [6]. However, an alternative pathway to DNA adducts has been the subject of extensive reviews, namely, the role of catechol estrogen quinones as initiators of breast and other human cancers [7,8]. After the initial oxidation to the catechol estrogens (CEs, 2-hydroxy-E2/E1 and 4-hydroxy-E2/E1) by cytochrome P450

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enzymes, further oxidation gave rise to the semiquinones/quinones that were the proximate carcinogens. The oxidative steps in the hydroxylation of E2 and E1 leading to the CEs themselves may well include epoxide formation as the corresponding epoxy-enone intermediates [9]. In fact, the facile conversion of the nonaromatic 1,2- and the 4,5-epoxyenones of E2/E1 to the catechol estrogens, 2- and 4-OH-E2/E1, respectively, was later demonstrated [10]. Although the chemical synthesis of these epoxy-enones was reported over twenty years ago [11], it remains to be determined whether any of the epoxy-estrogens are found in mammalian systems especially in the context of breast and ovarian cancers [12].

In studies on estrogen metabolism in domestic animals we have consistently encountered a metabolite of E1 which remained unidentified. One feature stood out, namely, it appeared as a metabolite of radiolabeled E1 ([^{3}H]-E1) but was not detected (UV 280 nm) when unlabeled E1 was the substrate in incubations with a number of non-hepatic tissues. Its presence varied from being a relatively prominent to a barely detectable, radioactive peak on chromatography. From the literature the occurrence of an epoxide seemed unlikely; however, as the investigation of its nature progressed a point was reached where we now propose that the metabolite is the 5α,6α-epoxide of E1. The following is a brief account of the work leading to this conclusion. A few examples are also given to illustrate the relatively widespread formation of this metabolite and, thereby, to draw further attention to its possible biological significance. Similar results have also been obtained in more limited studies with [^{3}H]-E2.

2. Materials and methods

2.1. Chemicals and reagents

The radioactive steroids (NEN) obtained from Perkin–Elmer (Shelton, CT, USA) were [2,4,6,7-^{3}H]-17β-estradiol (71.0 Ci/mmol) and [2,4,6,7-^{3}H]-estrone (74.1 Ci/mmol). Nonradioactive steroids were purchased from Steraloids Inc (Newport, RI, USA). 6α-Hydroxy-estrone (6α-OH-E1) was made by Dalton Chemical Laboratories Inc., Toronto, ON, Canada. Solvents from Caledon Laboratories, Ltd (Georgetown, ON, Canada) were glass-distilled and reagent grade; acetonitrile (HPLC grade 190) was used for HPLC. Sep-Pak C_{18} cartridges were purchased from Waters Scientific (Mississauga, ON, Canada). Medium 199 and supplements were supplied by Sigma. All other chemicals were analytical grade from Fisher Scientific (Toronto, ON, Canada).

2.2. Collection and preparation of tissues

2.2.1. Stallion

At castration of 8 stallions (3–17 years-old) the testes were placed on ice and taken to the laboratory where the epididymis and vas deferens were removed. Portions of the vas and mid regions of the caput, corpus and cauda epididymides were stripped of adventitious tissues, minced separately and transferred to a test tube containing physiological buffered saline (PBS). Three washes removed spermatozoa by suspension and gravity sedimentation. In each experiment replicates (2–4) of each tissue (≈250 mg) were dispensed for incubation within 3 h of the surgery.

2.2.2. Domestic boar (Sus scrofa)

Reproductive tracts (n > 10) were removed at slaughter from pubertal Yorkshire pigs (6 month-old) and at euthanasia, by intravenous injection of pentobarbital, from older breeding boars. Vas and epididymal tissues were prepared as above (*2.2.1.*). Prostate and seminal vesicles were also recovered from the older animals (n = 6) and dissected free of extraneous tissues before mincing finely with scissors. Equal amounts of tissue (100–300 mg, wet weight) from each source were dispensed for incubation.

2.2.3. Early equine conceptus

Mares from the research herd at the University of Guelph were used to recover conceptuses (Days 24–26), under conditions approved by the University Animal Care Committee. Conceptuses (n = 10) were collected by transcervical uterine lavage, as described previously [13]. The embryo proper (15–30 mg, wet weight) in PBS was dissected free of its extraembryonic membranes (yolk sac wall, allantois and amnion) under a dissecting microscope, using ophthalmic scissors and forceps. The trophoblast was divided into bilaminar (10–30 mg) and trilaminar (40–70 mg) components by dissection; these were incubated separately, without mincing, as was the case for the allantois (15–40 mg).

2.2.4. Mouse tissues

Mammary glands were dissected from mature nulliparous female mice, 4–6 months-old (n = 20). In all experiments, the tissues were pooled for each animal, minced with scissors and washed by decanting in PBS. About 85–300 mg wet weight of tissue were dispensed for incubation.

Additional tissues were collected from two mice (uterus, ovaries, brain, skeletal muscle and liver), washed and minced as above for the mammary gland. The amounts of tissues taken for incubation were (approximate wet weight, mg): uterus, 45–100; ovary, 10–40; brain, 100; muscle, 85–180; liver, 100.

2.2.5. Tissue incubation

Tissues were incubated for 2–4 h in culture medium (TC-199, 5 ml for >300 mg; 2 ml for <300 mg) in small glass flasks in a shaking water-bath at 37 °C under 5% CO_2 in air, after either [^{3}H]-E1/E2 (1×10^6 cpm; or 2×10^6 cpm for >300 mg) or non-radioactive E1 (50 or 100 μg) were added as substrates. Within 2 h from time of collection, incubations were done in duplicate for radiolabeled substrates and 2–6 replicates for non-radiolabeled E1. After incubation and removal of the media, the tissues were rinsed twice with 200 μl of fresh medium which was then added to the previously collected media. When the larger amounts of tissues were involved, the contents of the flasks were transferred to tubes for centrifugation to recover the media; in which case the tissues were washed twice with centrifugation in 1 ml TC-199 and the washings pooled with the spent media. Media and tissues were stored in glass vials at −20 °C until processed.

2.2.6. Analytical procedures

Steroids were recovered from the medium by solid-phase extraction (SPE, Waters C18 Sep-Pak column) as described previously [14]. Briefly, the incubation medium was diluted with water to a volume of 5 ml, applied to a primed column and the unconjugated and conjugated steroids were eluted with 5 ml of diethyl ether and 5 ml of methanol, successively. The amount of radioactive material in each SPE fraction was determined by liquid scintillation counting (LSC) of an aliquot in 4 ml of Ecolite scintillation cocktail (MP Biochemicals, Solon, OH). The eluates were taken to dryness under nitrogen at <45 °C. The dried residue was dissolved in 100 μl of acetonitrile:water (1:3) for injection on HPLC.

Chromatographic profiles of steroids from SPE were generated on a Waters HPLC System using a Nova-Pak C18 column with UV absorbance monitored at 280, 254 and 210 nm. Four solvent systems were used: (1) a binary solvent gradient (Waters # 8) of acetonitrile: water (28:72 to 90:10); (2) an isocratic solvent system of 30% acetonitrile in water; (3) an isocratic solvent system of 40% acetonitrile in water; and (4) a binary solvent gradient (Waters # 8) of acetonitrile: water (20:80 to 90:10). All systems were run for

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