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METABOLISM OF [^{3}H]EQUILIN IN NORMAL AND MALIGNANT HUMAN ENDOMETRIUM AND IN ENDOMETRIAL ADENOCARCINOMA TRANSPLANTED INTO NUDE MICE

BHAGU R. BHAVNANI and ALAN H. GERULATH
Department of Obstetrics and Gynaecology, St Michael's Hospital and University of Toronto, 30 Bond Street, Toronto, Ontario, Canada M5B 1W8

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Summary—One of the main components of conjugated equine estrogens is equilin sulfate and this estrogen in postmenopausal women is metabolized to 17β-dihydroequilin, 17β-dihydroequilenin and equilenin. To investigate the possibility that some of these estrogens may be formed directly in the target tissues, we studied the *in vitro* metabolism of [^{3}H]equilin in various types of normal and malignant human endometrium, including adenocarcinoma grown in athymic nude mice. The results indicate that normal and neoplastic human endometrium can form the above three metabolites. The highest level of 17β-reduced products were isolated from the normal secretory endometrium. Equilenin was the most abundant metabolite isolated from both the normal and malignant endometrium. The formation of [^{3}H]equilenin indicates the presence of a 6,8(9) steroid dehydrogenase-isomerase in the human endometrium. The formation of 17β-dihydroequilin in the endometrium may be of importance as this estrogen is 8 times more potent as a uterotrophic agent than equilin and estrone.

INTRODUCTION

The ring B unsaturated estrogens equilin, equilenin, 17α-dihydroequilin, 17α-dihydroequilenin, 17β-dihydroequilin and 17β-dihydroequilenin are excreted in the urine by pregnant mares and are formed by a squalene-cholesterol-independent biosynthetic pathway [1]. These estrogens in their sulphate ester form are components of conjugated equine estrogen preparations such as Premarin (Ayerst), which also contains sulphate esters of estrone, 17β-estradiol and 17α-estradiol. The 17β-reduced metabolites are present in trace amounts [2]. Several epidemiological studies indicate a strong association between endometrial cancer and conjugated equine estrogen replacement therapy [3–5], and this has been recently reviewed [6]. Furthermore, in one study, the increased risk of endometrial cancer was observed only with medications containing conjugated equine estrogens and not with estriol or estradiol [7]. Since conjugated equine estrogens consist of essentially equal amounts of ring B unsaturated estrogens and the classical estrogens, the role of each group of estrogens in the pathogenesis of endometrial cancer cannot be ascertained from epidemiological studies. However, the implications of the above observations are that some component(s) of the ring B unsaturated estrogens may play a key role in the increased risk observed with estrogen use.

Even though conjugated equine estrogens have been used for replacement therapy for over 40 yr, very little is known regarding the ring B unsaturated estrogen component of these drugs. We recently reported on the MCR (metabolic clearance rate) of equilin sulphate and equilin in postmenopausal women and demonstrated that equilin and equilin sulphate are metabolized *in vivo* to circulating sulphate esters of 17β-dihydroequilin, 17β-dihydroequilenin and equilenin, and that these metabolites are excreted in the urine as glucuronides [8, 9]. The *in vivo* formation of 17β-dihydroequilin is of importance, as this estrogen is several fold more potent uterotrophic agent than equilin or estrone [1]. Since it is well known that interconversion between estrone and estradiol can occur in the human endometrium, the objective of this study was to determine whether the formation of the biologically active estrogen 17β-dihydroequilin can occur in the normal and malignant human

endometrium. Since the amount of malignant endometrial tissue available for biochemical studies in most instances was very small, we have used the heterotransplantation of small pieces of human endometrial adenocarcinoma into athymic nude mice as a means of increasing the amount of tissue available for biochemical investigations [10, 11].

MATERIALS AND METHODS

Tissue samples

Specimens of normal and neoplastic human endometrium were obtained from patients undergoing hysterectomy or curettage. After removal of a part of the endometrial tissue for histology, the remaining tissue was rinsed with ice-cold Krebs–Ringer-phosphate buffer (pH 7.4) and was immediately used for incubations. Endometrial adenocarcinoma tumor pieces (1 × 2 × 2 mm) were transplanted subcutaneously into the flank of 6–8-week old athymic nude mice (Nu/Nu strain) bred and maintained in our animal facilities. Tumors were excised when they reached approximately 0.5–2.5 cm in diameter. Eleven endometrial tumors were transplanted: growth occurred in 8 (72%), of which 6 tumors were analyzed in this study. A poorly differentiated adenocarcinoma was maintained by serial transplantation in nude mice for five generations. Tumors from the first, second, third and fifth generation were analyzed.

Chemicals and isotopes

All solvents were analytical grade and were purchased from Fisher Scientific Company (Toronto). Biochemicals were obtained from Sigma Chemical Company (St Louis, Mo.). [2,4-^{3}H]equilin (SA 41 Ci/mmol) was obtained through custom synthesis (NEN) and was purified as described previously [12, 13]; its purity was over 95% when checked by isotope dilution technique.

Measurement of radioactivity

Radioactivity was measured in a Liquid Scintillation Spectrometer (Beckman, model LS 7500, Palo Alto, Calif.). The radioactive material in samples was measured by counting aliquots in 15 ml of toluene phosphor solution containing 0.4% Omnifluor (NEN). Crystalline samples were dissolved in 2 ml methanol to which were added 13 ml of toluene phosphor solution. All counts were corrected to 100% efficiency by external standardization.

Incubation procedure

Endometrial tissue (200 mg) was minced with fine scissors and incubated in 2 ml of Krebs–Ringer-phosphate buffer (0.1 M Na_2HPO_4, 0.15 μM KCl, and 0.15 μM NaCl, pH 7.4) containing 10 μM [^{3}H]equilin (5 μCi). The incubations were carried out in an atmosphere of O_2:CO_2 (95:5) at 37°C for 1 h. Control incubations were performed by using tissue which had been heat inactivated (80°C for 30 min).

Extraction and purification

At the end of the incubation, the tissue was homogenized in the medium and the mixture poured into a centrifuge tube containing 20 ml of ethanol and carrier equilin, equilenin, 17β-dihydroequilin and 17β-dihydroequilenin (1 mg of each). The mixture was vortexed and centrifuged for 10 min) (1000 g). The supernatant was decanted and the pellet resuspended in ethanol and recentrifuged. The process was repeated twice and the combined supernatant evaporated to dryness. The residue was dissolved in 100 ml of ethyl acetate and the extract washed with water (10 ml × 3) and then evaporated to dryness. The residue containing equilin and its metabolites was fractionated by paper chromatography using system A: n-heptane–benzene–methanol–water (10:5:8:2). The zones corresponding in mobility to equilin, equilenin, 17β-dihydroequilin and 17β-dihydroequilenin were eluted and rechromatographed as follows: equilin and equilenin were further purified by paper chromatography using system B: Isooctane–methanol–water (10:9:1), while 17β-dihydroequilin and 17β-dihydroequilenin were rechromatographed using system C: benzene–cyclohexane–methanol–water (1:2:3:3), as described previously [9, 13]. After the last chromatographic step, the amount of carrier steroids remaining were determined by measuring the optical density at 280 nm and the percent recovery calculated. The purified metabolites were characterized by reverse isotope dilution technique. Radiochemical purity was established by crystallization to constant specific activity both before and after the formation of a suitable derivative. The amount of each metabolite formed is corrected for procedural losses and is expressed as percent formed per hour.

RESULTS

Metabolism of [^{3}H]equilin in various types of endometrium

Approximately 75–85% of the radioactivity was recovered in the ether extract containing the phenolic steroids. The remaining radioactivity was water soluble and consists of steroid conjugates and was not processed. From the ether extract [^{3}H]17β-dihydroequilin, [^{3}H]17β-dihydroequilenin, [^{3}H]equilenin and [^{3}H]equilin were isolated and identified. The radiochemical purity of each of these metabolites was established and an example of the procedure is shown in Table 1. The amount of each metabolite isolated is shown in Table 2. The results indicate that both normal and neoplastic human endometrium can metabolize equilin to equilenin, 17β-dihydroequilin and 17β-dihydroequilenin. These results indicate that human endometrium can form the more potent estrogen 17β-dihydroequilin from equilin. The total percentage conversion of [^{3}H]equilin to the 17β-reduced steroids (17β-dihydroequilin + 17β-dihydroequilenin) in the proliferative, secretory and hyperplastic endometrium and in adenocarcinoma was 2, 7, 5 and 4% respectively. The amount of 17β-dihydroequilin and 17β-dihydroequilenin formed by the secretory endometrium was significantly higher than that formed by the proliferative endometrium. These metabolites were undetectable in the heat inactivated tissue. The formation of [^{3}H]equilenin indicates the presence of a 6,8(9) steroid dehydrogenase-isomerase in the human endometrium. The activity of this enzyme was quite variable and in some tissues, conversions as high as 20% were observed (Table 2). The amounts of [^{3}H]equilin remaining was also variable and the lowest amounts recovered were from the adenocarcinoma samples (mean 49.6%; range 23–67%).

Table 1. Proof of radiochemical purity of [^{3}H]labeled estrogens isolated following incubation of [^{3}H]equilin with an explant of human endometrial carcinoma tissue transplanted in athymic nude mice

Crystallization number	Specific activity (dpm/mmol × 10^6)	
	Equilin[a]	Equilin-3-acetate[b]
1	2.90	2.20
2	2.40	2.20
3	2.30	2.20
Calculated	3.00	2.30
	Equilenin[c]	Equilenin-3-acetate[b]
1	5.30	4.60
2	4.60	4.30
3	4.60	4.50
Calculated	5.70	4.60
	17β-Dihydroequilin[d]	17β-Dihydroequilin-3-methyl-ether[e]
1	0.60	0.40
2	0.50	0.40
3	0.50	0.40
Calculated	0.72	0.50
	17β-Dihydroequilenin[f]	17β-Dihydroequilenin diacetate[b]
1	1.90	1.60
2	1.50	1.60
3	1.60	1.50
Calculated	2.20	1.60

[a]A total of 5×10^5 dpm of [^{3}H]equilin was mixed with 45.2 mg of carrier equilin before crystallization. The calculated values are based on these figures.
[b]The third crystals were acetylated and the acetates formed were crystallized.
[c]A total of 5.65×10^5 dpm of [^{3}H]equilenin was mixed with 26.2 mg of carrier equilenin before crystallization. The calculated values are based on these figures.
[d]A total of 4.1×10^4 dpm of [^{3}H]17β-dihydroequilin was mixed with 15.4 mg of carrier 17β-dihydroequilin before crystallization. The calculated values are based on these figures.
[e]The third crystals were methylated and the methyl ether formed was crystallized.
[f]A total of 1.2×10^5 dpm of [^{3}H]17β-dihydroequilenin was mixed with 14.8 mg of carrier 17β-dihydroequilenin before crystallization.

Metabolism of [^{3}H]equilin in endometrial carcinoma explants grown in nude mice

Six endometrial adenocarcinoma samples were successfully implanted in nude mice and gave sufficient tissue for analysis. One of these tumors was transplanted through five generations; tissue from the first, second, third and fifth generation was analyzed (Table 3, samples 1–4). Samples from these serial tumor transplants were submitted for histology and

Table 2. *In vitro* metabolism of [^{3}H]equilin in normal, hyperplastic and malignant human endometrium

Type of endometrium (number)	17β-Dihydroequilin	17β-Dihydroequilenin	Equilenin	Equilin
Proliferative (9)	1.2 ± 0.2 (0.2–2.1)	0.9 ± 0.1 (0.26–1.2)	11.2 ± 2 (4.8–20)	65.4 ± 2 (51–73)
Secretory (9)	3.6 ± 0.5* (0.8–5.7)	3.0 ± 0.9** (0.6–9)	10.2 ± 2 (3.8–19)	58.9 ± 3 (41–65)
Hyperplasia (3)	2.3 ± 0.6 (1.7–3.4)	3.1 ± 1.5 (1.4–6.0)	8.5 ± 1 (6.5–11)	69 ± 4 (61–73)
Adenocarcinoma (11)	1.90 ± 0.3 (0.4–3)	2.0 ± 0.3 (0.5–4.3)	9.0 ± 1 (2.3–14)	50 ± 4 (23–67)

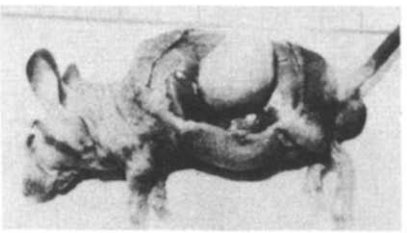
Results are given as ±SEM (range of values).
*Secretory vs proliferative $P < 0.001$.
**Secretory vs proliferative $P < 0.04$.

Table 3. *In vitro* metabolism of [^{3}H]equilin in explants grown in nude mice

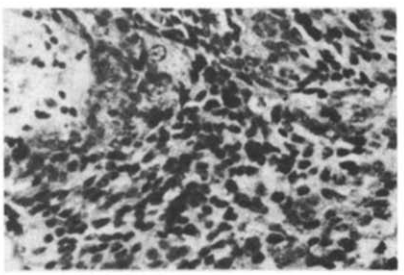
Number	17β-Dihydroequilin	17β-Dihydroequilenin	Equilenin	Equilin
1*	1.00	1.40	24.0	63.0
2*	0.60	0.75	16.0	60.0
3*	2.50	2.20	13.0	55.0
4*	0.70	1.70	13.0	28.0
5	0.90	0.90	9.0	51.0
6	0.80	0.90	16.0	54.0
7	3.10	1.20	9.0	73.0
8	2.60	1.00	13.0	66.0
9	1.60	0.60	9.0	70.0
Mean $\pm$ SD	1.50 $\pm$ 0.3	1.20 $\pm$ 0.2	13.5 $\pm$ 1.7	58 $\pm$ 4

Results are given as $\pm$SEM.
*Explants from the first, second, third, and fifth generation of the same primary adenocarcinoma.

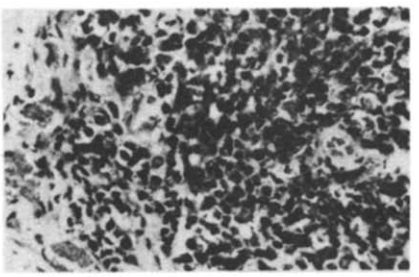
analyzed for estradiol and progesterone cytosol receptors (ER, PR). Histologic analysis (Fig. 1) indicated that the characteristic morphology of the primary tumor was maintained throughout the serial passage of the tumor in nude mice, however, the ER and PR cytosol receptor concentrations decreased with each generation; by the third generation, progesterone receptors were undetectable. ER (fmol/mg protein) primary 89; 2nd generation 33; 3rd generation 22; 5th generation 16; PR (fmol/mg protein) primary 160; 2nd generation 34; 3rd and 5th generation negative). Incubation of the explants with [^{3}H]equilin resulted as observed with the fresh tissue in the formation of 17β-dihydroequilin, 17β-dihydroequilenin and equilenin.



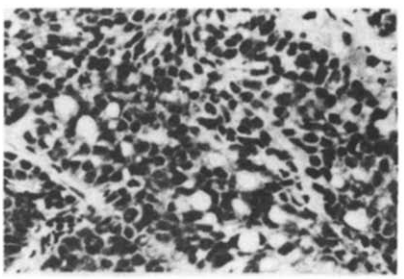
TRANSPLANTED CARCINOMA
IN NUDE MOUSE



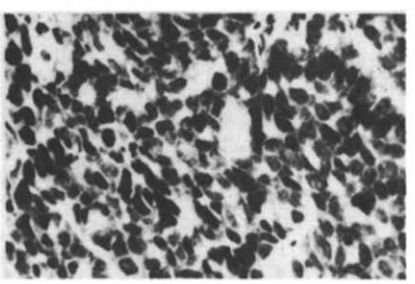
CARCINOMA - PATIENT



CARCINOMA - NUDE MOUSE
1st GENERATION



3rd GENERATION



5th GENERATION

Fig. 1. Histologic features of a poorly-differentiated adenocarcinoma of the human endometrium. The morphologic features remain unchanged on serial passage through nude mice.

However, amounts of the 17β-reduced metabolites formed were similar to those found in the proliferative endometrium and approximately 2-fold lower than those found in the secretory endometrium (Table 3). Though the formation of equilenin in these samples was higher (1.5-fold), this was not statistically significant. No difference was noted in the metabolism of equilin in the first to the 5th generation tumor explants (Table 3). However, the amount of [^{3}H]equilin not metabolized (28%), and the total amount of radioactivity recovered from the 5th generation tumor sample was lower. These results indicate that human endometrial tumor tissue grown in nude mice retains qualitatively its biochemical ability to metabolize equilin at least for the first 3–4 generations.

DISCUSSION

Recently it was demonstrated that the administration of [^{3}H]equilin or [^{3}H]equilin sulphate to postmenopausal women and men, resulted in formation of 17β-dihydroequilin, 17β-dihydroequilenin and equilenin, which were isolated from plasma and urine [8, 9]. The present investigation demonstrates that all three *in vivo* metabolites of equilin or equilin sulfate can be formed in normal and neoplastic human endometrium *in vitro*. The enzyme involved in the conversion of equilin to its 17β-reduced products is 17β-hydroxysteroid dehydrogenase (estradiol-dehydrogenase, E_2DH), an enzyme involved in the interconversion of estradiol and estrone in the human endometrium [14–18]. Alternatively, since multiple forms of this enzyme have been observed [19], the possibility that the 17β-hydroxysteroid dehydrogenase involved in the metabolism of ring B unsaturated estrogens is a distinct enzyme (isoenzyme), needs to be investigated. In the presence of NAD^+, the endometrial 17β-hydroxysteroid dehydrogenase converts estradiol to estrone; the reverse reaction, namely estrone→estradiol is of considerably lower magnitude, and under physiological conditions the oxidation reaction (estradiol→estrone) is favoured [18].

Equilin sulphate, one of the major components of conjugated equine estrogen preparations [1], is rapidly transformed *in vivo* to 17β-dihydroequilin and 17β-dihydroequilenin [8, 12]; we have in the present study, determined only the reverse reaction (reduction) i.e. equilin→17β-dihydroequilin + 17β-dihydroequilenin. The conversion of equilin to its 17β-reduced metabolites was highest in secretory endometrium. This is in keeping with the several fold higher 17β-hydroxysteroid dehydrogenase activity reported in the secretory endometrium [15, 18].

Results from the present study demonstrate that the endometrial 17β-hydroxysteroid dehydrogenase can also utilize an exogenous estrogen such as equilin, as substrate, in a manner similar to estrone. Though the interconversion between estradiol and estrone in the human endometrium is in the direction of estrone, this relationship needs to be established with the ring B unsaturated estrogens, i.e. 17β-dihydroequilin→equilin. We have now synthesized (unpublished data) high specific activity [^{3}H]17β-dihydroequilin and its metabolism in the human endometrium will be investigated in future studies. Whether 17β-dihydroequilenin is formed from 17β-dihydroequilin or from equilenin remains to be established. More detailed kinetic experiments under steady state conditions are needed to confirm these observations.

Though the metabolism of equilin in the adenocarcinoma explants grown in nude mice was qualitatively similar to the one observed with fresh tissue, the complete loss of progesterone receptors in the 3rd generation transplants and the progressive loss of estradiol receptors, indicates significant changes from the original tumor biochemistry, even though the histology of the tumor was not altered. Similar results have been previously reported by Satyashoroop *et al.* [11]. These authors further reported that the loss of steroid receptors on serial transplantation can be prevented by administration of exogenous estrogen to the nude mice. This approach, however, limits the usefulness of this model.

The most abundant metabolite identified in these experiments was equilenin. This is the first report, that we are aware of, in which the presence of the enzyme 6,8(9) steroid dehydrogenase (equilenin synthetase) in the human endometrium was demonstrated. However, the presence of this enzyme has been previously demonstrated in the rat liver [20]. Though, equilenin is a comparatively weak estrogen [11], it does possess the naphthalene ring structure and is therefore chemically more reactive than the other estrogens which have a benzenoid ring. It resembles more closely the polynuclear aromatic hydrocarbons, which are well known carcinogens [21, 22]. Though the actual mechanism is still unknown, conjugated equine estrogens and

other estrogens are thought to play a role in the etiology of endometrial carcinoma [3–7]. It has been suggested that the oncogenic potential of an estrogen depends on the extent of its metabolism to extremely labile catechol estrogens [23].

The formation of catechol estrogens 2- and 4-hydroxyestradiol in equal amounts has been reported to occur in human proliferative endometrium [24], and since equilin and equilenin can also form catechol estrogens [23], it is tempting to speculate that these equine estrogens can form the catechol derivatives in the human endometrium. The incubation conditions and the isolation procedures used in the present investigation are not suitable for the isolation of these labile estrogen metabolites. In the present investigation, the total amount of radioactivity recovered as unmetabolized substrate ([^{3}H]equilin) and the three metabolites, was between 50–80%. Since less than 15% of the total radioactivity was present in the aqueous phase (conjugate fraction), a significant amount of radioactive metabolites in some samples still remain to be identified.

In the Syrian hamster kidney model 17β-estradiol, estrone and equilin induce kidney tumors, while equilenin was inactive [25, 26]. Though 2-hydroxyestradiol and 2-hydroxyestrone were inactive in the above system, 4-hydroxyestradiol in contrast induced kidney tumors in 100% of the animals. Interestingly, equilenin only forms the 4-hydroxy metabolite [23]. However, its carcinogenic potential (if any) has not been determined.

It is also interesting to note that though the ring B unsaturated estrogens are belived to be native only to the pregnant mare, Salhanick and Berliner reported the presence of equilenin in human feminizing adrenal carcinoma and in this tumor, it was the most abundant estrogen present [27]. Thus, the formation of equilenin from equilin may be of importance in the human endometrium.

In conclusion, our results indicate that equilin is extensively metabolized by the human endometrium and that some of these metabolites are more potent uterotropic agents. Whether these metabolites play a direct or indirect role in the etiology of the carcinoma of the human endometrium remains to be investigated.

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