J. Steroid Biochem. Molec. Biol. Vol. 37, No. 2, pp. 301–303, 1990
Printed in Great Britain. All rights reserved

0960-0760/90 $3.00 + 0.00
Copyright © 1990 Pergamon Press plc

SHORT COMMUNICATION

IN VIVO CONVERSION OF NORETHISTERONE TO ETHYNYLOESTRADIOL IN PERIMENOPAUSAL WOMEN

M. J. REED,* M. S. ROSS, L. C. LAI, M. W. GHILCHIK[1] and V. H. T. JAMES
Department of Chemical Pathology and [1]The Breast Clinic, St Mary's Hospital Medical School, London W2 1PG, England

(Received 5 May 1990)

Summary—The extent to which norethisterone is converted to ethynyloestradiol is controversial. To investigate the conversion of norethisterone to ethynyloestradiol we have used a double isotope infusion technique to measure the conversion *in vivo*. The use of acids or bases was precluded to prevent possible artefactual formation of phenolic metabolites of norethisterone. Transfer constants for the conversion of norethisterone to ethynyloestradiol in two perimenopausal women were 2.26 and 2.34% as measured in blood and 2.27 and 0.38% in urine. Results from this study show that a small but significant proportion of norethisterone is converted to ethynyloestradiol *in vivo*.

Since the introduction of norethisterone for use in oral contraceptive therapy the extent, if any, to which this steroid is converted to ethynyloestradiol has remained controversial [1]. Initial studies by Breuer *et al.* [2] and Brown and Blair [3] reported the excretion of ethynyloestradiol after administration of norethisterone. Kamyab *et al.* confirmed the presence of [4-^{14}C]ethynyloestradiol in urine after the administration of [4-^{14}C]norethisterone [4]. Studies carried out by Townsley and Brodie, however, suggested that phenolic steroids detected in urine after administration of norethisterone might result from artefactual formation [5, 6]. These authors were able to demonstrate that an intermediate involved in the aromatization of androstenedione to oestrone, 19-norandrostenedione, was converted to 1β-hydroxy-19-norandrostenedione by placental and ovarian tissues. In the presence of acids or bases, used to extract phenolic steroids from urine, this 1β-hydroxy metabolite of androstenedione can be converted to an oestrogen.

In order to inhibit the artefactual conversion of 1β-hydroxymetabolites of norgestrel to phenolic products Sisenwine *et al.* developed a method in which urine was treated with sodium borohydride [7]. By reducing the 1-hydroxy-4-ene-one grouping in ring A of these metabolites the possibility of aromatization is prevented. Using such a procedure Sisenwine *et al.* found that while phenolic metabolites were still detectable in urine after administration of [4-^{14}C]norgestrel (0.17–0.27% of the administered dose), this was lower than the amount of administered dose detected in the phenolic fraction in the absence of treatment with sodium borohydride (0.63–0.88%).

Since these urinary investigations, however, studies have shown that human tissues are capable of converting norethisterone to ethynyloestradiol. Barbieri *et al.* using placental microsomes, and taking care to avoid the use of acids or bases, demonstrated that norethisterone was converted to ethynyloestradiol *in vitro* [8]. Conversion of norethisterone to ethynyloestradiol was linear with respect to time and microsomal protein content. Using human liver tissue Urabe *et al.* were similarly able to demonstrate conversion of norethisterone to ethynyloestradiol [9]. More recently Stepan *et al.* have used norethisterone to protect against menopausal bone loss [10]. They found that norethisterone (5 mg/day) resulted in a significant reduction in the rate of bone resorption. While this could be due to the androgenic or progestational effects of norethisterone on bone cells, they postulated that such protection could result from the metabolic transformation of norethisterone to ethynyloestradiol.

In order to investigate further this controversial aspect of the metabolism of norethisterone, we have measured the *in vivo* conversion of norethisterone to ethynyloestradiol using a similar double isotopic infusion technique to that developed to measure the peripheral conversion of androstenedione to oestrone in women.

SUBJECTS AND METHODS

Subjects

Two perimenopausal women with breast cancer were investigated after obtaining their informed consent. They had not received any medication for at least 1 month prior to the investigations.

Methods

Conversion of norethisterone to ethynyloestradiol was measured by the blood and urinary methods. [4-^{14}C]Norethisterone (54 mCi/mmol, obtained from Schering AG, Berlin) and [6,7-^{3}H]ethynyloestradiol [50 Ci/mmol, N.E.N., Du Pont, (England) Ltd] were purified before use by paper chromatography using the solvent system light petroleum:toluene:methanol:water (5:5:4:1 by vol). Norethisterone and ethynyloestradiol were infused in 5% ethanolic saline containing 4% human serum albumin at rates of 6.6 and 1.5 μCi/h for subject 1 and 2.9 and 1.2 μCi/h for subject 2 over a 12 h period. At the end of the infusion blood samples (2 × 50 ml) were taken and after centrifugation, plasma was stored at −20°C until processed. Urine was collected for 3 days from the start of the isotopic infusion. Samples were refrigerated at 4°C until the end of each 24 h period when urine was stored at −20°C until processed.

To whom correspondence should be addressed.

Table 1. Transfer constants ($[\rho]_{BB}^{Net\ EE}$) for the conversion of norethisterone (Net) to ethynyloestradiol (EE) in perimenopausal women

Subject	$^3H/^{14}C$ infused	$^3H/^{14}C$ EE	$[\rho]_{BB}^{Net\ EE}$
A	0.178	7.88	2.26
B	0.320	13.5	2.34

Measurement of $[\rho]_{BB}^{Net\ EE}$ and MCRs norethisterone and ethynyloestradiol

The conversion of norethisterone to ethynyloestradiol in blood was measured by a similar technique to that used to measure the conversion of androstenedione to oestrone [11, 12]. Briefly, after the addition of 200 μg of unlabelled norethisterone and ethynyloestradiol to monitor procedural losses, steroids were extracted with diethylether. After evaporation of the solvent the residue was dissolved in toluene:methanol (85:15, v/v) and subjected to Sephadex LH_{20} column chromatography. Eluted fractions containing norethisterone or ethynyloestradiol were pooled and steroids further purified by thin-layer chromatography (TLC) using aluminium oxide (F254) plates (Merck, Darmstadt, W. Germany) using chloroform as the solvent. Steroids were detected by u.v. absorption and the appropriate areas of the TLC plate were eluted with diethylether before further chromatography using this system. Recoveries of unlabelled norethisterone and ethynyloestradiol were measured spectrophotometrically (norethisterone 247 nM, ethynyloestradiol 250 nM). Recoveries for the two subjects were 22 and 57% for norethisterone and 33 and 30% for ethynyloestradiol. Radioactivity in the isolated norethisterone and ethynyloestradiol was measured using liquid scintillation spectrometry [11]. Transfer constants for the conversion of norethisterone to ethynyloestradiol were calculated by comparison of the $^3H:^{14}C$ ratio of infused steroids with that of ethynyloestradiol isolated from plasma [13]. In addition to measuring transfer constants, metabolic clearance rates (MCR) for norethisterone and ethynyloestradiol were determined from the rate of isotopic infusions and steady-state isotopic concentrations, corrected for procedural losses [13].

To validate the method 2000 dpm [4-^{14}C]norethisterone was added to 14 ml plasma which was then processed according to the procedure described. No radioactivity above the background level was detectable in the eluted area of the TLC plate corresponding to the location of ethynyloestradiol.

Measurement of $[\rho]_{BU}^{Net\ EE}$

Conversion of norethisterone to ethynyloestradiol was also measured by the urinary method. Pooled samples from the 3-day urine collection were prepared and steroid conjugates extracted by XAD-2 column chromatography [11]. Steroid conjugates were eluted with methanol and after evaporation of the methanol, hydrolyzed using a β-glucuronidase enzyme preparation (Sigma, Poole, England). Subsequent treatment with sodium borohydride, extraction and chromatography using an AG1-X2 anion exchange resin (Bio-Rad Laboratories Ltd) followed the procedure described by Sisenwine *et al.*[7]. Phenolic metabolites were eluted with 80% aqueous methanol and re-chromatographed using the AG1-X2 anion exchange resin. Phenolic metabolies were further purified by successive TLC using aluminium oxide plates with chloroform as the solvent. Transfer constants were calculated from a comparison of the $^3H:^{14}C$ ratio of infused steroids to the $^3H:^{14}C$ ratio of ethynyloestradiol isolated by this procedure.

Table 2. Metabolic clearance rate (MCR) of norethisterone (Net) and ethynyloestradiol (EE) in perimenopausal women

Subject	MCR–Net (1/24 h)	MCR–EE (1/24 h)
A	312	1809
B	279	1346

Table 3. Transfer constants $[\rho]_{BU}^{Net\ EE}$ for the conversion of norethisterone (Net) to ethynyloestradiol (EE) in perimenopausal women

Subject	$^3H/^{14}C$ Infused	$^3H/^{14}C$ ratios after: 1st TLC	2nd TLC	$[\rho]_{BU}^{Net\ EE}$ %
A	0.178	7.89	7.83	2.27
B	0.320	79.2	83.5	0.38

RESULTS

Significant levels of ^{14}C-radioactivity were detectable in the fractions associated with ethynyloestradiol extracted from blood and urine. Conversion of norethisterone to ethynyloestradiol measured in blood (Table 1) was similar for both subjects. The levels of [4-^{14}C]norethisterone and [^{3}H]ethynyloestradiol isolated from blood were used to calculate the MCR–norethisterone and MCR–ethynyloestradiol in these two subjects (Table 2). The MCR–ethynyloestradiol was 5–6 times higher than that of norethisterone.

Transfer constants measured by the urinary method are shown in Table 3. For one subject the value was similar to that determined by the blood method whereas the urinary value was lower than the corresponding blood value for the second subject.

DISCUSSION

Results obtained from this study suggest that a significant proportion of administered norethisterone is converted to ethynyloestradiol. Using techniques which avoid the use of acids or bases transfer constants were 0.4% measured by the urinary method for one subject and just over 2% for the other subject. The reason for the discrepancy in values measured by the blood and urinary method for one subject is not apparent. Values found for the conversion of norethisterone to ethynyloestradiol are within the range reported for the conversion of androstenedione to oestrone in postmenopausal women [12, 14].

If norethisterone is converted to ethynyloestradiol *in vivo* this could have important biological implications. As previously noted Stepan *et al.*[10] administered 5 mg norethisterone to postmenopausal women and found that this dose gave some protection against bone loss, though the effect was less than that achieved with 50 μg oestradiol administered transdermally. Given a 5 mg dose of norethisterone conversion of 0.4–2.27% as found in this study, would yield from approximately 20 to 100 μg ethynyloestradiol. Obviously these calculations do not take into consideration the extent of drug absorption or enterohepatic metabolism of norethisterone but do suggest that a biologically significant amount of ethynyloestradiol is formed from norethisterone by peripheral aromatization. As Stepan *et al.* [10] found that 5 mg norethisterone was less effective than 50 μg oestradiol at protecting women from bone loss, the amount of ethynyloestradiol formed from 5 mg of orally administered norethisterone is likely to be lower than that calculated, probably due to incomplete absorption of the drug.

In addition to protecting against bone loss, ethynyloestradiol formed from norethisterone might be expected to stimulate the synthesis of sex-hormone binding globulin (SHBG) and possibly stimulate the endometrium in postmenopausal women. With regards to a possible effect on SHBG synthesis, it is now accepted that the ability of orally administered oestrogens to stimulate SHBG is due to a first pass effect in the liver. No increase in plasma SHBG levels is seen when oestrogens are administered transdermally [25]. Therefore any ethynyloestradiol formed from norethisterone *in vivo* would not be expected to stimulate SHBG synthesis.

Whether ethynyloestradiol formed from norethisterone might stimulate the endometrium of postmenopausal women is more difficult to assess. Norethisterone has been shown to possess oestrogenic properties in a mouse uterine test, a finding which could be accounted for by conversion to ethynyloestradiol [26]. As far as we are aware, however, there are few reports of the administration of norethisterone alone to postmenopausal women. Obviously if norethisterone is used to protect menopausal women against bone loss then such studies will need to be carried out.

Much of the interest in originally studying the possible conversion of norethisterone to ethynyloestradiol arose from adverse side effects associated with oral contraceptive therapy and which are thought to be due to the oestrogenic component of the pill. It is of interest that in a recent study a 4-fold increase in the risk of breast cancer in women after 4 yr use of oestrogen plus progestin was reported [15], although an increased risk was not apparent in other studies.

As yet we have not examined possible sites in the body where norethisterone might be converted to ethynyloestradiol, but several studies have shown that the aromatase enzyme complex is present in adipose tissue [16, 17], skin [18] and muscle [19]. There is also some evidence that the liver might also be a site for the aromatization of androstenedione to oestrone [17, 20]. Results from the present study are supported by the findings of others who have demonstrated the *in vitro* conversion of norethisterone to ethynyloestradiol in human liver homogenates [9], hepatocytes [21] and ovarian tissue [22].

Values obtained for the MCR–norethisterone and MCR–ethynyloestradiol in this study are in agreement with those previously reported [23, 24].

In conclusion, in this study we have, for the first time, measured the extent of the conversion of norethisterone to ethynyloestradiol *in vivo*. Our results show that a small but significant degree of conversion does occur. This finding is in keeping with results obtained *in vitro* with human tissues and may account for the ability of norethisterone to protect postmenopausal women from excessive bone resorption.

Acknowledgement—We are grateful to Schering A.G., Berlin for the [4-^{14}C] norethisterone.

REFERENCES

1. Fotherby K. F. and James F.: Metabolism of synthetic steroids. *Adv. Steroid Biochem. Pharmac.* **3** (1972) 67–165.
2. Breuer H., Dardenne U. and Nocke W.: Ausscheidung von 17-ketosteroiden, 17-ketogen steroiden und ostrogen beim mencschen und gaben von 17-athynyl-19-nortestosteron-estern. *Acta Endocr.* **33** (1960) 10–26.
3. Brown J. B. and Blair H. A. F.: Urinary oestrogen metabolites of 19-norethisterone and its esters. *Proc. R. Soc. Med.* **53** (1960) 433.
4. Kamyab S., Fotherby K. and Klopper A. I.: Metabolism of [4-^{14}C]norethisterone in women. *J. Endocr.* **41** (1968) 263–272.
5. Townsley J. D. and Brodie H. J.: A new placental metabolite of oestr-4-ene-3,17-dione: A possible source of error in oestrogen estimation. *Biochem. J.* **101** (1966) 25c–27c.
6. Townsley J. D. and Brodie H. J.: Mechanism of oestrogen biosynthesis. IV Ovarian metabolism of estr-4-ene-3,17-dione. *Biochim. Biophys. Acta* **144** (1967) 440–445.
7. Sisenwine S. F., Liu A. L., Kimmel H. B. and Ruelius H. W.: Phenolic metabolites of DL-norgestrel: a method for the removal of 1-hydroxylated metabolites, potential sources of phenolic artifacts. *Acta Endocr.* **76** (1964) 789–800.
8. Barbieri R. L., Petro Z., Canick J. A. and Ryan K. J.: Aromatization of norethisterone to ethinylestradiol by human placental microsomes. *J. Clin. Endocr. Metab.* **57** (1983) 299–303.
9. Urabe M., Yamamoto T., Yoshiji S., Honjo H. and Okada H.: Aromatization of norethisterone to ethynyloestradiol in human adult liver. *Steroids* **50** (1987) 607–608.
10. Stepan J. J., Pospichal J., Schreiber V., Kanka J., Presl J. and Pacovsky V.: Evidence that the progestational agent norethisterone protects against menopausal bone loss. *Programme 71st Meeting of The Endocrine Society*, Abstr. 444.
11. Reed M. J., Hutton J. D., Beard R. W., Jacobs H. S. and James V. H. T.: Plasma hormone levels and oestrogen production in a menopausal woman with endometrial carcinoma and an ovarian thecoma. *Clin. Endocr.* **11** (1979) 141–150.
12. Reed M. J., Hutton J. D., Baxendale P. B., James V. H. T., Jacobs H. S. and Fisher R. P.: The conversion of androstenedione to oestrone and production of oestrone in women with endometrial cancer. *J. Steroid Biochem.* **11** (1979) 905–911.
13. Baird D. T., Horton R., Longcope C. and Tait J. F.: Steroid dynamics under steady-state conditions. *Recent Prog. Horm. Res.* **25** (1969) 611–656.
14. Grodin J. M., Siiteri P. K. and MacDonald P. C.: Source of oestrogen production in postmenopausal women. *J. Clin. Endocr. Metab.* **36** (1973) 207–214.
15. Berkvist L., Adami H-O., Persson I., Hoover R. and Schairer C.: The risk of breast cancer after estrogen and estrogen progestin replacement. *New Engl. J. Med.* **321** (1989) 293–297.
16. Siiteri P. K. and MacDonald P. C.: Role of extraglandular estrogen in human endocrinology. In *Handbook of Physiology*, Section 7, *Endocrinology* (Edited by G. B. Astwood and R. O. Greep). American Physiological Society, Washington (1973) p. 615.
17. Frost P. G., Reed M. J. and James V. H. T.: The aromatization of androstenedione by human adipose and liver tissue. *J. Steroid Biochem.* **13** (1980) 1427–1431.
18. Schweikert H. U., Milewich L. and Wilson J. D.: Aromatization of androstenedione by cultured human fibroblasts. *J. Clin. Endocr. Metab.* **43** (1976) 785–795.
19. Longcope C., Pratt J. H., Schneider S. H. and Fineberg S. E.: Aromatization of androgens by muscle and adipose tissue *in vivo*. *J. Clin. Endocr. Metab.* **46** (1978) 146–152.
20. Smuk M. and Schwers J.: Aromatization of androstenedione by human adult liver *in vitro*. *J. Clin. Endocr. Metab.* **45** (1977) 1009–1012.
21. Yamamoto T., Kitawaki J., Shoroshita K., Urabe M., Sasaki J., Fukuoka M. and Naitoh K.: The confirmation of norethisterone aromatization in primary human hepatocytes by vitafiber-II cell culture system. *Acta Obstet. Gynaec. Jap.* **40** (1988) 87–89.
22. Yoshiji S., Yamamoto T. and Okadu H.: The aromatization of norethisterone by human ovarian specimens. *Acta Obstet. Gynaec. Jap.* **39** (1987) 1422–1423.
23. Mills T. M., Lin T. J., Hernandez-Ayup S., Greenblatt R. B., Ellegood J. O. and Mahesh V. B.: The metabolic clearance rate and urinary excretion of oral contraceptive drugs. *Am. J. Obstet. Gynaec.* **120** (1974) 764–772.
24. Longcope C. and Williams K. I. H.: The metabolism of synthetic estrogens in non-users and users of oral contraceptives. *Steroids* **25** (1975) 121–133.
25. Chetkowski R. J., Meldrum D. R., Steingold K. A., Randle D., Lu J. K., Eggena P., Hersham J. M., Alkjaersig N. K., Fletcher A. P. and Judd H. L.: Biologic effects of transdermal estradiol. *N. Engl. J. Med.* **314** (1986) 1615–1620.
26. Baker B. L., Eskin T. A. and August L. N.: Direct action of synthetic progestins on the hypophysis. *Endocrinology* **92** (1973) 965–972.