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Transactivation of Progestin- and Estrogen-Responsive Promoters by 19-Nor Progestins in African Green Monkey Kidney CV1 Cells

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New and more potent progestins and antiprogestins suitable for reproductive therapy and contraception are currently the target of intensive research. The design of such drugs has been hampered by the complex technology required for screening these compounds at the molecular level. To solve this problem, we developed an in vitro cell system that allows detection of the progestagenic effects of a given compound using a PRE_2-TATA-CAT reporter vector transiently introduced in a cell line stably transfected with the rabbit progesterone receptor (PR). The African Green Monkey Kidney CV1 (AGMK-CV1) cell line was chosen because these cells do not express endogenous steroid receptors; the selected clone stably expressing the rabbit PR has been maintained in our laboratory for more than 2 yr without detectable losses in PR content and progestagenic response. The presence and function of the PR were assessed by immunohistochemical and saturation analyses as well as by monitoring transactivation of the PRE_2-TATA-CAT reporter gene. In this cell line, the PR is expressed at a concentration of 0.170 fmol/mg of protein, and the receptor is localized within the cell nucleus in either the presence or absence of the potent synthetic progestin R5020. This PR-expressing cell system allowed study of the in vitro progestational activity of several 19-nor progestins. The antiprogestin RU486 inhibited CAT activity induced by R5020; norethisterone (NET), levonorgestrel (LNG), and gestodene (GSD) induced PRE_2-TATA-CAT activity at concentrations similar to those of R5020, whereas NET A-ring–reduced metabolites induced CAT activity at an extent lower than (5α-NET) or similar (3β,5α-NET) to that of the precursor compound. The PRE_2-TATA-CAT induction by 17β-estradiol was also analyzed and no crossreactivity was detected. However, when the ERE-VitA2-TK-CAT (estrogen-responsive element-vitellogenin A2-thymidine kinase promoter-CAT) reporter vector and the estradiol receptor α or β were cotransfected, CAT activity was induced in the presence of 17β-estradiol, and NET tetrahydro-reduced derivatives. The results indicate that this AGMK-CV1-PR cell assay system appears to be suitable for measuring the effects of different synthetic progestins at the transcriptional level. In this assay system, NET, LNG, and GSD exhibit potent progestational effects at the transcriptional level. In the particular case of NET, the assay system allowed us to determine that the single or multiple hormonal transcriptional effects of this compound are partially mediated by its A-ring-reduced derivatives.

Key Words: 19-Nor progestins; norethisterone; estrogen receptor; progesterone receptor.

Introduction

The progestagenic action of progesterone and synthetic progestins is mainly mediated by the progesterone receptor (PR), which is intracellularly located in the target tissues *(1,2)*. This receptor belongs to the superfamily of nuclear transactivators *(3)*; in their activated state, these transactivators act as transcription factors capable of inducing target gene transcription after triggering a cascade of events, including allosteric changes in the receptor molecule, receptor dimerization, increase in receptor phosphorylation, release of heat-shock proteins, and interaction of the steroid receptor complex with specific hormone-responsive elements located in the regulatory regions of the progesterone-responsive genes *(4,5)*. The PR exists as two distinct molecular isoforms that derive from a single copy gene. Isoform B contains 933 amino acids (~114 kDa), and the truncated variant, isoform A (~94 kDa), lacks the N-terminal 164 amino acids *(6–8)*. It seems that the rabbit PR (rPR) is an exception among all species studied, since it is expressed exclusively as the long B isoform *(9,10)*.

The characterization of the mode of action of progestins is an issue of particular interest because of the wide use of

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these compounds as contragestational and therapeutic agents *(11–13)*. This requires a rigorous evaluation of the net biologic effects and intrinsic properties of both the original compound and its metabolic bioconversion products. Although progestins exert their progestational activity by binding to the PR, they may also interact with other steroid hormone receptors *(14)*. Norethisterone (NET) and levonorgestrel (LNG) belong to the first generation of synthetic progestins; these compounds are still widely employed as contraceptives *(15)*. Both 19-nor progestins may be bioconverted to 5α-, 3α,5α-, and 3β,5α-reduced metabolites, which may potentially exhibit particular agonistic or antagonistic properties *(16–19)*. The A-ring-reduced metabolites of NET and LNG mainly interact with the PR and estrogen receptor (ER) *(20)*; the 5α-reduced metabolites bind to the PR, whereas the 3β,5α-reduced metabolites bind to the ER and therefore exert distinct hormonal effects *(17,21,22)*. Although in a recent study Kuhnz et al. *(23)* found that 19-nor progestins may also be bioconverted to ethynyl-estradiol, the intrinsic estrogenic property of some of these compounds is still a controversial issue.

During the last decade, several in vitro studies employing cotransfected cell systems have been used to characterize the molecular mechanisms underlying the biologic effects of different synthetic progestins *(18,21,22,24–30)*. However, the mechanisms subserving the tissue- and promoter-specific agonistic and antagonistic effects of these compounds still remain incompletely understood. To gain further insights into the mode of action of synthetic progestins at the molecular level, we developed a sensitive in vitro assay that employs African Green Monkey Kidney CV1 (AGMK-CV1) cells stably expressing the PR (CV1-PR), in which measurement of chloramphenicol acetyltransferase (CAT) gene *(31,32)* transactivation from a progesterone-responsive reporter vector (PRE2-TATA-CAT) *(22)* allows easy estimation of the progestational and antiprogestational effects of a given compound. This cell line exclusively expresses the PR at constant concentrations, avoiding crossreactivity with other steroid receptors that may potentially bind to progestins and progesterone-responsive elements (PREs), as is the case of the glucocorticoid receptor in HeLa cells or the ER and PR in T47-D and MCF-7 cells *(33)*. An additional advantage of this cell assay system is that it allows examination of the hormonal activities of different compounds under identical conditions of receptor content, overcoming the potential limitations imposed by the relatively low amounts of PR yielded by transiently transfected cell systems *(34)*. Using this sensitive cell assay system, we assessed the molecular effects of NET, LNG, and gestodene (GSD) (a third-generation synthetic progestin) as well as of NET A-ring–reduced metabolites on PR-mediated actions. In addition, we examined the actions of NET and its tetrahydro-reduced metabolites on ERα- and ERβ-mediated transactivation of an estradiol-responsive construct transfected into AGMK-CV1 cells.

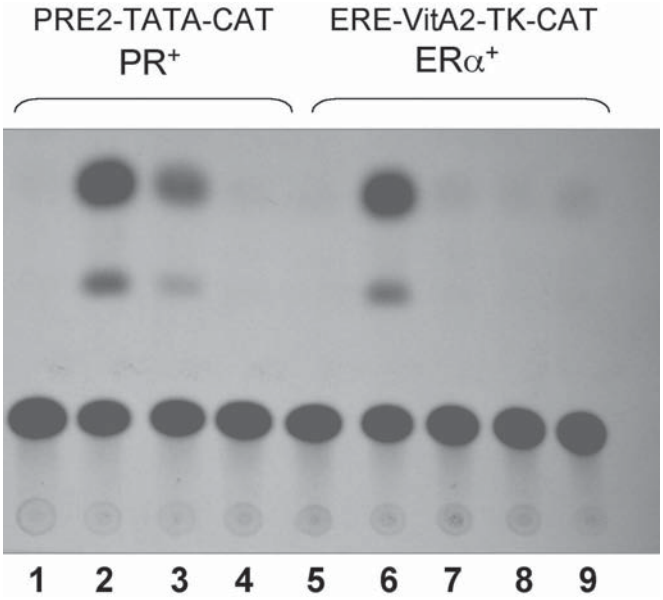

Fig. 1. Representative crossreactive analysis of rPR and human ER. AGMK-CV1 cells were transiently transfected with 500 ng of the rPR or the human ERα cDNAs, the responsive plasmids (PRE2-TATA-CAT or ERE-VitA2-TK-CAT, respectively), and the β-galactosidase expression plasmid. Cultures were maintained for 48 h in the absence or presence of 10^{-8} *M* E_2 or promegestone (R5020) and then assayed for CAT enzyme activity after normalization for β-galactosidase activity. Antihormones (RU486 or 4-hydroxy-tamoxifen [TAM]) were added at a 100-fold molar excess concentration. Lane 1, EtOH; lane 2, R5020; lane 3, R5020 plus RU486; lane 4, E_2; lane 5, EtOH; lane 6, E_2; lane 7, E_2 plus 4-hydroxy-TAM; lane 8, 4-OH-TAM; lane 9, R5020.

Results

Response of AGMK-CV1 Cells Cotransfected with rPR and PRE2-TATA-CAT PR-Responsive Reporter Gene

Given that CV1 cells do not express steroid receptors and that some factors are required for transcriptional activation in the presence of steroid-receptor complexes, we tested the responsiveness of the transfected PRE2-TATA-CAT and estrogen-responsive element-vitellogenin A2-thymidine kinase promoter-CAT (ERE-VitA2-TK-CAT) reporters to synthetic progestins in the presence of transiently expressed rPR. As expected, when CV1 cells were cotransfected with the rPR cDNA and the PRE_2-TATA-CAT reporter, CAT activity was strongly induced by 10^{-8} *M* concentrations of the synthetic progestins. By contrast, no induction was detected in the presence of estradiol (E_2) when CV1 cells were cotransfected with the ERE-VitA2-TK-CAT reporter and the rPR cDNA. However, when the ERE-VitA2-TK-CAT reporter was cotransfected with the human ERα, CAT activity was strongly inducible by 10^{-9} *M* E_2 but not by 10^{-8} *M* R5020, the potent progesterone (P_4) agonist (Fig. 1). The low background promoter activity and high levels of progestin-induced activity of the PRE_2-TATA-CAT reporter gene prompted us to establish a rPR-harboring cell line for

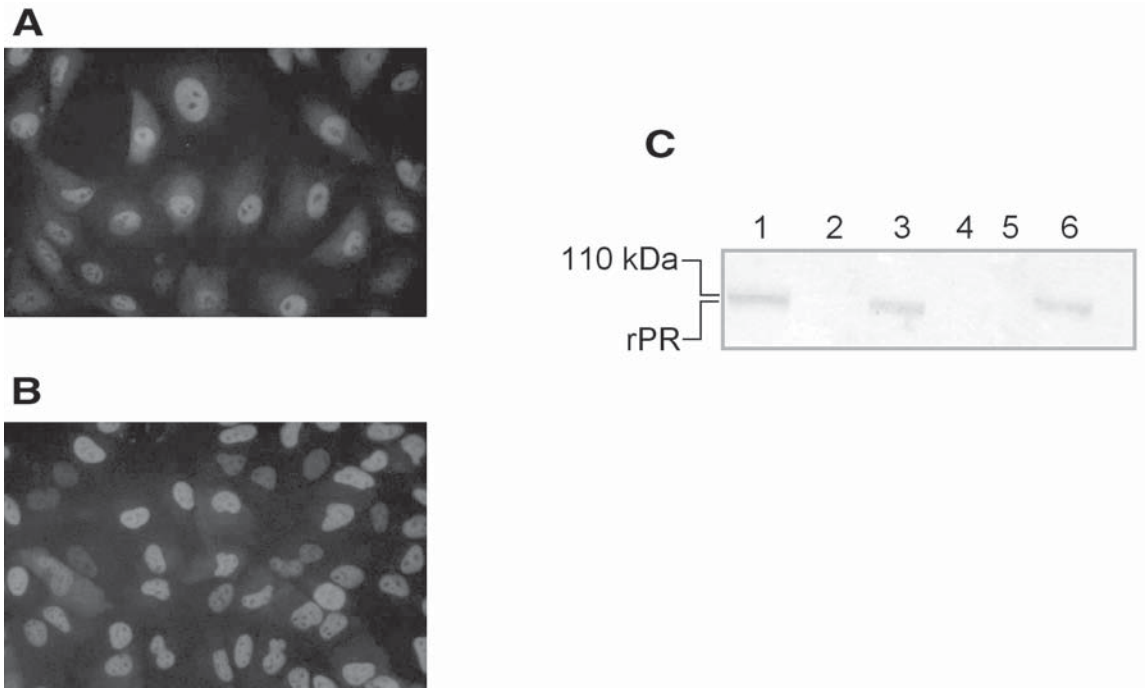

Fig. 2. Immunologic localization of rPR in CV1-PR cells and Western blot analysis of rPR in six cell clones. (**A**) In basal conditions, immunoreactive rPR was mainly identified within the cell nucleus; (**B**) after treatment with 10^{-8} *M* P_4, immunoreactive rPR was exclusively localized in the nucleus; (**C**) Western blot analysis of the rPR stably expressed by CV1-PR cells. One hundred micrograms of cytosol proteins was separated in sodium dodecylsulfate polyacrylamide gel electrophoresis and transferred to immobilon-P membranes. Transferred proteins were blocked and incubated with a mixture of anti-rPR MAbs and then identified with horseradish peroxidase–conjugated rabbit antimouse immunoglobulins. The immunodetected PR bands migrated at 110 kDa. Lanes 1–6 correspond to six different cell clones cotransfected with the rPR expression vector and the pKSV-neo construct harboring the neomycin resistance gene. Only clones 1, 3, and 6 were reactive to the anti-PR Let126 and MI60 MAbs.

screening and analysis of the progestational activity of some synthetic steroids.

Stably Transfected CV1-PR Cells Express a Functional rPR

Since the PREs interact not only with the PR but also with glucocorticoid, mineralocorticoid, and androgen receptors *(35,36)*, and considering the fact that synthetic progestins are able to bind these receptors *(20,37)*, a model exclusively expressing the PR was designed in order to determine the hormonal activity induced by the activated rPR. For this purpose, we stably transfected the AGMK-CV1 steroid receptor–negative cells with the rPR. Stably transfected cell clones positive for rPR were obtained by geneticin selection, and cells were analyzed for PR by immunocytochemical and immunoblot analysis (Fig. 2). In basal conditions, immunoreactive rPR was mainly identified within the cell nucleus (Fig. 2A), whereas in the presence of 10^{-8} *M* P_4, the stably expressed PR was exclusively nuclear (Fig. 2B). Figure 2C shows a representative Western blot of six different clones. Only clones 1, 3, and 6 were reactive to the anti-PR Let126 and MI60 monoclonal antibodies (MAbs). Because of its adequate PR expression levels (170 fmol/mg of cytosolic protein) and ability to transactivate the PRE_2-TATA-CAT reporter when treated with R5020 but not with the P_4 antagonist RU486, clone 1, named CV1-PR, was used for further studies.

Competition Binding of 19-Nor Progestins for rPR in CV1-PR Cells

To analyze the interaction of synthetic progestins with the stably expressed PR, cytosol preparations from CV1-PR cells were incubated with 1 n*M* [^{3}H] ORG 2058 at 4ºC in the presence or absence of increasing concentrations of different steroid competitors (ORG 2058, P_4, NET, 5α-NET, 3β,5α-NET, LNG, 5α-LNG, RU486, and E_2). As shown in Fig. 3, ORG 2058, P_4, LNG, RU486, NET, and 5α-NET were efficient competitors for progesterone-specific binding sites in CV1-PR cell extracts, whereas 3β,5α-NET and E_2 were not. This finding correlated with the progestagenic action of these steroids in the transactivation assays (discussed subsequently).

Stably Transfected CV1-PR Cells Are Responsive to 19-Nor Progestins

To determine the progestational activity of NET, LNG, GSD, and NET A-ring–reduced derivatives at the transcriptional level, CV1-PR cells were transfected with the PRE2-

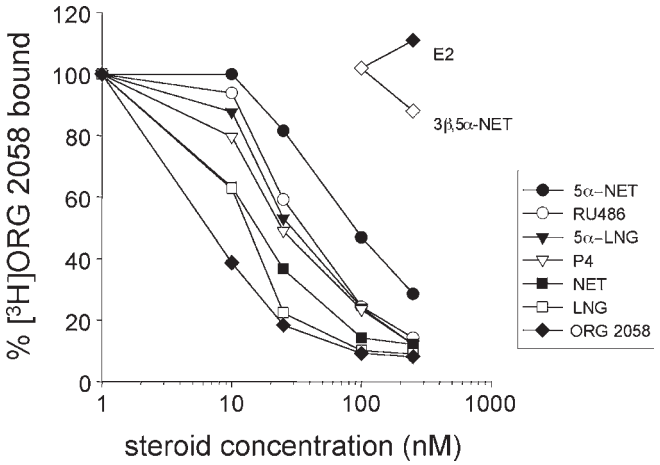

Fig. 3. Competition assay of different 19-nor progestins for rPR binding. Increasing doses of progestins were added to cytosol suspensions containing [^{3}H] ORG 2058, and the mixture was incubated at 4ºC for 18 h. Bound and free fractions were separated by the addition of a dextran-coated suspension. Nonspecific binding was determined in the presence of 1000-fold excess unlabeled ORG 2058. Binding of 3β,5α-NET at a 250 n*M* concentration was negligible. Each point represents the mean of three experiments in triplicate incubations.

TATA-CAT reporter gene and subsequently treated with increasing doses of each progestin. As shown in Fig. 4A, all progestins stimulated PRE2-TATA-CAT gene transcription in a dose-dependent manner. According to this assay, R5020 and GSD were the most potent inducers (fold induction: ~19 and ~25, respectively). The A-ring-reduced metabolites of NET induced lower progestagenic responses than NET when tested at nanomolar concentrations (10^{-8} *M*) (Fig. 4B). However, when tested at micromolar doses, 3β,5α-NET induced significant CAT activity, with a fold induction factor similar to that observed for the precursor compound (NET) (fold induction: ~14 and ~11, respectively) (not shown). This finding was surprising considering that binding of this particular compound to the PR was minimal when tested up to a concentration of 250 n*M (18,38)*.

NET and Its Tetrahydro-Reduced Metabolites Transactivate ERE-VitA2-TK-CAT Reporter Vector Through ERα and ERβ

The estrogenic properties of NET and its metabolites were determined by transiently cotransfecting the ERE-VitA2-TK-CAT vector and the human ERα or ERβ into AGMK-CV1 cells. Cells were then treated with NET, 3β,5α-NET, 3α,5β-NET, and E_2 at various doses. The results showed that all progestins were able to stimulate ERα-mediated transactivation of the ERE-VitA2-TK-CAT reporter vector when tested at several concentrations (Fig. 5A) and that this transactivation was efficiently inhibited by 100-fold excess of the antiestrogen TAM. As previously reported *(18, 38)*, the 3β,5α tetrahydro-reduced metabolite of NET was a potent agonist for the ERα; in this assay, this derivative was the most potent compound among all progestins tested (Fig. 5A). The estrogenic activity of NET and its 3α,5α-reduced derivative was lower than that exhibited by E_2. The results obtained using the ERβ as a transactivator demonstrated that the ERβ transactivated the ERE-VitA2-TK-CAT reporter vector in the presence of E_2 in a manner similar to that of the ERα, although to a lower extent (Fig. 5B). Both A-ring tetrahydro-reduced NET derivatives were able to induce ERβ transactivation of the ERE-VitA2-TK-CAT reporter when tested at micromolar concentrations (Fig. 5B); however, the extent to which these NET derivatives activated the estrogen-responsive vector through this particular ERβ was significantly lower than that observed with the ERα.

Discussion

In the present study, we validated and applied an in vitro cell system expressing the rPR to evaluate the progestational and antiprogestational activity of several 19-nor progestins and NET A-ring–reduced derivatives. A transfection procedure was used on AGMK-CV1 cells to generate clones stably expressing the rPR. Since expression of rPR in the CV1-PR cell clones was regulated by the SV40 promoter present in the pKSV10 expression vector (which may be distinctly expressed by each cell clone), the expression level of rPR among individual cell clones showed some variations; in this regard, the analysis of several cell lines over prolonged periods of culture did not yield significant changes in the expression levels of the PR. In contrast to what has been previously observed in cytosolic samples from uterine tissue, in which three rPR bands with an M_r of 110, 79, and 65 kDa were apparent *(9,38)*, we only identified a single 110-kDa rPR band in the stably transfected CV1-PR cell line. This difference is probably owing to the absence of receptor proteolysis in the CV1-PR cells.

It has been shown that synthetic progestins exert a wide variety of progestagenic activities in the hypothalamus and/or the pituitary, including inhibition of the midcycle gonadotropin surge *(39,40)*. In particular, NET is able to induce expression of the uteroglobin gene in prepubertal rabbits *(41,42)*, an action that has been widely used as a molecular marker for progestational activity *(43,44)*. When NET is further reduced in its A-ring, the molecule loses PR affinity and acquires affinity to both the androgen receptor and the ER *(18,20,40)*. On the other hand, the introduction of a methyl group at position C-18 in the NET molecule yields LNG, a 19-nor progestin with high progestagenic and androgenic properties *(11,12,28)*. GSD, a potent progestagenic compound devoid of androgenic properties *(45,46)*, is a synthetic progestin closely related to LNG, differing only by the presence of a double bond between its carbons 15 and 16. Although the variabilities exhibited by these compounds in progestagenic potency as well as in intrinsic andro-

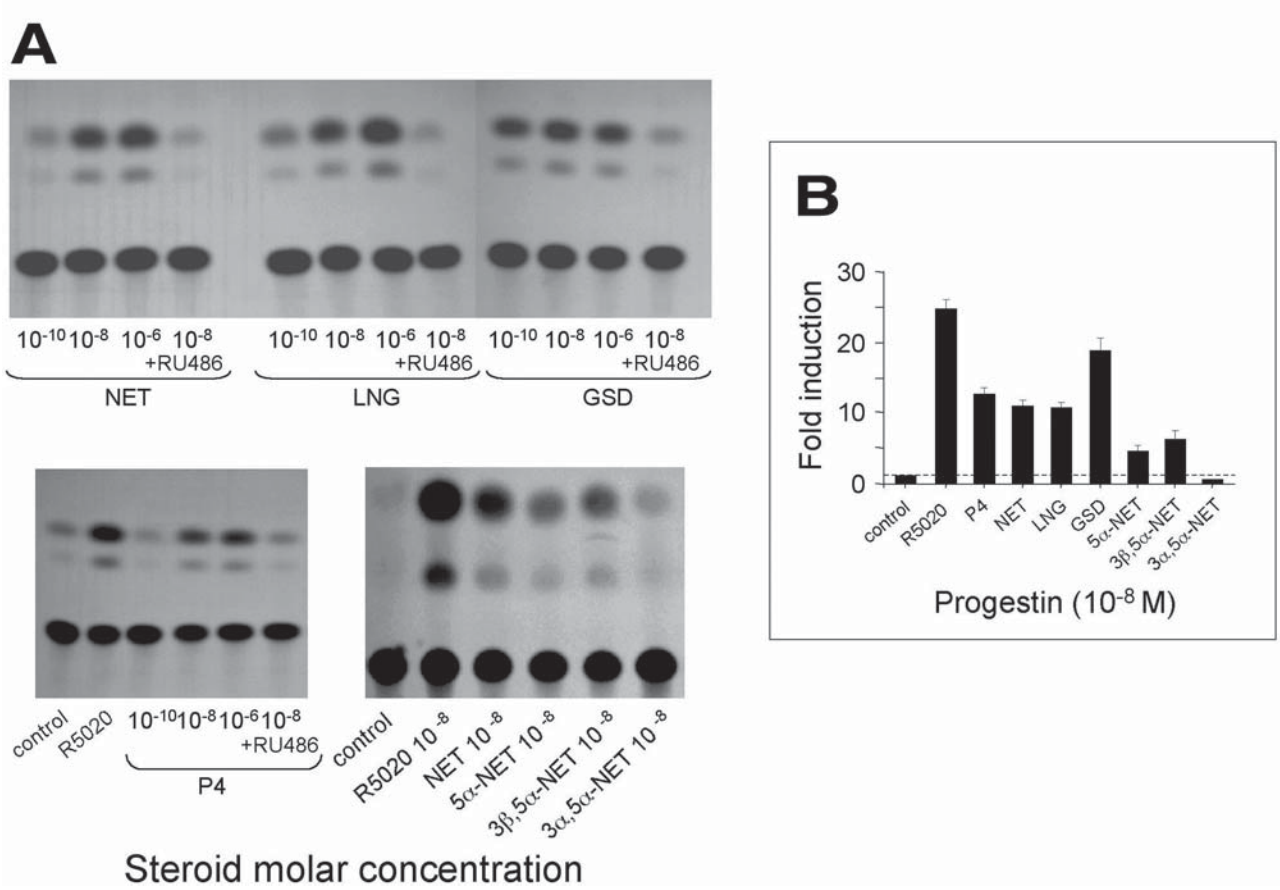

Fig. 4. 19-nor progestins and NET A-ring–reduced derivative-stimulated transcription of PRE2-TATA-CAT reporter vector. (**A**) Representative TLC from CAT activity showing PRE2-TATA-CAT induction by different progestins. CV1-PR cells were transfected with the PRE2-TATA-CAT reporter vector and the β-galactosidase expression plasmid used as internal control. Cultures were maintained for 48 h in the presence of the indicated progestins. The antiprogestin RU486 was added at a 100-fold molar excess (10^{-6} *M*). In some experiments, R5020 was used as a positive control. The autoradiograms are representative of three independent experiments. (**B**) Potency of different progestins to promote PRE2-TATA-CAT reporter vector transcription (means ± SEM from three separate experiments). Fold induction was calculated as the ratio of acetylated chloramphenicol generated in 60 min at 37ºC by 100 μg of proteins from progestin-treated cells over untreated control cells, after correction for β-galactosidase activity. Intraassay variation was ≤10%. Fold induction factor was calculated at 10^{-8} *M* progestin concentration.

genic and estrogenic activities may be apparently subserved by these chemical modifications *(47)*, 19-nor progestins are metabolized in vivo to 5α- or 5β-hydroxy and/or to 3α, 5α- and 3β,5α-tetrahydro-reduced derivatives, which are also biologically active *(40,48)*.

Employing the CV1-PR cell system, we found that NET, LNG, GSD, and the A-ring-reduced metabolites of NET exert their progestational activity through the interaction with an intracellular PR protein and the subsequent binding of the progestin-activated PR to the PREs located upstream of a P_4-regulated reporter vector (PRE2-TATA-CAT) *(22)*. All progestins tested were able to transactivate the PRE2-TATA-CAT reporter in a dose-dependent fashion, an effect that was completely inhibited by the addition of 100-fold molar excess RU486. These data confirm and extend those of previous studies *(4,35)* showing that stimulation of transcription by the progestin-activated PR requires the presence of PREs in the template. We also demonstrated that NET, LNG, and GSD are compounds with a progestagenic activity three to sixfold higher than P_4, with GSD the most potent agonist as disclosed by the lower doses required to induce CAT activity.

The observation that the tetrahydro-reduced derivative 3β,5α-NET induced CAT activity in the CV1-PR cell line contrasts with the findings of a previous study showing that this derivative does not apparently bind the PR *(38)*. However, significant transcription of the progesterone-responsive gene by 3β,5α-NET was only achieved at pharmacologic doses of this compound. This finding was interesting particularly considering that reverse bioconversion of tetrahydro-reduced NET derivatives to NET does not occur in any progestin target cell. Furthermore, we have recently found that exposure of AGMK-CV1 cells to [^{3}H]-NET results in ~83% bioconversion of this progestin to its [^{3}H]-3α,5α-NET derivative within the first 24 h and that neither [^{3}H]-5α-NET, [^{3}H]-3β,5α-NET, nor [^{3}H]-NET are identified in

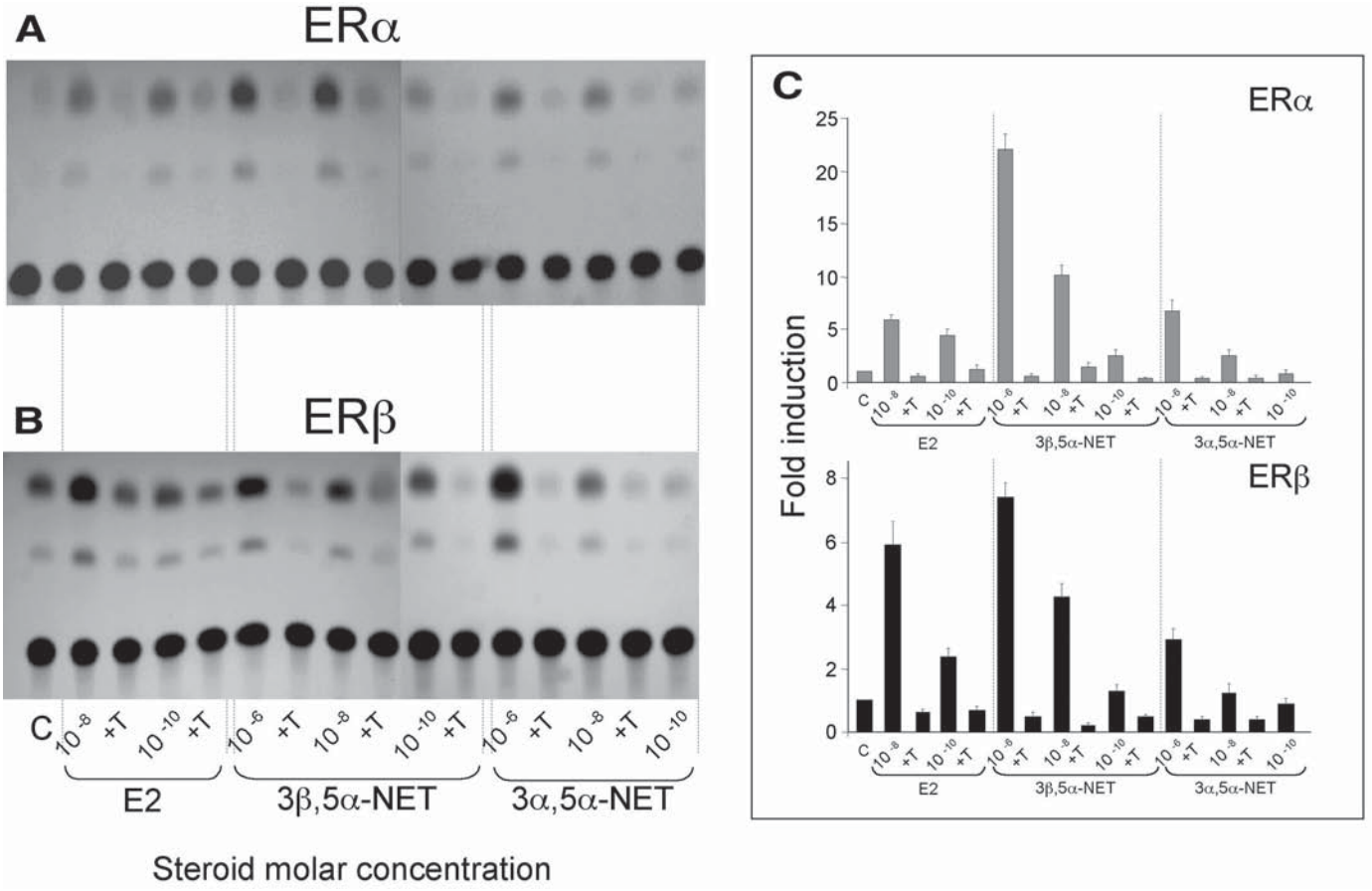

Fig. 5. Transactivation of ERE-VitA2-TK-CAT reporter vector by E_2, NET, and NET tetrahydro-reduced derivatives. CV1 cells were transiently transfected with the (**A**) ERα or (**B**) ERβ expression vector, the ERE-VitA2-TK-CAT reporter vector, and the β-galactosidase expression plasmid. Cultures were maintained for 48 h in the presence of 10^{-10}–10^{-6} *M* concentrations of the indicated steroids. TAM (T) was added at 10 n*M*. The autoradiograms shown are representative of three independent experiments. (**C**) Potency of different E_2 and tetrahydro-reduced NET derivatives to promote ERE-VitA2-TK-CAT reporter vector transcription (means ± SEM from three separate experiments). Fold induction is the ratio of acetylated chloramphenicol formed by 100 μg of proteins from steroid-treated cells over untreated control (C) cells.

proportions >10% after an incubation period of 48 h (unpublished data). These data concurrently suggest that minimal binding of the 3β,5α-NET derivative to the PR (as found in the present study) is apparently sufficient to activate the receptor and induce transcription of the PRE2-TATA-CAT reporter gene in CV1 cells. Additional studies are required to further clarify the mechanisms subserving the transcriptional effect of this particular NET derivative. Since 3β,5α-NET inhibits uteroglobin synthesis in vivo through a mechanism probably involving the ER *(41,42)*, it would also be interesting to test whether this NET derivative may selectively exert antiprogestational effects in CV1-PR cells coexpressing an ER-responsive expression vector.

Employing AGMK-CV1 cells, we additionally demonstrated that the tetrahydro-reduced derivatives of NET exert estrogenic activities mediated by both ERα and ERβ. These results confirm and extend those of previous studies in which NET stimulated the growth and promoted proliferation of MCF-7 and T-47D ER-positive cells *(49,50)* as well as transactivation of an ERE-responsive gene *(51)*. In AGMK-CV1 cells, NET and its A-ring tetrahydro-reduced metabolites transactivated the ERE-VitA2-TK-CAT reporter vector in the presence of the ERα and ERβ. Since NET does not bind the ERs, it is possible that the minimal (albeit measurable) estrogenic effect of NET had occurred via its bioconversion to more active tetrahydro-reduced compounds with increased affinity for the ER *(18,40)*. Although the 3β, 5α-NET derivative induced the most significant ERα-mediated transactivation of the ERE-VitA2-TK-CAT reporter in AGMK-CV1 cells, we have found that only ~4% of [^{3}H]-NET is bioconverted to [^{3}H]-3β,5α-NET in this cell line (unpublished data). Therefore, induction of ERE-VitA2-TK-CAT transactivation by NET probably occurred by the action of the 3α, 5α-NET derivatives and the minute amounts of the 3β,5α-NET progestins derived from endogenous bioconversion. The possibility that some bioconversion of NET to ethy-

nyl-estradiol had occurred is remote, considering that the presence of a carbon atom in position 19 is a structural prerequisite for A-ring steroid aromatization *(52)*.

The present study allowed confirmation of the intrinsic estrogenic activity previously reported for 3β,5α-NET *(18, 38)* and additionally demonstrated that this action may also be mediated through the ERβ, as has been recently documented in HeLa cells *(53)*. Furthermore, pharmacologic doses of the 3α,5α-NET metabolite, previously reported to be unable to bind the ER in rat uterine cytosols *(38)*, effectively transactivated the ERE-VitA2-TK-CAT reporter in the presence of either the ERα or ERβ, thus emphasizing the high sensitivity of this cell assay system to detect a given hormonal effect in a setting of low or negligible ligand-receptor binding. Alternatively, this apparent discrepancy between steroid binding and gene transactivation may be owing to differences in steroid receptor conformations and/or coactivator/corepressor content between naturally occurring and transfected cell systems.

In summary, the results presented herein demonstrate that the CV1-PR cell line is a useful tool to determine the hormonal effects of a number of synthetic progestins when transfected with steroid receptors and adequate responsive genes. In this assay system, NET, LNG, and GSD exhibit potent progestational effects at the transcriptional level. In the case of NET, its A-ring tetrahydro-reduced derivatives are responsible, in part, for the PR-, ERα-, and ERβ-mediated transactivation of progesterone- and estrogen-responsive genes. The fact that 19-nor progestin derivatives may also exert estrogenic effects is important considering that estrogens have been largely associated with breast and endometrial cancer *(25,26,54,55)* and that these compounds are widely used as contraceptive agents. Nevertheless, the impact of the in vivo bioconversion of 19-nor progestins into compounds with estrogenic potency on estrogen-dependent tumors still needs to be more deeply investigated.

Materials and Methods

Chemicals

Levonorgestrel, NET, 17β-estradiol, progesterone, and TAM were obtained from Sigma (St. Louis, MO). A-ring-reduced metabolites (5α-NET, 3β,5α-NET, and 3α,5α-NET) were kindly provided by Dr. Gustavo A. García (School of Chemistry, National University of Mexico, Mexico); 5α-NET was obtained by lithium-ammonia reduction of NET *(56)*, whereas 3β,5α-NET and 3α,5α-NET were synthesized through sodium borohydride and L-selectride reduction under anhydrous conditions of 5α-NET, respectively *(20,38)*. Chemical purity of NET and its derivatives was assessed by their melting points, high-performance liquid chromatographic behavior, infrared absorption, and H-nuclear magnetic resonance. The physical and spectroscopic constants of these compounds have been previously described *(20,38)*. RU486 was kindly provided by Roussell Uclaf (Romanville, France). Promegestone and GSD were obtained from Organon (Oss, The Netherlands). (Dichloroacetyl-1-^{14}C)-chloramphenicol (2.04 GBq/mmol), [6,7-^{3}H]-ORG 2058, and unlabeled ORG 2058 were from Amersham Pharmacia Biotech (Buckinghamshire, UK). All progestins were 99% pure. All media and antibiotics for cell culture were purchased from Life Technologies (Gaithersburg, MD).

Gene Constructs

The rPR cDNA expression vector (pKSV-rPR) *(57)* as well as the human ERα cDNA expression vector were kindly provided by Professor Edwin Milgrom (INSERM, Le Kremlin Bicêtre, France). The human ERβ *(58)* was kindly donated by Professor Jan-Åke Gustaffson (Karolinska Institute, Sweden). The reporter vectors used in this study were the PRE2-TATA-CAT *(41)* and the ERE-VitA2-TK-CAT (ERE fragment -331 to –297) *(59)*. The β-galactosidase plasmid (pSV-βGal) (Promega, Madison, WI) was used as a control plasmid.

Cell Culture and Transfections

AGMK-CV1 cells were cultured at 37ºC in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% fetal calf serum, 2 m*M* L-glutamine, 0.1 U/mL of insulin, and 100 IU/mL of penicillin in a 5% CO_2 atmosphere. For transient transfections, cells were transfected by the calcium phosphate–DNA coprecipitation method as previously described *(60)*. Briefly, cells (5×10^5) were plated in 100-mm Petri dishes in DMEM medium. After 24 h, cells were rinsed with phosphate buffer (0.05 *M*)–saline (0.9%) (phosphate-buffered saline [PBS]), pH 7.4, and incubated with phenol red–free medium containing 10% dextran–charcoal stripped calf serum. Since CV1 cells are devoid of steroid receptors, they were cotransfected with 2 μg of expression vectors encoding the rPR, the ERα, or the ERβ; 1.0 μg of pSV-β-Gal; and 5 μg of the PRE_2-TATA-CAT or ERE-VitA2-TK-CAT reporter vectors. In all experiments, the amount of transfected DNA was adjusted to 20 μg by the addition of herring sperm DNA. Twenty-four hours after transfection, a 1-min shock of dimethylsulfoxide (10% in serum-free medium), was given and the cells were rinsed with PBS and refed with stripped medium. Thereafter, steroids diluted in ethanol were added using a maximal final concentration of 0.001% ethanol. Twenty-four hours after the steroid treatments, the cells were harvested in buffer A (15 m*M* Tris-HCl, pH 8.0; 60 m*M* KCl; 15 m*M* NaCl; 1 m*M* dithiothreitol (DTT); and 2 m*M* EDTA) and processed for the CAT assays. For the stable transfection of the rPR, CV1 cells were established by cotransfection of the rPR expression vector with a pKSV-neo construct harboring the neomycin resistance gene and subsequent cloning under selective geneticine (500 μg/mL) pressure. Single-cell clones were obtained by dilution titration of positive selected cell pools. The CV1-PR cell clone

was maintained up to 24 mo under geneticine pressure (200 μg/mL), without detectable losses in PR expression or progestagenic response.

Detection of rPR by Immunocytochemistry

Immunocytochemical detection of the rPR in the stably rPR-transfected cells was performed as described by Guiochon-Mantel et al. *(57)*. Briefly, ~5×10^5 cells plated on fibronectin-precoated chamber slides (Nunc, Naperville, IL) were incubated for 2 h with the anti-rPR MAbs Let126 and MI60 *(61)*. Immunofluorescence analysis was performed using a fluorescein-conjugated rabbit antimouse antibody (Dakopatts, Glostrup, Denmark) at a 1:40 dilution. Photographs were taken on a Leitz microscope with a Fujichrome 1600 ASA film. Subcellular localization of the rPR was performed by observing ~100 cells. Staining was considered as nuclear or cytoplasmic when it was exclusively located in the nucleus or in the cytoplasm or when staining was stronger in the nucleus than in the cytoplasm and vice versa.

Western Blot Analysis

Western blot analysis was performed as described by Pasapera et al. *(41)* with some modifications. Total protein extracts from stably transfected AGMK-CV1-PR cell clones were obtained by freeze thawing. Proteins (100 μg) were separated by polyacrylamide gel electrophoresis and transferred to nylon membranes (18 h, 30 V at 4ºC) in a buffer containing 25 m*M* Tris and 192 m*M* glycine, pH 8.3. Membranes were blocked with 10% (w/v) nonfat dry milk in 10 m*M* PBS, pH 7.2, for 1 h at room temperature and then incubated for 60 min with MAbs MI60 and Let126 *(61)* in 10 m*M* PBS with 10% (w/v) nonfat dry milk at 1:300 and 1:400 dilutions, respectively. The membranes were washed five times in PBS and thereafter in Tris-buffered saline (TBS) (150 m*M* NaCl; 50 m*M* Tris-HCl, pH 7.2). After the last wash, membranes were incubated with peroxidase-conjugated rabbit antimouse immunoglobulins (Dako, Carpintería, CA) (1:1000 in TBS containing 10% [w/v] nonfat dry milk) for 1 h at room temperature. Color was developed using a solution containing 0.6 mg/mL of diaminobenzidine (Life Technologies) in 10 m*M* Tris-HCl (pH 7.6) and 1 μL/mL of 30% H_2O_2.

Binding Assays

Progestin binding to the PR was measured by a modification of the previously described charcoal-dextran method *(20)*. Briefly, cells were harvested by trypsinization and centrifugation at 450*g* for 5 min. The cells were washed with PBS, and the resultant pellet was redisolved in TEDM buffer (20 m*M* Tris-HCl buffer, pH 7.4; 1.5 m*M* EDTA; 0.25 m*M* DTT; and 10 m*M* sodium molybdate) and frozen at –70°C. The cell pellet was allowed to thaw, and the cells were homogenized in TEDM buffer supplemented with 10% gycerol (v/v) and aprotinin (100 μg/mL) using a Dounce all-glass homogenizer. The homogenate was centrifuged at 105,000*g* for 45 min at 4ºC, and the supernatants (cytosol) were collected and used for binding assays. Cytosols were incubated with 20 n*M* [6,7-^{3}H]-ORG 2058 (specific activity of 45 Ci/mmol; Amersham) in the absence or presence of increasing concentrations of steroid competitors. After a 16- to 20-h incubation period (at 4ºC), a charcoal-dextran suspension was added and incubated for 15 min. The suspension was finally centrifuged and aliquots of the supernatant were counted. Nonspecific binding and total binding were measured by the simultaneous addition of TEGM buffer with or without unlabeled ORG 2058.

CAT Assays

Whole-cell extracts were obtained by freeze thawing and centrifugation. Protein concentration was determined by the bicinchoninic acid assay (Pierce, Rockford, IL). CAT activity was measured as described by Pothier et al. *(32)*, with minor modifications. Briefly, cell extracts (100 μg) were incubated in buffer A with 5 μL of (dichloroacetyl-1-^{14}C)-chloramphenicol and acetyl-CoA (4 m*M*) for 1 h at 37ºC. Acetylated and nonacetylated forms of (dichloroacetyl-1-^{14}C)-chloramphenicol were separated by thin-layer chromatography (TLC) in chloroform:methanol (95:5) for 45 min. TLC plates were exposed to Kodak Biomax-ML film (Kodak, México D.F., México) 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 subtrate *(62)*.

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