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Norethisterone is bioconverted to oestrogenic compounds that activate both the oestrogen receptor α and oestrogen receptor β in vitro

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Abstract

In the present study, we used [^{3}H]norethisterone to explore the bioconversion of this compound to A-ring reduced metabolites in African Green Monkey Kidney CV-1 cells and breast cancer T-47D cells. Additionally, we analyzed the capability of each norethisterone tetrahydro-reduced compound to bind the human oestrogen receptors α and β and transactivate an oestrogen-sensitive reporter gene. The results showed that norethisterone is mainly metabolized to 3α,5α-norethisterone (>85% of total [^{3}H]norethisterone added) by CV-1 and T-47D cells, and that both A-ring tetrahydro-reduced metabolites exhibit different capabilities to displace [^{3}H]17β-oestradiol from the oestrogen receptor α and β, being 3α,5α-norethisterone the weakest competitor. We also found that 3α,5α-norethisterone and 3β,5α-norethisterone activate both oestrogen receptors at nanomolar concentrations and that the transactivation induced by the oestrogen receptor α was generally higher (1.7- to 4.0-fold) than that provoked by the β receptor isoform. In oestrogen receptor α-transfected CV-1 and T-47 D cells, the oestrogenic-like potency of the 3β,5α-tetrahydro-reduced form was similar to that exhibited by 17β-oestradiol and 2.5- to 4.0-fold higher than that shown by the 3α,5α-reduced compound; conversely, in the oestrogen receptor β system the potency of the natural ligand was higher than that presented by the 3β,5α-tetrahydro-reduced metabolite. In CV-1 cells expressing the oestrogen receptor β, the transactivation potency of 3β,5α-norethisterone was ~ 2-fold higher than that exhibited by its 3α,5α-tetrahydro-reduced isomer, whereas in T-47D cells the potency of the 3α,5α-tetrahydro-reduced compound was slightly higher than that shown by the 3β,5α A-ring reduced norethisterone metabolite. These results demonstrate that CV-1 and T-47D cells possess the enzymatic machinery to bioconvert norethisterone into the 5α-reduced, 3α-hydroxylated form and that neither 3α,5α- or 3β,5α-norethisterone exhibit preference or selectivity towards a particular oestrogen receptor isoform to induce a particular oestrogenic effect in these cell lines.
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Keywords: 19-Nor progestins; Norethisterone; Oestrogen receptor

1. Introduction

The characterization of the oestrogenic properties and mode of action of synthetic progestins is an issue of particular interest because of the wide use of these compounds as contragestational and therapeutic agents (Kuihl, 1996; Rebar and Zeserson, 1991; Stiruk-Ware, 1999). In the evaluation of the net biological effects of a given synthetic progestin, it is important to unambiguously distinguish the effects that may be attributed to the action of the original compound from those resulting from its metabolic bioconversion products. Although progestins exert their progestational activity by binding to the progesterone receptor, their bioconversion products may interact with other steroid hormone receptors, including the androgen receptor and the oestrogen receptor (Pérez-Palacios et al., 1992; Lemus et al., 2001), leading to potentially undesirable effects in some tissues like the breast and the endometrium.

Norethisterone belongs to the first generation of synthetic progestins; this compound is still widely employed as a contragestational agent as well as in hormone replacement therapy (Ravinder et al., 1997). The bioconversion of norethisterone into 5α-norethisterone, 3α,5α-norethisterone and 3β,5α-norethisterone A-ring reduced metabolites, may

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potentially confer to the resulting product particular agonistic or antagonistic properties (Negro-Vilar and Pérez-Palacios, 1991; Markiewickcs and Gurpide, 1994; Dijkema et al., 1998; Schoonen et al., 2000). These A-ring reduced metabolites of norethisterone may interact with either the progesterone receptor or the oestrogen receptor (Chávez et al., 1985). 5α-Norethisterone and 3β,5α-norethisterone bind to the progesterone and oestrogen receptors, respectively, whereas the interaction of 3α,5α-norethisterone with either receptor is apparently negligible (Chávez et al., 1985; Schoonen et al., 2000). Therefore, these metabolites exert distinct hormonal effects (Markiewicks et al., 1992; Schoonen et al., 1995; Pasapera et al., 1995a,b). Although Kuhnz and colleagues (1997) found that 19-nor progestins might be also bioconverted to ethinyl-oestradiol, the intrinsic oestrogenic property of some of these compounds is still a matter of some controversy.

Several in vitro studies employing co-transfected cell systems have been used to characterize the mechanisms underlying the biological effects of different synthetic progestins at the transcriptional level (Jordan et al., 1993; Markiewickcs and Gurpide, 1994; Botella et al., 1995; Pasapera et al., 1995a; Schoonen et al., 1995, 1998, 2000; Kemppainen et al., 1999; Lemus et al., 2000). Although the in vitro metabolism of several 19-nor progestins by tissue explants or homogenates has been reported (Lemus et al., 1992, 2001; Blom et al., 2001), the role of particular bioconversion pathways and cell-specific co-activators in defining the promoter-specific agonistic or antagonistic effects of these compounds in the intact cell still remains incompletely understood.

In preliminary experiments, we found that norethisterone induced in vitro transactivation of an estrogen-regulated reporter vector transiently co-transfected with the oestrogen receptor α or oestrogen receptor β in African Green Monkey Kidney CV-1 cells. This finding was surprising because of the known inability of this particular progestin to bind and activate the oestrogen receptor. Since the tetrahydro-reduced compounds of norethisterone may transactivate an oestrogen-responsive vector in these cells (Pasapera et al., 2001), we investigated whether the extent of norethisterone bioconversion to these compounds by CV-1 cells and T-47D cells (derived from a natural target tissue for oestrogen actions) may explain such oestrogenic effect of norethisterone. Herein we report the results of this series of studies.

2. Material and methods

2.1. Chemicals

Norethisterone, 17β-oestradiol and 4-hydroxy-tamoxifen were obtained from Sigma (St. Louis, MO, USA). A-ring reduced metabolites (5α-norethisterone, 3β,5α-norethisterone, and 3α,5α-norethisterone) were synthesized as previously described (Bowers et al., 1958; Pasapera et al., 2001). Diethylstilboestrol was obtained from Sigma. (Dichloroacetyl-1-^{14}C)-chloramphenicol (specific activity, 53.1 mCi/mmol), [2,4,6,7-^{3}H]17β-oestradiol ([^{3}H]17β-oestradiol; specific activity 93 Ci/mmol), and [^{3}H]norethisterone (19-nor-17α-ethinyltestosterone; specific activity 14.9 Ci/mmol) were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). All steroids were 99% pure. All media and antibiotics for cell culture were purchased from Life Technologies (Gaithersburg, MD, USA). ICI 182,780 (7α-[9-[(4,4,5,5,5,pentafluoropentyl)sulphinyl]nonyl]-estra-1,3,5(10)-triene-3,17β-diol) was kindly provided by Dr. Jean Francois Savouret (INSERM, Le Kremlin Bicêtre, France).

2.2. Gene constructs

The human oestrogen receptor α cDNA inserted in the expression vector pKSV-10 (Amersham Pharmacia) was kindly provided by Professor Edwin Milgrom (INSERM, Le Kremlin Bicêtre, France) (Savouret et al., 1991). The human oestrogen receptor β (Pace et al., 1997), inserted in the expression vector pSG5 (Stratagene, La Jolla, CA, USA) was kindly donated by Professor Jan-Åke Gustaffson (Karolinska Institute, Sweden). The reporter vector used in this study contained the Vitellogenin A2 oestrogen-responsive element (fragment −331 to −297) linked to the thymidine kinase promoter and the cDNA of the chloramphenicol acetyltransferase (ERE-VitA2-TK-CAT) (Klein-Hitpass et al., 1986; Pasapera et al., 2001). The β-galactosidase plasmid (pSV-β-galactosidase) (Promega, Madison, WI, USA) was used as a control plasmid.

2.3. Cell culture and transfections

African Green Monkey Kidney CV-1 and T-47D cells were kindly provided by Dr. Jean Françoise Savouret (Le Kremlin Bicetre, France). CV-1 cells do not express endogenous oestrogen or progestin receptors (Pasapera et al., 2001), whereas the particular clone of T-47D cells employed in the present study expresses negligible amounts of these receptors as disclosed by receptor binding assays (not shown). Cells were cultured at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 0.2 U/ml insulin (T-47D cells only) and 100 U/ml penicillin in a 5% CO_2 atmosphere. Plasmids were amplified in JM-109 *Escherichia coli* bacterial strain (Promega) and purified by anion exchange chromatography employing the QIAGEN ENDO-free plasmid purification kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Plasmid DNA was quantified by measuring absorbance at 260 nm, and its quality was verified by agarose gel electrophoresis and ethidium bromide staining. Plasmid DNAs (1 μg/μl) were transiently transfected into cells by liposome-mediated DNA transfer using lipofectamine (Life Technologies). Briefly, cells were plated at an initial density of 1.5×10^5 cells per well in 6-well cell culture plates for 24 h at 37 °C

and 5% CO_2. Cells were then rinsed with phosphate buffer (0.05 M)–saline (0.9%), pH 7.4, and incubated with phenol red-free DMEM containing 10% dextran–charcoal stripped calf serum. CV-1 and T-47D cells were co-transfected with 0.25 μg of an expression vector encoding either the oestrogen receptor α or the oestrogen receptor β, 0.25 μg of pSV-β-galactosidase and 1 μg of the ERE-VitA2-TK-CAT reporter vector. In all experiments, the amount of transfected DNA was adjusted to 2 μg/ml by the addition of pSG5 plasmid DNA; lipofectamine was adjusted to 7.5 μl/ml OPTI-MEM (Life Technologies). Twenty-four hours after the start of transfection, cells were refed with carbon-charcoal stripped medium and the steroids diluted in ethanol (maximal concentration, 0.001%) were added. Twenty-four hours after the steroid treatments, the cells were harvested in buffer A (15 mM Tris–HCl pH 8.0, 60 mM KCl, 15 mM NaCl, 1 mM dithiothreitol and 2 mM EDTA) and processed for the CAT assays.

2.4. Metabolism of norethisterone in CV-1 and T-47D cells

Cells were plated in 6-well plates (5×10^5 cells/well) and cultured in stripped DMEM under a 5% CO_2 atmosphere at 37 °C for 24 h. Thereafter, the cells were rinsed with fresh medium and [^{3}H]norethisterone (at a 34 nM concentration) was added. At incubation times periods of 0.10, 0.30, 0.50, 1, 2, 4, 8, 12, 24 and 48 h, cells and media (~ 2 ml) were collected from triplicate wells and immediately frozen at − 20 °C until steroid extraction.

2.5. Isolation and measurement of radioactive metabolites

Radiolabelled norethisterone and its A-ring reduced metabolites were extracted first with an equal volume of ethylic ether containing 500 μg of norethisterone and each A-ring reduced metabolite as carriers, and then with another volume of ethylic ether without carriers. The organic layer was separated, evaporated to dryness under a stream of nitrogen and the extract was chromatographed in thin layer chromatography plates (Silica gel 60 F_{254} Merck, Darmstadt, Germany). The plates were developed in benzene, then in benzene/ethyl acetate (80:20, v/v) and finally in benzene/methanol (95:5); norethisterone, 5α-norethisterone, 3β,5α-norethisterone and 3α,5α-norethisterone were localized on the plates under ultraviolet absorption measurements at 254 or 366 nm whenever the Oërtel reagent [sulphuric acid/ethanol (2:1)] was added. The areas corresponding to each compound were eluted into scintillation vials and the radioactivity was counted after adding 5 ml of scintillation liquid (Scintiverse II, Fisher Scientific, NJ, USA) to dried eluates.

2.6. Receptor binding assay

Receptor binding assays were performed as described by Kumar and Chambon (1988), with minor modifications. Briefly, 1×10^6 CV-1 cells were plated in 10-mm Petri dishes and transfected with 10 μg of each oestrogen receptor cDNA by the lipofectamine method. Twenty-four hours after transfection, cells were harvested by tripsinization and whole cell extracts were obtained after freeze-thawing in TEGM buffer [20 mM Tris–HCl pH 7.4, 1 mM EDTA, 10 mM sodium molybdate, 400 mM KCl, 350 mM sucrose and 1 × protease inhibitors cocktail (Roche, Germany)]. Fifty microliters of cell extracts were incubated in triplicate wells (96-well plates) with 8.0 nM [^{3}H]17β-oestradiol and increasing concentrations (1–1000 nM) of each non-radiolabelled compound (17β-oestradiol, norethisterone, 5α-norethisterone, norethisterone tetrahydro-reduced metabolites and ICI 182,780) for 18 h at 4 °C. The reaction was stopped by the addition of 100 μl of a dextran–charcoal suspension and the plates were centrifuged at $1800 \times g$ for 10 min. The radioactivity present in 50 μl aliquots of the supernatants was determined in a Packard Tricarb 2900 TR spectrometer (Packard Instrument, Meriden, CT, USA). The concentration of each unlabelled compound required to displace 50% of [^{3}H]17β-oestradiol from the α and β oestrogen receptors (ED_{50}) was calculated by linear regression analysis after logit-log transformation of the data (Zambrano et al., 1999).

2.7. CAT assays

Whole-cell extracts were obtained by freeze-thawing and centrifugation as previously described (Pasapera et al., 2001). Protein concentration was determined by the BCA assay (Pierce Chemical, Rockford, IL, USA). CAT activity was measured as described by Pothier et al. (1992), with minor modifications. Briefly, cell extracts (100 μg) were incubated in 150 μl buffer A with 5 μl of (dichloroacetyl-1-^{14}C)-chloramphenicol and acetyl-CoA (4 mM) for 1 h at 37 °C. The acetylated and non-acetylated forms of radiolabelled chloramphenicol were separated in thin layer chromatography silica-gel plates in a system of chloroform/methanol (95:5) for 45 min. The plates were then exposed to Kodak Biomax-MR film (Kodak, México D.F., Mexico) for 24 h at room temperature and the radioactive spots were analyzed by densitometry. For each assay, three series of experiments were performed in triplicate incubations. β-Galactosidase activity was measured in cell extracts using chlorophenol red β-D-galactopyranoside as substrate (Eustice et al., 1991).

3. Results

3.1. Metabolic bioconversion of [^{3}H]norethisterone in CV-1 and T-47D cells

Incubation of CV-1 and T-47D cells with [^{3}H]norethisterone resulted in a time-dependent bioconversion of

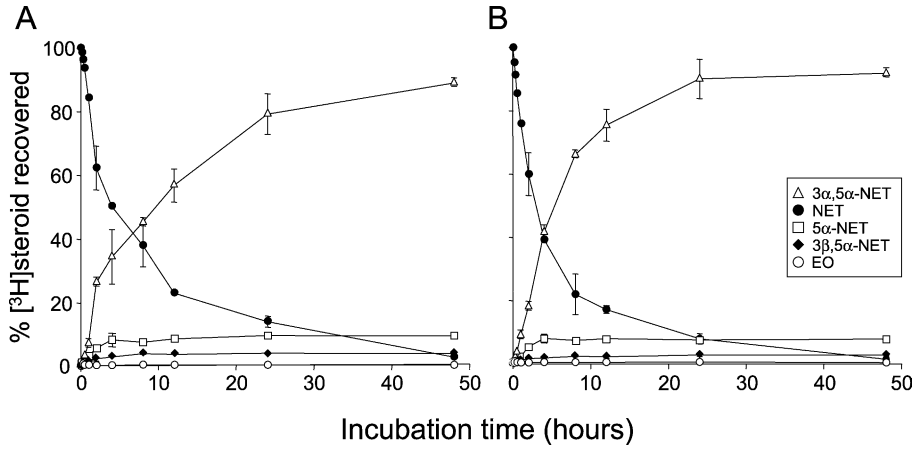

Fig. 1. Metabolic conversion of [^{3}H]norethisterone in CV-1 and T-47D cells. CV-1 (A) and T-47D cells (B) were plated in 6-well plates and cultured in stripped DMEM in the presence of [^{3}H]norethisterone as described in Material and methods. After incubation periods of 0.10, 0.30, 0.50, 1, 2, 4, 8, 12, 24 and 48 h, cells and media were collected and the metabolic conversion products were identified by thin layer chromatography. Each time point represents the mean ± S.D. from triplicate incubations. The results shown are representative of two separate experiments. NET, norethisterone; EO, ethinyl-oestradiol.

[^{3}H]norethisterone into two different metabolites, [^{3}H]3α, 5α-norethisterone, which represented 80–89% of total radioactivity recovered, and [^{3}H]5α-norethisterone (~ 8% of total recovered); production of [^{3}H]3β,5α norethisterone was negligible (<4%), and no [^{3}H]ethinyl-oestradiol was detected (Fig. 1). Maximal level of norethisterone transformation to [^{3}H]3α,5α-norethisterone in CV-1 and T-47D cells occurred at 48 and 24 h, respectively. In both cell lines, [^{3}H]norethisterone concentrations began to decrease in the incubation medium within the initial 60 min of exposure, thereafter declining in an exponential manner throughout the ensuing 47 h.

3.2. Competition binding of norethisterone and its A-ring reduced metabolites for the oestrogen receptors α and β

To analyze the ability of norethisterone and its metabolites to interact with both oestrogen receptor isoforms, cytosol preparations from CV-1 cells transfected with the oestrogen receptor α and β cDNAs were incubated with 8.0 nM [^{3}H]17β-oestradiol for 18 h at 4 °C, in the presence or absence of increasing concentrations of the steroid competitors (norethisterone, 5α-norethisterone, 3α,5α-norethisterone, 3β,5α-norethisterone, ICI 182,780 and 17β-oestradiol). As shown in Fig. 2, 3β,5α-norethisterone, ICI 182,780 and

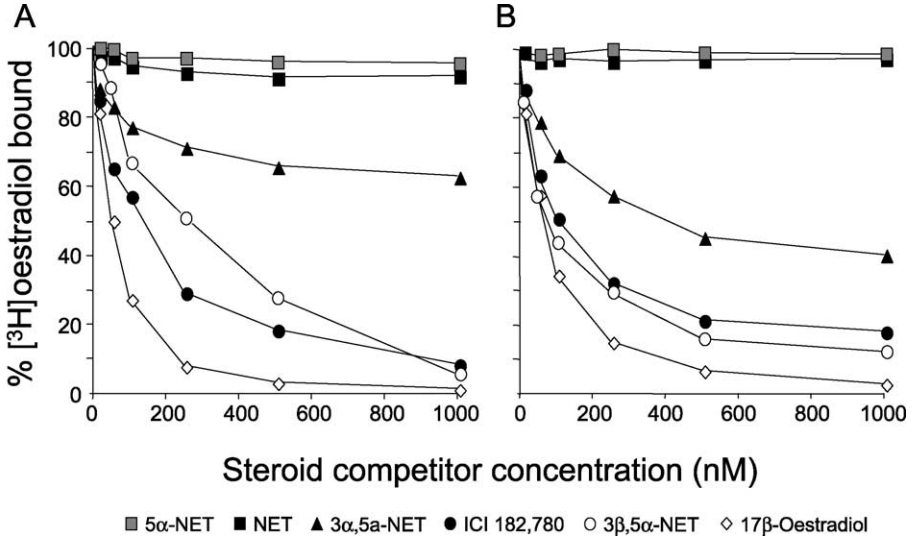

Fig. 2. Competition assay of A-ring reduced metabolites of norethisterone for binding to the oestradiol receptor α and oestradiol receptor β. Increasing concentrations of norethisterone and its A-ring reduced metabolites were added to [^{3}H]17β-oestradiol-containing cytosolic preparations from CV-1 cells transiently expressing the oestrogen receptor α (A) or β (B). The mixture was then incubated at 4 °C for 18 h. Bound and free fractions were separated by the addition of a dextran-charcoal coated suspension. Non-specific binding was determined by the addition of 1000-fold excess unlabelled 17β-oestradiol. Each point represents the mean of three separate experiments; dispersions are omitted for clarity. NET, norethisterone.

17β-oestradiol efficiently competed for the oestrogen receptors α and β, whereas 3α,5α-norethisterone was a weak competitor. Neither norethisterone or 5α-norethisterone were able to displace [^{3}H]17β-oestradiol from the receptor. The relative affinity (as indicated by their corresponding ED_{50}) of both, 3α,5α- and 3β,5α-tetrahydro-reduced compounds for the oestrogen receptor β was higher than for the oestrogen receptor α (Table 1). This finding correlated with the oestrogenic effects exhibited by these steroids in the transactivation assays (see below).

3.3. Norethisterone and its tetrahydro-reduced metabolites transactivate the oestrogen-sensitive reporter vector through the oestrogen receptor α and oestrogen receptor β in CV-1 cells

In preliminary experiments, the response of CV-1 and T-47D cells transiently expressing the ERE-VitA2-TK-CAT reporter and the oestrogen receptor α to 17β-oestradiol, diethylstilbestrol and 4-hydroxy-tamoxifen was tested. In both cell lines, CAT activity was strongly induced by 10^{-8} M 17β-oestradiol and diethystilbestrol, whereas 4-hydroxy-tamoxifen was marginally active in CV-1 cells and moderately active in T-47D cells (Fig. 3).

The oestrogenic properties of the tetrahydro-reduced metabolites of norethisterone in CV-1 cells transiently co-transfected with the ERE-VitA2-TK-CAT vector and the human oestrogen receptor α or oestrogen receptor β expression vectors are shown in Fig. 4. The transactivation assays showed that both compounds were able to stimulate oestrogen receptor α-mediated transactivation of the reporter vector when tested at 10^{-8} and 10^{-6} M concentrations and that this transactivation was efficiently inhibited by 100-fold excess of ICI 182,780 (Fig. 4A). Although both tetrahydro-reduced metabolites behaved as agonists for the oestrogen receptor α, the transactivation potency of 3β,5α-norethisterone was higher (~ 8- to 13-fold) than that exhibited by the 3α,5α-reduced metabolite (Fig. 4C). As previously observed in other cell lines (Delaunay et al., 2000), the transactivation potency of the oestrogen receptor β in CV-1 cells exposed to 17β-oestradiol was lower than that exhibited by the activated oestrogen receptor α (Fig. 4B). Both A-ring tetrahydro-reduced norethisterone-derived compounds were able to induce oestrogen receptor β-mediated transactivation of the ERE-VitA2-TK-CAT reporter when added at two different concentrations (10^{-8} and 10^{-6} M). However, as found with 17β-oestradiol, the extent to which both compounds activated the oestrogen-responsive vector through this particular receptor was lower than that observed with the oestrogen receptor α (Fig. 4C).

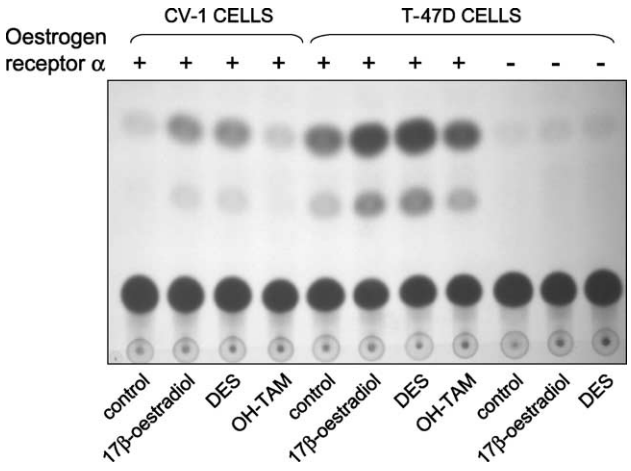

Fig. 3. Transactivation of the ERE-VitA2-Tk-CAT reporter vector in CV-1 and T-47D cells transiently expressing the oestrogen receptor α. Transfected cells were incubated for 24 h in the presence or absence (control) of oestrogens [17β-oestradiol and diethylstilbestrol (DES)] or the anti-oestrogen 4-hydroxy-tamoxifen (OH-TAM) added at a 10^{-8} M concentration. In both cell lines, CAT activity was strongly induced by 17β-oestradiol and diethystilbestrol, whereas 4-hydroxy-tamoxifen was marginally active in CV-1 cells and moderately active in T-47D cells. Nevertheless, after normalization CAT activity induced by this anti-oestrogen was similar to those found in control cells (not shown).

Table 1
Effective dose (ED_{50}) and relative receptor binding activity of norethisterone, its A-ring reduced metabolites and ICI 182,780 for the oestrogen receptors α and β

Steroid	Oestrogen receptor α		Oestrogen receptor β	
	ED_{50} (nM)	Relative activity	ED_{50} (nM)	Relative activity
17β-Oestradiol	38 ± 0.18	1.0	50 ± 0.30	1.0
Norethisterone	N.D.	N.D.	N.D.	N.D.
5α-Norethisterone	N.D.	N.D.	N.D.	N.D.
3α,5α-Norethisterone	1720 ± 90	0.02	456 ± 3.0	0.11
3β,5α-Norethisterone	183 ± 14	0.21	65 ± 1.0	0.77
ICI 182,780	93 ± 0.94	0.44	106 ± 0.70	0.47

Extracts from CV-1 cells transfected with the expression vector for human oestrogen receptor α or β, were incubated in the presence of 8.0 nM [^{3}H]17β-oestradiol and increasing concentrations (1–1000 nM) of unlabelled competitors. Free steroid was separated from receptor-bound steroid by carbon-charcoal extraction. Data are presented as means ± S.D. of three separate experiments.

3.4. Norethisterone and its tetrahydro-reduced metabolites transactivate the ERE-Vita2-TK-CAT reporter vector through the oestrogen receptor α and oestrogen receptor β in T-47D cells

The estrogenic properties of norethisterone and its A-ring reduced metabolites were determined in breast cancer-derived T-47D cells transiently co-transfected with the ERE-VitA2-TK-CAT vector and the human oestrogen receptor α or β expression plasmids and then treated as previously described for CV-1 cells. The results are shown in Fig. 5. The transactivation assays showed that the tetrahydro-reduced metabolites of norethisterone stimulated oestrogen receptor α- and oestrogen receptor β-mediated transcriptional activation of the reporter vector when tested at three different dose levels (10^{-9}, 10^{-8} and 10^{-6} M

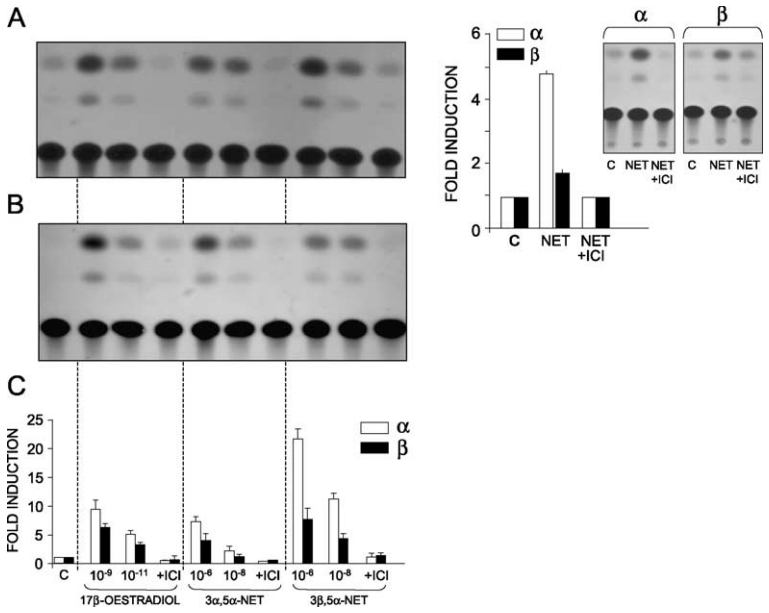

Fig. 4. Transactivation of the ERE-VitA2-TK-CAT reporter vector by norethisterone and its tetrahydro-reduced metabolites in CV-1 cells. CV-1 cells were transiently transfected with the human oestrogen receptor α (A) or β (B) expression vectors, the ERE-VitA2-TK-CAT reporter vector and the β-galactosidase expression plasmid used as internal control. Cultures were maintained for 48 h in the presence of the indicated steroids. ICI 182,780 (ICI) was added at a 100-fold molar excess of each oestrogen analogue dose. The autoradiograms are representative of three independent experiments. (C) Potency of 17β-oestradiol and the tetrahydro-reduced forms of norethisterone to promote oestradiol-sensitive reporter vector transcription. The data represent the mean ± S.D. of three independent experiments in triplicate. Fold induction was calculated as the ratio of acetylated chloramphenicol generated in 60 min at 37 °C by 100 μg of proteins from steroid-treated cells over untreated control cells, after correction for β-galactosidase activity. The intra-assay variation was typically ≤ 10%. Inset: Potency of norethisterone (NET; 10^{-6} M concentration) to promote oestrogen-responsive reporter vector transcription. C, control; α, oestrogen receptor α; β, oestrogen receptor β.

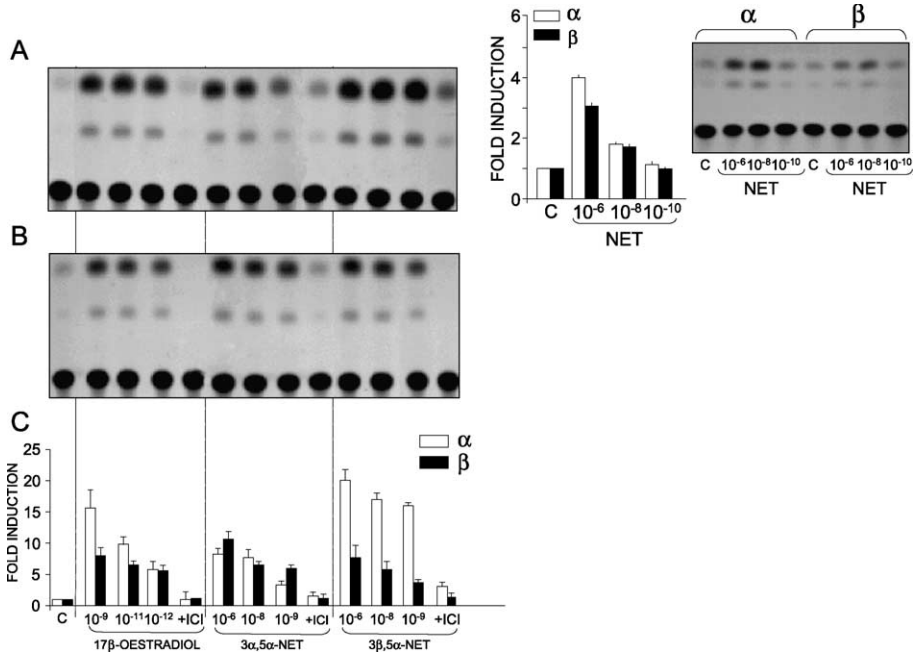

Fig. 5. Transactivation of the ERE-VitA2-TK-CAT reporter vector by norethisterone and its tetrahydro-reduced metabolites in T-47D cells. (A) Transactivation of the oestrogen-responsive reporter vector in T-47D cells transiently expressing the human oestrogen receptor α. (B) Transactivation of the reporter vector in cells expressing the oestrogen receptor β. (C) Potency of 17β-oestradiol and the tetrahydro-reduced forms of norethisterone to promote oestradiol-sensitive reporter vector transcription; the data represent the mean ± S.D. of three independent experiments in triplicate. See legend for details. Inset: Potency of norethisterone (NET) to promote oestrogen-responsive reporter vector transcription. C, control; α, oestrogen receptor α; β, oestrogen receptor β.

concentrations). In these cells, the potency of 3α,5α-norethisterone to induce oestrogen receptor β-mediated transactivation of the reporter vector was slightly higher than that observed with the activated oestrogen receptor α (Fig. 5C).

4. Discussion

In the present study, we analyzed the metabolic bioconversion of [^{3}H]norethisterone in two different cell types, the African Monkey Kidney-derived CV-1 cells, which are not natural targets for oestrogens and T-47D cells, which are derived from a tumor of the mammary gland, a well-known target for oestrogen actions (Gustafsson and Warner, 2000). The results suggest that the A-ring of norethisterone is probably a target for 5α-steroid reductase and 3α-hydroxysteroid dehydrogenase activities in both cell lines, being 3α,5α-norethisterone the predominant resulting compound. Variations in composition, transmembrane topology, catalytic properties and relative amounts of the enzymes involved in this process may explain the time-related differences in bioconversion rates between the two cell lines (Chetyrkin et al., 2001). The observation that 3α,5α-norethisterone but not 5α-norethisterone or 3β,5α-norethisterone was the main metabolite of norethisterone, strongly suggests that as found in oestrogen target tissues such as the vagina and uterus (Blom et al., 2001), 5α-reduction is the rate-limiting step in the bioconversion of norethisterone to tetrahydro-reduced forms by both cell lines. These findings also confirm the absence of 3β-hydroxisteroid dehydrogenase activity in T-47D cells (Gingras et al., 1999) and additionally demonstrate that the CV-1 cell line does not expresses this metabolic pathway. The metabolic bioconversion of norethisterone to metabolites with intrinsic oestrogenic potency (see below) supports the view that the oestrogenic effects of norethisterone may be owing to its rapid biotransformation into oestrogenic compounds rather than to intrinsic oestrogenic properties, as was initially proposed (Larrea et al., 1987; Pérez-Palacios et al., 1992; Pasapera et al., 1995b; Rabe et al., 2000).

Both tetrahydro-reduced norethisterone metabolites competed with [^{3}H]17β-oestradiol for binding to the oestrogen receptors α and β, with a higher relative binding affinity of the two compounds for the oestrogen receptor β. Further, in both receptors the binding activity of 3α,5α-norethisterone was lower than that exhibited by its 3β,5α-tetrahydro-reduced counterpart, finding that correlated with the ability of the two compounds to transactivate the oestrogen-responsive reporter vector present in CV-1 and T-47D cells. The ability of 3α,5α-norethisterone to bind both oestrogen receptors contrasts with a previous study in which no 3α,5α-norethisterone binding was detected in rat uterine cytosols when tested up to 250 nM concentrations (Vilchis et al., 1986). Our findings also disagree with a recent study in which the 3β,5α-tetrahydro-reduced metabolite behaved as a weak competitor for 17β-oestradiol binding to the oestrogen receptor β in HeLa cells (Larrea et al., 2001). Differences in relative amounts of expressed receptors and oestrogen competitors may explain these apparent discrepancies. In addition, the allosteric transition in oestrogen receptor conformation provoked by the tetrahydro-reduced metabolites of norethisterone, may render the receptor able to specifically interact with a particular set of co-activators differentially expressed in HeLa cells versus breast- or kidney-derived cells.

In this work, we demonstrate that both tetrahydro-reduced metabolites of norethisterone were able to transactivate the oestrogen responsive vector in CV-1 and T-47D cells transiently expressing either the oestrogen receptor α or oestrogen receptor β. In particular, micromolar concentrations of 3β,5α-norethisterone induced clearly detectable oestrogen receptor α- and β-mediated transactivation of the oestrogen reporter gene in CV-1 cells, whereas in T-47D cells transactivation of the reporter was achieved even at nanomolar concentrations of the ligand. The observation that the 3β,5α-tetrahydro-reduced compound showed no preference or selectivity towards a particular oestrogen receptor in the cell lines employed, contrasts with recent findings in HeLa cells, in which exposure to 10^{-9}–10^{-7} M concentrations of 3β,5α-norethisterone failed to transactivate an oestrogen-sensitive reporter gene controlled by a promoter similar to that present in the reporter vector employed in the present study (Larrea et al., 2001). These apparent discrepancies may be owing to differences in amounts of oestrogen receptor expression vectors transfected and/or cell lines employed as targets (Giannoukos et al., 2001). In fact, it has been shown that the transcriptional activity of the activation function-1 and activation function-2 oestrogen receptor domains may vary depending on the coactivators present in a given cell type (Tora et al., 1989; Cowley and Parker, 1999; Jones et al., 1999; Delaunay et al., 2000; McKenna and O'Malley, 2000). The overall findings additionally offer a plausible explanation for the previously reported effects of norethisterone on MCF-7 and T-47D oestrogen receptor positive cells (Jeng et al., 1992; Kalkhoven et al., 1994) as well as its apparent ability to transactivate an oestrogen responsive gene (Rabe et al., 2000), which probably occurred via conversion to more active tetrahydro-reduced compounds. The possibility that some bioconversion of norethisterone to ethinyl-oestradiol had occurred is remote, considering that the presence of a carbon atom in position 19 is a structural pre-requisite for A-ring steroid aromatization (Short, 1964). In fact, Blom et al. (2001) were unable to identify ethinyl-oestradiol as a bioconversion product of norethisterone in tissue fragments from uterus, vagina and aorta.

In summary, it was shown in this study that norethisterone is preferentially metabolized to the oestrogenic 3α,5α tetrahydro-reduced form in CV-1 cells and T-47D cells and that this compound and its 3β,5α-tetrahydro-reduced isomer may transactivate an oestrogen responsive gene through both the oestrogen receptor α and oestrogen receptor β. Thus, this

study provides a molecular basis for some of the differential transcriptional effects exhibited by both oestrogen receptor isoforms when activated by norethisterone and its A-ring reduced metabolites in cell lines derived from distinctly different tissues. The role of the in vivo bioconversion of 19-nor progestins to compounds with varying oestrogenic potencies on the development of estrogen-dependent undesirable effects, still remains to be fully defined.

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