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Receptor Independent Effects on DNA Replication by Steroids

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Abstract There is now convincing evidence associating estrogens with an increased risk of some cancers. However, the absence of a complete correlation between estrogen receptor binding and the biological activity of these estrogens has suggested the possibility of other mechanisms of action. The effect on DNA replication of several hormones that are putatively involved in breast cancer was tested at a physiological concentration. The studies were conducted in a HeLa cell-free system by using a plasmid containing a specific mammalian origin of replication (DHFR *ori*β) as template DNA. A series of related steroids produced an entire range of activity from enhancement to inhibition of in vitro DNA replication. These studies indicate a new possible target, which may help to better understand the effect of these hormones in breast cancer. Furthermore, the results show that this in vitro DNA replication system provides an evaluative assay for the effects of compounds on hormone-responsive cancers independent of some hormone receptors. J. Cell. Biochem. 70:323–329, 1998.

Key words: steroids; DNA replication; carcinogenesis; proliferation; cell-free system

Steroid hormones, in particular estrogens, induce cell proliferation and regulate the human mammary epithelial morphogenesis. In addition, these hormones have been associated with neoplastic changes. Estrogens have been shown to be promoters of mammary carcinogenesis in both human and experimental models. The therapeutic use of estrogens (e.g., hormone replacement therapy, oral contraceptives) has been associated with an enhancement in the risk of breast cancer [Meirik et al., 1986; Hulka, 1990]. Other evidence supporting the link of estrogens to breast cancer is their ability to generate mammary tumors in rodents [Bradlow et al., 1985] and their mitogenic effects on mammary human tissue [Osborne et al., 1993; Fishman et al., 1995] and established breast cancer cell lines [Darbre and King, 1988].

Despite intensive research, the molecular and cellular mechanisms through which estrogens influence carcinogenesis are not completely understood. The carcinogenicity of estrogens has been attributed primarily to their principal mechanism of action, which is mediated through the estrogen receptor (ER). However, there is evidence of a lack of correlation between binding of estrogens to the estrogen receptor and the biological activity [Raynound et al., 1985; Korach et al., 1987], suggesting that estrogen action may not be mediated exclusively through the receptors.

Several nonreceptor-mediated mechanisms have been suggested, including direct chemical interactions of certain estrogens with DNA and/or proteins [Bucala et al., 1982; Liehr and Roy, 1990; Liehr et al., 1991; Telang et al., 1992]. In addition, estrogens may interact with the DNA structure by insertion between base pairs into partly unwound double-stranded DNA [Hendry and Mahesh, 1995].

A common feature during the development of carcinogenesis is cell proliferation. Because estrogens affect cell proliferation, the DNA replication apparatus is therefore one of the potential targets. To help elucidate the effect of estrogens on DNA replication, we employed a mammalian in vitro DNA replication system

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that is both progesterone- and estrogen-receptor negative [Walker et al., 1984; Diaz-Perez et al., 1996]. This Hela cell-free system is used to replicate a bacterial plasmid containing a specific mammalian origin of DNA replication, in this case the origin from the hamster dihydrofolate reductase (DHFR) locus. The DHFR origin, oriβ, is one of the most thoroughly characterized origins of bidirectional replication (OBR) and is located approximately 17-kb downstream of the *dhfr* locus [Burhans et al., 1990]. It has been shown to be an origin of replication by various mapping techniques, including the nascent assay based on DNA polymerase chain reaction [Vassilev et al., 1990] and Okazaki fragment distribution [Burhans et al., 1990]. Zannis-Hadjopoulos et al. [1994] showed by the *Dpn* I resistance assay [Peden et al., 1980] that pX24 plasmid containing the DHFR oriβ was able to replicate autonomously in human cells and in an in vitro cell free-system, which used human HeLa cell extracts [Pearson et al., 1991]. This system was used in this study. Moreover, we recently demonstrated by "earliest-labeled DNA fragment labeling" that initiation of replication in this cell free-system begins preferentially within the oriβ-containing fragment of pX24 plasmid [Diaz-Perez et al., 1996].

This in vitro DNA replication assay allows the study of the possible interactions between various compounds with either DNA or the protein(s) that are involved in DNA synthesis. The present data suggest a direct nonreceptor-mediated effect of several steroids on DNA replication.

MATERIALS AND METHODS

Cell Extracts and Plasmids

HeLa S3 nuclei and cytosol were purchased from Cellex Biosciences (Minneapolis, MN). The extracts were prepared as described by Pearson et al. [1991]. The protein concentrations of the nuclear and cytosolic extracts were 3.8 mg/ml and 11 mg/ml, respectively.

Plasmid pX24 of Dr. J. Hamlin (University of Virginia, Charlottesville) was provided by Dr. M.L. DePamphilis (NIH, Bethesda, MD). This plasmid pX24 contains a 4.8-kb *Xba* I fragment of the *ori*β region of DHFR, inserted into the *Xba* I site of pUC13 [Burhans et al., 1990]. Plasmid 30.4 consists of a pBluescript clone containing a random breast tumor cDNA insert of 0.7 kb and without autonomous replication activity [Nielsen et al., 1994].

Biochemicals

Steroids were purchased from Sigma (Mississauga, Ontario, Canada) and Steraloids Inc. (Wilton, NH), [α-^{32}P] dCTP and [α-^{32}P] dTTP were obtained from NEN-DuPont (Boston, MA), *Dpn* I was obtained from New England Biolabs (Beverly, MA), and 6,7 17β-[^{3}H] was obtained estradiol from Armesham (Oakville, Ontario, Canada). Stock solutions of the steroids were made in ethanol. Dilutions were done in water, and the final ethanol concentration never exceeded 0.1%.

Estrogen Receptor Binding Assay

The level of estrogen receptors in the nuclear and cytoplasmic extracts was determined by using the steroid-binding assay. Aliquots of nuclear or cytoplasmic HeLa extracts (50 µg of protein) were incubated with 20 nM 17β-[^{3}H]estradiol for 2 h at room temperature in the presence or absence of unlabeled estradiol (4 µM). Nonspecific binding was defined as binding remaining in the presence of 4 µM 17β-estradiol. Bound ligand was separated from free ligand by the dextran-coated charcoal method and determined by liquid scintillation counting. A value higher than 10 fmol/mg cytosol or nuclear protein indicated detectable ER and was designated as positive [Oude Elferink et al., 1991].

In Vitro Replication Assay

In vitro replication was carried out as previously described [Diaz-Perez et al., 1996]. Standard in vitro reactions were basically composed of equimolar amounts of a supercoiled plasmid, either pX24 or plasmid 30.4 (150 ng and 74 ng, respectively), HeLa nuclear and cytoplasmic extracts, an ATP regenerating system, PEG, a mixture of nucleotides (ATP, CTP, GTP, UTP, dATP, dGTP, dTTP, and dCTP), and 10 µCi of [α-^{32}P] dCTP and [α-^{32}P] dTTP. The experiments were conducted in two different ways: (1) preincubation of the experimental compounds with HeLa cell extracts, followed by adding the DNA template and precursor nucleotides to allow replication to occur; or (2) preincubation of the experimental compounds with the template DNA and the precursor nucleotides, followed by

adding the remaining components of the in vitro reaction to initiate DNA replication. Preliminary experiments indicated that preincubations with either HeLa cell extracts or DNA and nucleotide precursors produce significant different results than simultaneous exposure to extracts, DNA, and nucleotide precursors.

After DNA isolation and purification, DNA replication was measured by using the *Dpn* I resistance assay [Peden et al., 1980]. *Dpn* I cleaves only fully methylated input DNA at the sequence $G^{m}ATC$. Both plasmids (pX24 and 30.4) were propagated in dam^{+} (deoxyadenosine methylase) bacteria and thus fully methylated. Because HeLa cell extracts lack this enzyme, the products of one round of DNA replication that occur in this in vitro system [Pearson et al., 1991] are hemimethylated and thus resistant to *Dpn* I cleavage. To ensure complete digestion of the DNA with *Dpn* I, we used λ DNA as an internal control in all reactions, and the digestion products were verified by ethidium bromide staining (data not shown). DNA forms II (circular) and III (linear) were quantitated as previously described [Diaz-Perez et al., 1996] by densitometry of a phosphoimager screen using the Fuji BAS 2000 analyzer. We used physiologically relevant concentrations (10 nM) of the steroids [Hayes et al., 1996].

Dose–Response Experiments

HeLa cell extracts or DNA and precursor nucleotides were preincubated with different concentrations (0.1 nM, 1 nM, 10 nM, or 100 nM) of the estrogens, 17β-estradiol, estrone, and estriol. Assays of DNA replication proceeded as described earlier.

RESULTS

Measurement of Estrogen Receptors in HeLa S3 Cells

HeLa S3 cells have been reported to be estrogen receptor negative [Walker et al., 1984]. Because our in vitro replication system uses HeLa cell extracts, we decided to verify the estrogen receptor levels in nuclear and cytoplasmic extracts from HeLa cells to eliminate the possibility of receptor-mediated mechanism as a mode of action of these estrogens in this system. The estrogen receptors were measured by a ligand-binding assay. The results confirmed that both the nuclear and cytoplasmic extracts were estrogen-receptor negative (<8 fmol/mg protein) and consequently suitable for use in our in vitro DNA replication system.

Determining an Effective Hormone Concentration

To determine an effective hormone concentration, we analyzed a range of estrogen concentrations, from 0.1 to 100 nM. This range represents intracellular concentrations that can be reached in vivo. Replication was carried out as explained in *Materials and Methods*. Different concentrations (0.1–100 nM) of the parent estrogen, 17β-estradiol, and two of its principal metabolites, estrone and estriol, were preincubated with either HeLa cell extracts (Fig. 2A) or DNA and nucleotide precursors (Fig. 2B) to determine an effective hormone concentration. A typical autoradiograph of in vitro replication products after the in vitro assay is shown in Figure 1. The results demonstrated a concentration-dependent effect of 17β-estradiol, estrone, and estriol on DNA replication. *Dpn* I-resistant bands corresponding to forms II and III of DNA were quantitated by subtracting the background and were normalized for the amount of DNA in ethidium bromide gels, as previously described [Diaz-Perez et al., 1996; Nielsen et al., 1994].

Preincubation of the estrogens with HeLa cell extracts (Fig. 2A) showed a small and similar effect at 0.1 nM and 1 nM for the three estrogens; however, at 10 nM, we observed an important and distinct enhancement on DNA synthesis for all of the estrogens used, i.e., approximately 1.7-fold for estrone and 17β-estradiol and 3.6-fold for estriol. At 100 nM estriol and estrone exhibited a decline in in vitro DNA synthesis of pX24, whereas 17β-estradiol increased by approximately 2.5-fold by comparison to the 0.1-nM level.

Preincubation of estrogens with DNA (Fig. 2B) at 0.1 nM and 1 nM affected in vitro DNA replication of pX24 in a manner similar to that described earlier. At 10 nM concentration, however, the estrogens showed different effects. The estrogen 17β-estradiol resulted in an increase of approximately 1.9-fold, estriol had no effect, and estrone resulted in a decrease of DNA synthesis in comparison with the 0.1-nM level. Consequently, the dose–response experiments showed that the 10-nM level was an effective hormone concentration to compare one compound with another.

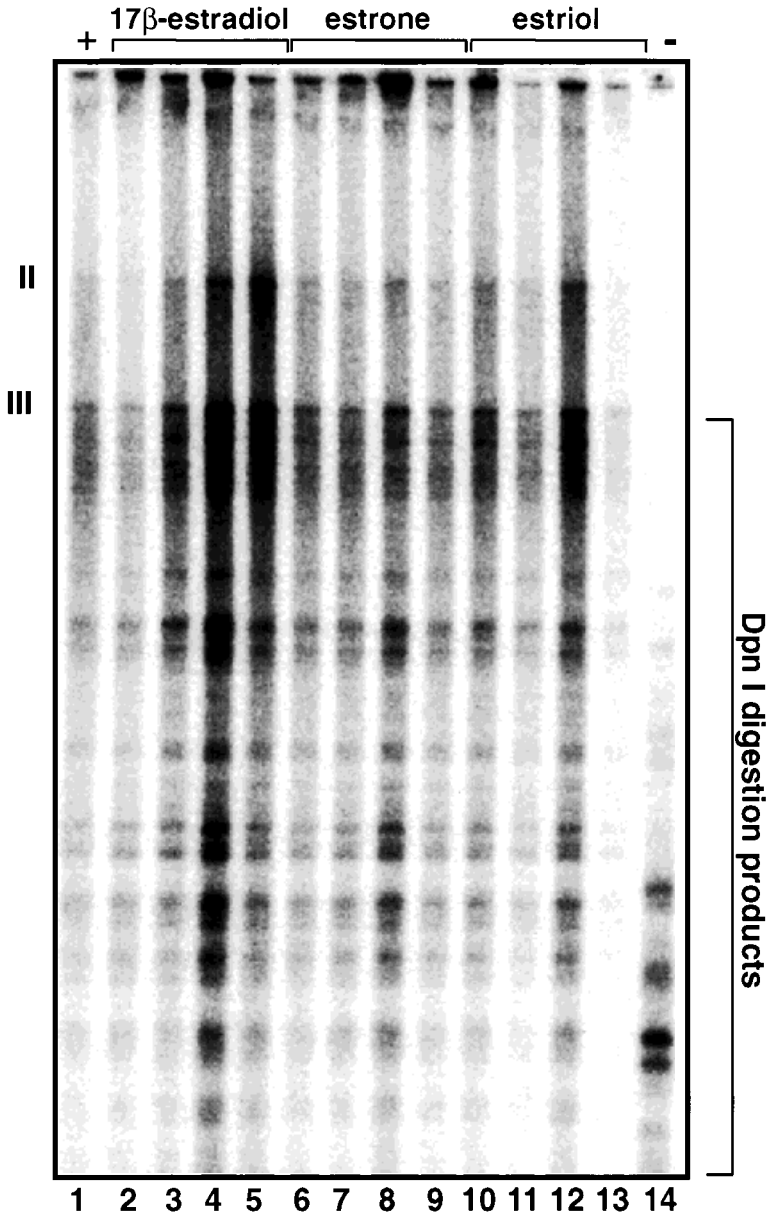

Fig. 1. In vitro DNA replication in the presence of different concentrations (0.1–10 nM) of estrogens. HeLa cell extracts were preincubated for 15 min at 30°C with H_2O (**lane 1:** pX24, positive control), 17β-estradiol (**lane 2:** 0.1 nM, **lane 3:** 1 nM, **lane 4:** 10 nM, and **lane 5:** 100 nM), estrone (**lane 6:** 0.1 nM, **lane 7:** 1 nM, **lane 8:** 10 nM, and **lane 9:** 100 nM), or estriol (**lane 10:** 0.1 nM, **lane 11:** 1 nM, **lane 12:** 10 nM, and **lane 13:** 100 nM). In vitro DNA replication reactions were carried out as explained in Materials and Methods. *Dpn* I-resistant bands corresponding to forms II (relaxed circular) and III (linear) of DNA (indicated in the figure) were quantified by subtracting background data and normalized for the amount of DNA in ethidium bromide gels. **Lane 14** denotes the negative control, plasmid 30.4, which did not replicate.

Effects of Steroids on In Vitro DNA Replication

To analyze the possible interaction of the hormones with either the replication proteins or the DNA, they were preincubated with either HeLa cell extracts or DNA and nucleotide precursors, respectively. Pretreatment of HeLa cell extracts with the hormones (Table I; 10 nM) resulted in an enhancement (progesterone, 17β-estradiol, estrone, estriol, 16keto-β-estradiol, 2-OH-estradiol, 4-OH-estradiol, 16α-OH-estrone, 2-OH-estrone, 4-methoxy-estrone) of DNA synthesis by comparison with the untreated control. The increase in DNA synthesis ranged from 1.4-fold (17β-estradiol) to 3.6-fold (estriol). In contrast, 2,3-methoxy-estrone produced an inhibitory effect on in vitro DNA syn-

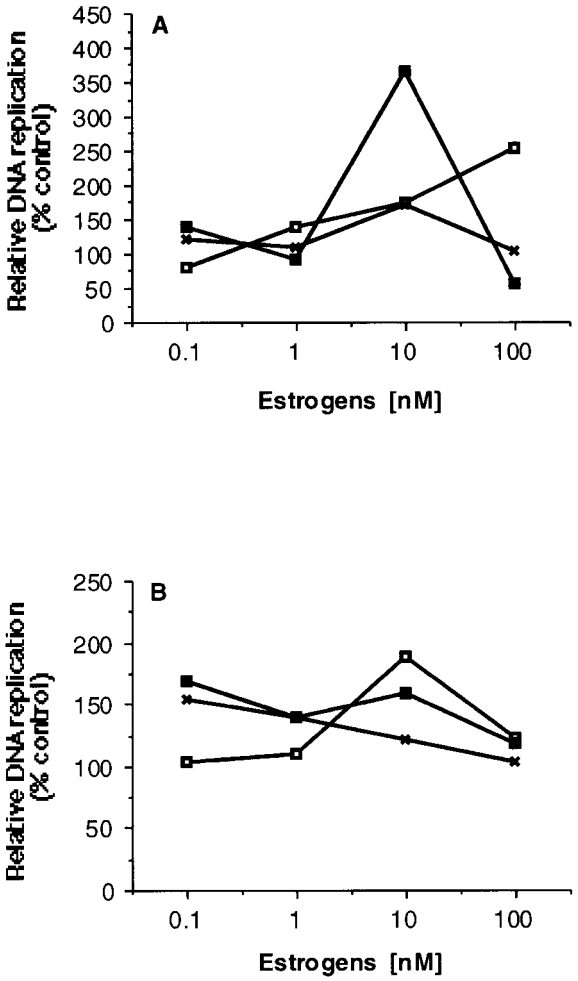

Fig. 2. Determining an effective hormone concentration. Estrogens (17β-estradiol, estrone, and estriol) were preincubated with either HeLa cell extracts (**A**) or DNA and precursor nucleotides (**B**). All the in vitro conditions are the same as described in Materials and Methods. The data are expressed as the percentage of DNA replication activity (vs. that of untreated controls, 100%) versus different concentrations (0.1–100 nM) of hormones. Open square, 17β-estradiol; ×, estrone; solid square, estriol.

TABLE I. Effect of Hormones on In Vitro DNA Replication*

	Extracts			DNA		
Compound	% DNA	S.D.	n	% DNA	S.D.	n
Testosterone	99	12	3	73	18	4
Progesterone	208	30	3	115	9	3
17β-Estradiol	173	56	3	179	14	2
17α-Estradiol	112	29	2	119	22	2
Estrone	172	29	3	109	17	2
Estriol	363	117	2	145	19	2
16-Epiestriol	105	30	2	118	27	2
17-Epiestriol	110	16	2	103	19	2
16,17-Epiestriol	114	10	2	201	6	2
16Keto-β-estradiol	150	19	4	99	26	2
2-OH-estradiol	267	67	3	52	16	2
4-OH-estradiol	227	53	3	53	6	2
16α-OH-estrone	166	23	2	151	26	3
16β-OH-estrone diacetate	100	20	3	157	41	2
β-Estradiol-17 acetate	86	19	2	68	18	2
Estrone acetate	90	35	2	111	3	2
2-OH-estrone	156	47	3	92	20	4
4-Methoxy-estrone	153	27	2	53	0	2
2,3-Methoxy-estrone	70	6	2	63	0	2

*The in vitro DNA replication assay was done as described in Figure 1. The compounds were preincubated with HeLa cell extracts or with DNA. Incorporation is completely dependent on a plasmid containing an origin of replication. Data are expressed as the percentage of the non-drug-treated control (100%) and represent the average of at least two separate experiments. %DNA, percentage of DNA replication; n, number of assays; S.D., standard deviation.

thesis, whereas some steroids (testosterone, 17α-estradiol, 16-epiestriol, 17-epiestriol, 16,17-epiestriol, 16β-hydroxy estrone, β-estradiol-17 acetate, estrone acetate) did not alter DNA synthesis levels significantly above those of the control in this system.

However, when input DNA was pretreated with the hormones (10 nM; Table I) 17β-estradiol, 16,17-epiestriol, 16α-hydroxy-estrone, 16β-hydroxy estrone diacetate, and estriol resulted in an enhancement of DNA replication, whereas pretreatment with testosterone, 2- and 4-hydroxy estradiol, β-estradiol-17 acetate, and 4-methoxy estrone produced an inhibition of DNA synthesis. Enhancement of DNA synthesis differed from 1.5-fold (16α-hydroxy-estrone, estriol) to 2.0-fold (16,17-epiestriol). The inhibitory effect ranged between 30% (testosterone) and 50% (2- and 4-hydroxy estradiol, 4-methoxy estrone). The rest of the compounds had no significant effect when compared with the control.

DISCUSSION

Previous studies [Diaz-Perez et al., 1996] have demonstrated that the mammalian in vitro DNA replication system used in this study could be used to identify enhancers and inhibitors of DNA replication. In the present study, some of the steroid compounds resulted in an increase of DNA synthesis, some resulted in an inhibition of DNA synthesis, others had opposite effects depending on whether they were preincubated with DNA or HeLa cell extracts, and some had no effect in this in vitro system. The

results showing an enhancement of DNA replication by some of the compounds were consistent with results of previous in vivo studies. The estrogens 17β-estradiol, estriol, and 16α-hydroxy-estrone enhanced DNA synthesis in mouse mammary epithelial cells [Telang et al., 1992; Suto et al., 1993] and in the human mammary carcinoma cell line MCF-7 [Schneider et al., 1984]. Moreover, Li et al. [1995] found that 17β-estradiol, 4-hydroxy estradiol, estrone, and 16α-hydroxy-estrone increased in vitro cell proliferation of primary renal epithelial hamster cells in culture. In addition, Fishman et al. [1995] observed that 16α-hydroxy-estrone promotes the expression of a transformed phenotype in human cells. Conversely, in vitro studies have indicated that the 2- and 4-hydroxy metabolites of estradiol and estrone inhibited growth rates of human breast cancer cells [Schneider et al., 1984], which is consistent with our results on DNA replication after pretreatment of DNA. However, other studies have shown a proliferative outgrowth of hamster kidney cells by 4-hydroxy estradiol [Li et al., 1995], which is consistent with the effects observed when the protein extract was preincubated with the compound. A discrepancy exists regarding the effect of progesterone. Some evidence has shown an antiproliferative effect of progesterone [Santen et al., 1990], but studies on rodent mammary tissue have demonstrated that progesterone induced proliferation [Fishman et al., 1995], as was observed in the pretreatment of the steroid with the protein extracts.

The estrogen 17α-estradiol had no effect on DNA replication in our system. This result is also consistent with that of previous studies, which have shown that 17α-estradiol does not promote tumor development in hamster and that treatment corresponds with low cell proliferation activity [Li et al., 1995].

The HeLa cell extracts used in the present study lack estrogen receptors; therefore, these hormones, at physiologically relevant concentrations, enhanced or inhibited DNA replication by a process different from that of receptor-mediated mechanisms. Thus, this in vitro DNA replication assay has allowed the identification of a possible additional mode of action for these hormones: the direct interaction of steroids with the replication machinery independently of steroid receptors. There may be a dichotomy of results with certain estrogen compounds, depending on whether the pretreatment was performed with extracts or DNA. In these cases, it is also interesting to note a similar dichotomy between steroid receptor binding and biological effects for in vivo studies. For example, certain super estrogenic ligands bind poorly to the estrogen receptor [Raynound et al., 1985]. Conversely, certain strong binding ligands can have poor estrogenic activity [Korach et al., 1987]. Clues as to the origin of the differences in vivo may be demonstrated by the present in vitro DNA replication studies. The combined nonreceptor-mediated modes of action may enjoy different emphasis or impact depending on cell type or state, favoring action on DNA or on replication proteins in extracts according to the existing conditions. These results may help in understanding the role of these agents in the development and treatment of breast cancer.

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