Effect of neonatal exposure to the antioestrogens nafoxidine and CI-628 upon the development of the uterus in the prepubertal rat

P. S. Campbell and P. M. Satterfield

Department of Biological Sciences, The University of Alabama in Huntsville, Huntsville, AL 35899, U.S.A.

Summary. Neonatal Sprague–Dawley rats were injected with the antioestrogens nafoxidine or CI-628 on Day 3 of life alone or in combination with oestradiol benzoate 24 h later. Oestrogen-stimulated glucose oxidation and cytoplasmic oestrogen binding sites of the uteri were assessed at 21–23 days of age. Neither antioestrogen antagonized the prepubertal uterine impairments produced by neonatal oestradiol treatment. Both antioestrogens administered alone produced deficits which mimicked those produced by neonatal oestrogenization. However, the agonist property of each antioestrogen was differentially expressed: treatment with CI-628 reduced prepubertal oestrogen binding sites in the uterus, but nafoxidine exposure decreased the sensitivity of the uterus to oestradiol stimulation of glucose oxidation. It is postulated that CI-628 directly affects the uterus to reduce production of oestrogen receptor protein, while nafoxidine affects the development of the uterine phosphogluconate oxidative pathway indirectly through impaired ovarian function. However, antioestrogens blocked the neonatal oestradiol-induced reduction in the oestrogen-stimulated production of actomyosin in the adult uterus. Therefore, while both CI-628 and nafoxidine are clearly agonists in the neonatal rat, each appears to exhibit cell-specific agonist and antagonist properties.

Keywords: uterus; antioestrogen; oestrogen receptor; glucose metabolism; neonate; rat

Introduction

It has been previously demonstrated (Gellert *et al.*, 1977; Campbell, 1980) that neonatal administration of oestradiol benzoate results in impaired uterine growth responses to exogenous oestradiol in the prepubertal rat. This reduction in uterine responsivity is the result of a decrease in available cytoplasmic oestrogen binding sites in the uterus with a concomitant decrease in the nuclear retention of the receptor–oestrogen complex by uterine nuclei (Gellert *et al.*, 1977; Campbell, 1980). Additionally, neonatal exposure to oestradiol benzoate impairs the capacity of the uterus to metabolize glucose under oestrogen stimulation (Campbell, 1980). The latter effect augments the severity of disturbance of uterine function by neonatal oestrogenization in contrast to that produced by neonatal androgenization.

The purpose of the experiments reported herein was therefore to determine whether the antioestrogens CI-628 and nafoxidine would allay the effects of neonatal oestrogenization by oestradiol benzoate or whether their agonist properties would prevail over their antagonistic action.

Materials and Methods

Preparation of animals and materials. Rats of the Sprague–Dawley strain (SAF/SDD) were obtained from Southern Animal Farms (Prattville, AL) and maintained in a controlled environment in the UAH animal facility with a lighting

schedule of 13 h light:11 h dark (lights on 07:00 h). Commercially pelleted food (Purina) and water were provided *ad libitum*.

Young were designated 1 day old on the morning of discovery, and litters were culled to a maximum of 8 females with only one treatment group housed with a given mother. All experiments used animals from a minimum of two different litters. Neonates were injected subcutaneously (s.c.) with peanut oil vehicle (0·1 ml), 50 µg CI-628 (α-{4-pyrrolidinoethoxy}-phenyl-4-methoxy-α-nitrostilbene) or 50 µg nafoxidine (1-2-{p-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthy)phenoxyl}ethyl pyrrolidine HCl) on Day 3 of life. On Day 4 additional injections of 100 µg oestradiol benzoate or peanut oil vehicle were administered. Other rats were neonatally ovariectomized on Day 3 of life under hypothermia-induced anaesthesia. Rats were killed at 21–23 days of age by cervical dislocation for measuring uterine cytoplasmic oestrogen binding sites and oestrogen-stimulated glucose metabolism. Some neonatally-treated rats were allowed to reach adulthood and between 60 and 80 days of age were bilaterally ovariectomized under ether anaesthesia for subsequent actomyosin determination.

Oestradiol benzoate, diethylstilboestrol (DES), oestradiol-17β and all laboratory chemicals were obtained from Sigma Chemical Co. (St Louis, MO). The antioestrogens nafoxidine and CI-628 were generously provided by the Upjohn Company (Kalamazoo, MI) and Warner-Lambert/Parke-Davis (Detroit, MI), respectively. [6,7-^{3}H] Oestradiol (sp. act. 49–51 Ci/mmol) was obtained from the New England Nuclear Corporation (Boston, MA) and had a radiochemical purity of 98%. D-[(U)-^{14}C]glucose (sp. act. 1·19–1·92 µCi/µg; radiochemical purity 99%) was also acquired from NEN. A Beckman LS-100C scintillation counter was used to determine the radioactivity of the samples using Aquasol-2 (NEN) as the scintillation cocktail. Tritium was counted at 38% efficiency and carbon-14 was counted at 75% efficiency.

Determination of cytoplasmic oestrogen binding sites. Soluble oestrogen binding sites were measured by the charcoal–dextran assay (Korenman, 1968). Each uterus was quickly removed, stripped of adhering fat and connective tissue and homogenized in 2 ml ice-cold TE buffer (10 mM-Tris–1·5 mM-EDTA, pH 7·4) using a motor-driven Kontes Duall glass homogenizer. After centrifugation at 1000 g, the cytosol of each sample was divided into two 0·5 ml samples labelled A and B. Tube A received 10^{-8} M-[^{3}H]oestradiol while tube B received the labelled oestradiol plus 100-fold excess DES. The assay tubes were incubated for 60 min at 0–4°C with vortexing every 15 min. At the end of incubation an equal volume of 1·0% charcoal–0·05% dextran suspension was added to adsorb unbound [^{3}H]oestradiol and the tubes were incubated for another 15 min. After the charcoal–dextran particles were pelleted, a 200 µl sample of supernatant was removed from each tube and added to 10 ml scintillation cocktail to determine specific binding. At least 95% of the specific oestrogen binding sites in the immature rat uterus are occupied when oestradiol is present in the assay buffer at 10^{-8} M (Anderson *et al.*, 1973).

Oestrogen-stimulated glucose metabolism. The oxidation of [U-^{14}C]glucose to ^{14}CO$_2$ was determined by the method of Nicolette & Gorski (1964). Animals were injected s.c. with 0·1 µg oestradiol-17β and uteri were obtained 3 h later. The dose of oestradiol used is the minimal dose required to promote maximum stimulation of glucose oxidation for this time interval (Anderson *et al.*, 1973). The uteri were stripped of adhering connective tissue and placed into 25 ml Erlenmeyer flasks containing 2 ml Eagles medium (Difco; Detroit, MI) and 10^{-6} M-[U-^{14}C]glucose (0·5 µCi total activity). The flasks were aerated with 95% O$_2$–5% CO$_2$ and sealed with rubber serum stoppers containing filter paper wicks soaked with 0·25 ml methyl benzonium hydroxide to trap evolved ^{14}CO$_2$. The flasks were incubated 1 h at 37°C after which 1·5 ml 5 N-H$_2$SO$_4$ was injected through the serum stoppers to halt the reaction and promote the release of carbon dioxide from the incubation medium. After an additional 2 h incubation at 37°C, the ^{14}CO$_2$ which had been absorbed onto the filter paper wick was determined by placing the wick into 10 ml Aquasol-2 and counting the radioactivity.

Determination of actomyosin. Ovariectomized adult rats were injected s.c. for 6 consecutive days with 5 µg oestradiol-17β/100 g body weight. Uteri were obtained 24 h after the last injection and kept frozen at −20°C until the actomyosin content could be analysed. The extraction and estimation of actomyosin was carried out according to the procedure described by Needham & Williams (1963). To quantitate actomyosin content, 2 or 3 uteri from the same treatment group were placed into an ice-cold solution containing three times the uterine volume of 0·5 M-KCl buffered with 0·04 M-K$_2$HPO$_4$ (pH 7·1) and 1/50 of the volume of 0·1 M-ATP adjusted to pH 7·4 with Trizma base. The uteri were homogenized using a Polytron homogenizer and the homogenate was centrifuged at 1000 g for 10 min at 4°C. The supernatant was decanted and reserved for later actomyosin estimation. The pellet was resuspended at two times the uterine volume with the buffered KCl solution and then centrifuged again for 10 min. The two supernatant pools were combined and stored overnight at 4°C to permit ATP degradation.

Viscosity measurements were made the next day using an Ostwald viscosimeter to estimate actomyosin content of the uteri. Uterine supernatant was added to the viscosimeter and flow rates of the supernatant between two fiducial marks were determined at 25°C in the presence or absence of 0·1 M-ATP–MgCl$_2$. The concentration of actomyosin in each uterine fraction was then calculated on the basis of the difference in the two viscosities determined in the presence or absence of ATP according to an equation described by Needham & Williams (1963).

Total uterine protein was measured by the Folin–Ciocalteau method described by Lowry *et al.* (1951) using crystalline bovine serum albumin as the standard.
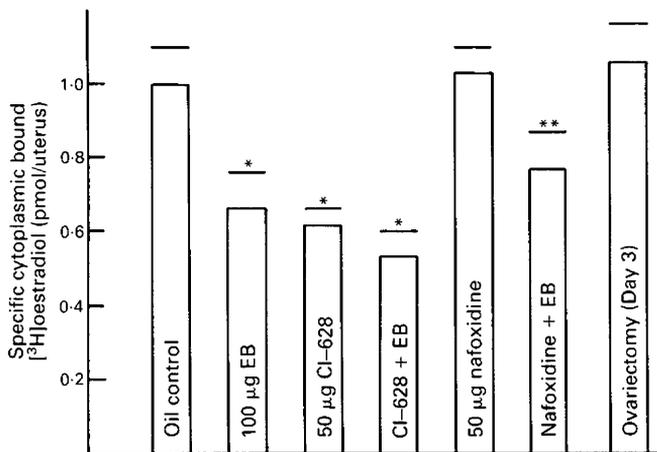
Fig. 1. Cytoplasmic oestrogen binding sites in uteri of prepubertal rats neonatally treated with oestradiol benzoate (EB), antioestrogen (CI-628 or nafoxidine) or ovariectomized. Each value is the mean ± s.e.m. of 8 different determinations. All treatments were compared to the vehicle-injected controls by Dunnett's T statistics. *$P < 0.025$; **$P < 0.05$.

Results

As illustrated in Fig. 1, neonatal treatment with 100 µg oestradiol benzoate or 50 µg CI-628 alone resulted in a decreased availability of cytoplasmic oestrogen binding sites in the uterus of the prepubertal rat. While nafoxidine alone did not affect later expression of oestrogen receptor protein in the uterus, the subsequent neonatal exposure to oestradiol at 24 h after nafoxidine injection resulted in a prepubertal expression of oestrogen receptor production similar to that seen in response to the neonatal exposure to oestradiol benzoate alone.

Figure 2 indicates that oestrogen stimulation of glucose oxidation in the prepubertal rat uterus was significantly reduced from control levels ($P < 0.025$) in animals which had been treated neonatally with oestradiol benzoate or nafoxidine alone or with oestradiol benzoate in combination with either of the antioestrogens. The mean value for each of these treatments did not differ from the average of the unstimulated control group which was injected with saline instead of oestradiol 3 h before assay. The metabolic response to oestradiol in the treatment groups resembled that observed in rats which had been neonatally ovariectomized. Thus, only neonatal treatment with CI-628 alone did not produce an adverse effect on this uterine response.

An experiment to determine the extent to which the antioestrogens CI-628 or nafoxidine deplete specific cytoplasmic oestrogen binding site availability 24 h after neonatal injection was also conducted (Table 1). Nafoxidine given as a single 50 µg injection 24 h before assay for cytoplasmic oestrogen binding by the charcoal–dextran procedure reduced the measured estimation of control uterine cytosolic receptor by 81·5%. The same dose of CI-628 produced a 72·8% depletion of cytoplasmic oestrogen binding sites in the uterus.

The ovarian weights of prepubertal rats treated neonatally with steroid hormone ester and/or antioestrogen are given in Table 2. A significant reduction ($P < 0.05$) in ovarian weights was evident for all treatment groups when compared to the peanut oil vehicle-injected controls. This reduction in prepubertal ovarian weight in treatment groups is largely coincident with reduced oestrogen binding sites in the uterus and the impairment of oestrogen-stimulated glucose metabolism. However, only the relationship between the relative magnitude of ovarian weight reduction as a consequence of the different neonatal treatments and the degree to which uterine glucose metabolism is compromised exhibits a significant correlation coefficient ($P < 0.05$).
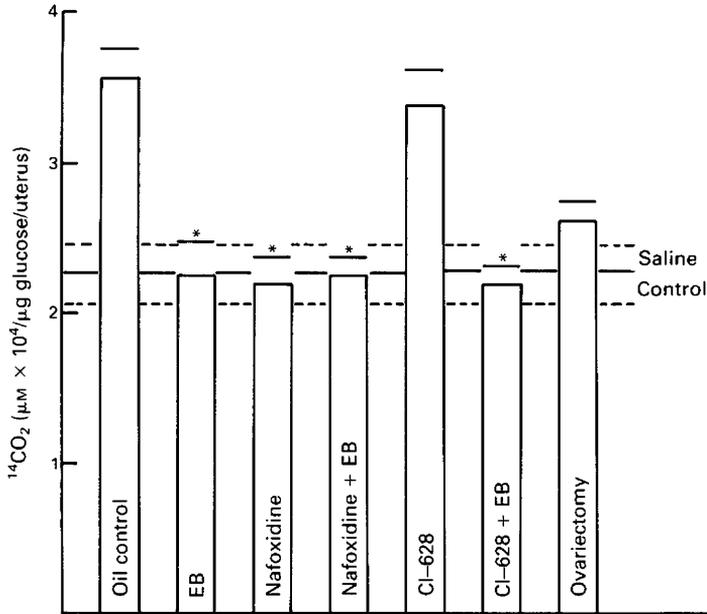
Fig. 2. In-vitro determination of oestradiol-stimulated glucose metabolism in uteri of prepubertal rats neonatally ovariectomized or treated with oestradiol benzoate (EB) or antioestrogen (nafoxidine or CI-628). Each value is the mean ± s.e.m. of at least 8 separate experiments. The broken lines represent the upper and lower s.e.m. limits for the mean basal rate of glucose oxidation (saline-injected controls). *$P < 0.025$ compared with control value (Dunnett's T).

Table 1. Depletion of cytoplasmic oestrogen binding sites in uteri of prepubertal rats injected with antioestrogen

Treatment	No.of rats	[³H]Oestradiol bound per uterus after injection (pmol/24 h)	% Depletion of cytoplasmic oestrogen binding sites
Peanut oil	8	1·51 ± 0·20	0·0
50 μg CI-628	8	0·41 ± 0·09	72·8
50 μg Nafoxidine	8	0·28 ± 0·03	81·5

Values are mean ± s.e.m.

The effect of neonatal treatment with oestradiol benzoate alone or 24 h after prior treatment with nafoxidine or CI-628 upon oestrogenic induction of uterine actomyosin in adulthood is presented in Table 3. Uteri from animals treated neonatally with oestradiol benzoate exhibited a significant reduction ($P < 0.05$) from control actomyosin production. In rats in which both antioestrogens had been administered 24 h before neonatal oestradiol benzoate injection, intermediate organ concentrations of actomyosin were evident after oestrogen treatment in adulthood. However, when uterine actomyosin content was expressed on the basis of total uterine protein, the relative actomyosin content in animals treated neonatally with antioestrogen plus oestradiol benzoate is similar to that seen in control animals.

Table 2. Relationship between ovarian weight (mean $\pm$ s.e.m.) and the availability of uterine cytoplasmic oestrogen binding sites and uterine glucose metabolism in animals treated neonatally with oestradiol benzoate (EB), nafoxidine (NAF) and CI-628

Neonatal treatment	No. of rats	Ovarian wt (mg)	A*	B*
Oil (control)	26	$13\cdot5 \pm 0\cdot6$		
100 µg EB	22	$6\cdot2 \pm 0\cdot4^{**}$	+	+
50 µg NAF + 100 µg EB†	17	$7\cdot6 \pm 0\cdot3^{**}$	+	+
50 µg NAF	16	$7\cdot0 \pm 0\cdot3^{**}$	−	+
50 µg CI-628 + 100 µg EB†	18	$5\cdot6 \pm 0\cdot4^{**}$	+	+
50 µg CI-628	16	$8\cdot5 \pm 0\cdot4^{**}$	+	−

*A = relationship between ovarian weight and oestrogen binding sites; correlation coefficient = $0\cdot51$; B = relationship between ovarian weight and glucose metabolism; correlation coefficient = $0\cdot87$ ($P < 0\cdot05$). A positive (+) relationship indicates that both ovarian weight and the experimental uterine parameter varied in a parallel fashion from control. A negative (−) relationship indicates that only the ovarian weight or the experimental uterine parameter alone is significantly different from control.
†Injections given 24 h apart.
**$P < 0\cdot005$ (Dunnett's T test) compared with control.

Table 3. Uterine actomyosin content in adult rats treated neonatally with oestradiol benzoate (EB) or with the combination of nafoxidine (NAF) or CI-628 plus EB

Treatment	No. of rats	Actomyosin	
		mg/g uterus	mg/mg total protein
Oil control	7	$3\cdot25 \pm 0\cdot24$	$0\cdot074 \pm 0\cdot007$
100 µg EB	7	$1\cdot11 \pm 0\cdot15^{*}$	$0\cdot030 \pm 0\cdot005^{*}$
50 µg NAF + 100 µg EB†	4	$2\cdot10 \pm 0\cdot82$	$0\cdot068 \pm 0\cdot034$
50 µg CI-628 + 100 µg EB†	5	$2\cdot32 \pm 0\cdot54$	$0\cdot072 \pm 0\cdot020$

Values are mean $\pm$ s.e.m.
†Injections spaced 24 h apart.
*$P < 0\cdot05$ (Neuman–Keuls statistics) compared with control value.

Discussion

It is evident from the results that neonatal exposure to the antioestrogens CI-628 or nafoxidine does not allay the effects of subsequent neonatal oestradiol benzoate treatment upon later prepubertal expression of uterine oestrogen receptor protein or metabolic enzymes of the phosphogluconate oxidative pathway. However, while the antagonist properties of these antioestrogens are not effective on these two measures, the two antioestrogens are acting quite differently with respect to their agonist properties. Nafoxidine simulates oestrogen in its disruptive effect upon the development of the uterine glucose metabolic pathway, while CI-628 simulates oestradiol benzoate action on the impairment of cytoplasmic oestrogen binding sites in the prepubertal rat uterus. This differential effect may be a reflection of variable cell specific agonist properties of antioestrogens (Markaverich *et al.*, 1981; Clark *et al.*, 1982; Dohler *et al.*, 1984).

The ovarian weight reduction observed in the prepubertal rat treated neonatally with steroid ester or antioestrogen probably represents feedback suppression of gonadotrophin secretion due to the introduction of exogenous agonist (Clark *et al.*, 1973; Gellert *et al.*, 1977; Campbell, 1980). Additionally, previous reports have demonstrated a causal relationship between prepubertal ovarian weights and uterine sensitivity to oestrogen-stimulated growth or glucose oxidation (Campbell, 1980; Campbell & Modlin, 1987). In this regard, neonatal treatment with CI-628, unlike nafoxidine, is without effect upon development of the uterine phosphogluconate oxidative pathway, and CI-628 treatment does not result in the reduction of prepubertal ovarian weights to the same extent as do other treatments. Furthermore, the production of oestrogen receptor protein is clearly not dependent upon endogenous oestrogen since neonatal ovariectomy does not affect this measure (Clark & Gorski, 1970). The effect of neonatal exposure to oestradiol benzoate or CI-628 upon the reduction of oestrogen binding sites in the prepubertal uterus probably, therefore, represents a direct tissue response to the agonist. Hence, neonatal exposure to agonist may 'turn off' oestrogen receptor production and decrease the production of normal levels of enzymes in the metabolic pathway for glucose oxidation for entirely different reasons. The early impairments in the development of the reproductive tract observed in this study are consistent with the induction of reproductive anomalies in adulthood as a result of perinatal exposure to antioestrogens such as clomiphene, nafoxidine and tamoxifen (Gellert *et al.*, 1971; Clark & McCormack, 1977; Chamness *et al.*, 1979; McCormack & Clark, 1979; Clark *et al.*, 1982; Dohler *et al.*, 1986).

Neonatal treatment with nafoxidine or CI-628 before neonatal injection of oestradiol benzoate 24 h later, however, did prevent the oestradiol-induced reduction of oestrogen-stimulated production of actomyosin in adult rat uteri. The hyperoestrogenization of the uterus due to antioestrogen exposure occurs principally in the endometrium: the antioestrogens do not affect the myometrium acutely or developmentally on histological or biochemical bases while oestradiol affects luminal epithelium, stroma and myometrium (Markaverich *et al.*, 1981; Clark *et al.*, 1982). Therefore, in tissue in which both antioestrogens lack agonist properties, their antagonist actions appear to prevail.

Although we observed some apparent cytoplasmic oestrogen binding sites remaining after neonatal antioestrogen injection, these residual binding sites may not be available for subsequent nuclear translocation (Williams & Gorski, 1974). Moreover, depletion of all cytoplasmic receptors does not appear to be a prerequisite for the antagonistic action of the antioestrogens (Hayes *et al.*, 1981). However, the mechanism of action of the antioestrogens upon reproductive tract development is complicated by the presence of specific antioestrogen binding sites distinct from oestrogen receptors in a number of tissues in the rat, including the uterus and ovary (Sutherland *et al.*, 1980; Winneker & Clark, 1983; Sudo *et al.*, 1983). Indeed, it has been proposed that antioestrogen interaction with such binding sites may alter biological potencies and pharmacokinetics to produce differential effects (Sudo *et al.*, 1983). Clearly, the possibility that antioestrogen binding sites may mediate some of the effects of the antioestrogens needs to be examined.

Furthermore, previous reports have indicated that antioestrogens are uterotrophic in the neonatal rat (Clark *et al.*, 1982; Clark & Guthrie, 1983). The results described herein also confirm the prominent agonist properties of the antioestrogens CI-628 and nafoxidine on neonatal reproductive tract tissues. Each antioestrogen produces prepubertal reproductive deficits that differentially mimic those produced by oestradiol benzoate, while neither antioestrogen blocks the oestradiol-induced impairment which is not produced when the antioestrogen is administered alone to the neonatal rat.

Since some antioestrogens are being used clinically to induce ovulation in anovulatory women and to inhibit breast cancer, the dual potential of these compounds needs to be recognized to avoid unexpected and perhaps undesirable side effects. The results reported in this work therefore reaffirm and extend the caution raised previously in using these drugs during pregnancy and/or the perinatal period (Clark & McCormack, 1977; McCormack & Clark, 1979; Chamness *et al.*, 1979). In addition to the structural and functional reproductive anomalies previously observed in

adulthood, it is evident from this study that early, prepubertal alterations at the molecular level also occur as a consequence of exposure to antioestrogens early in life.

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