Morphometric studies of neonatal estrogen imprinting in the mature mouse prostate

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Abstract

Estrogens play an important role in prostate physiology and neonatal exposure to estrogens has profound effects on the mature structure and hormonal sensitivity of rodent prostate. We aimed to determine the long-term effects of neonatal estrogens on the ductal architecture, morphology and hormonal sensitivity of the mature mouse prostate. Newborn mice (day 1–2) were administered a single injection (s.c.) of estrogens (estradiol benzoate (EB), diethylstilbestrol (DES)) with or without concomitant anti-estrogens (tamoxifen (TAM) or ICI 182 780 (ICI)) TAM or ICI alone, GnRH-antagonist (GnRH-A) or vehicle. At 7 weeks of age, ventral prostates (VP) were microdissected to estimate branch tip numbers and processed for stereologic analysis of volume fractions and diameters of various tissue components. Estrogens induced

permanently reduced branching morphogenesis leading to reduced VP weights and these effects were fully reproduced by GnRH-A, consistent with an indirect effect. Stereologically, neonatal estrogens induced epithelial and stromal hyperplasia and significantly reduced ($P < 0.05$) the diameters of VP glandular tubules and lumen compared with controls and these regressive effects were not reversed either by TAM or ICI. These studies confirm that a single neonatal dose of both DES and EB produces imprinting in the mature mouse prostate and indicate that neonatal estrogen effects involve both direct as well as indirect effects. In addition, both TAM and ICI act as partial agonists to the estrogen receptor in the ventral prostate of neonatal mouse.

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Introduction

Estrogens are involved both in the normal development of human prostate as well as subsequent adult prostate disease (Santti *et al.* 1994). Prostate diseases like benign prostatic hyperplasia (BPH) and prostate cancer are highly prevalent but their origins are not well understood. However, hormonal factors early in life seem to influence the occurrence of late-life prostate diseases. For example, androgen deficiency in men either due to castration or genetic mutations protects against late-life prostate disease (Quigley *et al.* 1995) while men with genetic 5 α -reductase deficiency develop a rudimentary prostate that does not develop late-life prostate disease (Imperato-McGinley *et al.* 1992). The mechanism of such latent effects may be explained by the phenomenon of hormonal imprinting in which the phenotypic expression of the imprinted characteristic appears after a long delay.

Estrogens during early life may alter development of the prostate, which thus may be predisposed for abnormal function and disease in later life. Exposure of experimental animals to estrogens during critical periods of development have been reported to lead to altered

structure (Chung & MacFadden 1980, Gaytan *et al.* 1986, Naslund & Coffey 1986, Zhao *et al.* 1992, vom Saal *et al.* 1997) and function (Higgins *et al.* 1981, Prins *et al.* 1993) of the prostate. Neonatal estrogens induce long-term modifications in the accessory sex glands of rodents including epithelial changes (Arai 1970, Pylkanen *et al.* 1991, 1993), changed hormonal sensitivity (Rajfer & Coffey 1978, 1979), alterations in the androgen receptor expression (Prins 1992, Prins & Birch 1995), auto-up-regulation of estrogen receptor expression (Prins & Birch 1997) and urethral dysfunction (Lehtimaki *et al.* 1996). Although the concept of neonatal imprinting was suggested some years ago, the molecular basis of such long-term changes in hormonal sensitivity is not well understood. This study aimed to investigate the structural and molecular basis of neonatal estrogen imprinting using a mouse model. This may have important implications for prostatic diseases that originate early in life. More specifically, this study aims to demonstrate if estrogens have direct or indirect (via suppression of gonadotropins) effects on the prostate and, if so, whether the effects of estrogens are mediated via the estrogen receptor.

Materials and Methods

Animals

Mice used in this study belonged to the hypogonadal (*hpg*) colony. The *hpg* mutation, a large truncation of the gene encoding gonadotropin-releasing hormone (GnRH) and its associated peptide (GAP) (Mason *et al.* 1986, Seeburg *et al.* 1989), was identified as a spontaneous mutation transmitting an autosomal recessive sterile phenotype in mice representing functional gonadotropin deficiency (Cattanach *et al.* 1977). The *hpg* colony is maintained at the University of Sydney by breeding from fertile heterozygotes originally from F1 hybrids of two inbred strains C3H/HeH and 101/H.

Genotyping was performed by PCR of DNA isolated from tail snips and amplification of a region of genomic DNA using three oligonucleotide primers placed strategically around the large deletion in the GnRH gene. The products were resolved on agarose gel and three genotypes (homozygous hypogonadals (*hpg/hpg*), heterozygotes (N/*hpg*) and homozygous normal (N/N) mice) are distinguished based on band sizes. Heterozygous and homozygous normal mice of this strain are phenotypically identical and were utilized for this study. Earlier studies have confirmed that growth and development of most androgen-dependent organs in age-matched heterozygote and homozygous males are identical (Crawford *et al.* 1993).

All mice were housed in groups of between three and four in standard plastic mouse cages, maintained under controlled conditions (lights on 0700–1900 h, temperature 20–24 °C), and allowed to feed *ad libitum*. All operative procedures were performed under anesthesia administered by an injection (0.01 ml/g body weight, i.p.) of a solution containing 5 mg/ml each ketamine (Parke-Davis, Caringbah, NSW, Australia) and xylazine (Bayer Australia Ltd, Botany, NSW, Australia).

Chemicals

Diethylstilbestrol (DES) was obtained from Research Plus Steroid Laboratories (Denville, NJ, USA) estradiol benzoate (EB, 1,3,5 (10)-estratrien-3, 17 β -diol 3-benzoate) from Steraloids inc. (Wilton, NH, USA), tamoxifen (TAM) from ICN Biomedicals inc. (Costa Mesa, CA, Ohio, USA) and GnRH-antagonist (GnRH-A, ORG 30276) from Organon, Oss, Netherlands. Collagenase was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA), arachis (peanut) oil from Sigma Chemical Co. (St Louis, MO, USA) and heparin from Delta West (Perth, WA, Australia). ICI 182 780 was kindly provided by Professor Rob Sutherland (Garvan Institute, Australia).

Experimental design

A preliminary experiment was done to establish the dose for DES and EB administration. Mice were administered

(s.c.) a range of doses (5–50 μ g) of estrogens dissolved in arachis oil on the first or second day of life. The maximally tolerated dose of 5 μ g was used for the present study. Higher doses elicited toxic effects resulting in high mortality rates of neonates.

On the first or second day of life, phenotypically normal mice were administered a single s.c. injection (5 μ g/pup) of estrogens (EB, DES) with or without (5 μ g) TAM or ICI 182 780 (ICI), TAM alone, ICI alone, GnRH-A or arachis oil in 20 μ l volume. The dose of ICI used was similar to that used for reduction of uterine weight in immature rats (Wakeling *et al.* 1991).

At 7 weeks of age, mice were weighed, killed by cardiac exsanguination under anesthesia, organs (ventral prostate (VP), seminal vesicles (SVs) and epididymis) excised and serum stored at –20 °C for hormone level determinations. SVs were weighed both before and after manual expression of fluid and organ weights were corrected for body weights before statistical analysis. Some mice from each group ($n=4$) underwent whole body perfusion to obtain perfusion fixed tissue for stereologic studies.

Both DES and EB were studied to investigate if DES had additional non-estrogenic effects besides acting as an estrogen. The use of a non-steroidal anti-estrogen TAM was intended to clarify if the actions of estrogens are mediated via the estrogen receptor. If the effects were fully blocked and similar for both drugs, this would mean that both DES and estradiol act predominantly via the estrogen receptor. However, if TAM blocked estradiol effects fully but not as fully for DES, this would suggest that DES might have additional non-estrogen receptor mediated effects on the prostate. Since TAM is known to be a partial agonist a new, more potent anti-estrogen-ICI 182 780, was studied for its estrogen receptor-blocking effects. GnRH-A was studied to control for indirect effects of estrogens via suppression of endogenous gonadotropins and testicular testosterone (T) secretion (where estrogens form via aromatization of T).

Hormone assays

T was measured in serum samples after hexane–ethyl acetate extraction, reconstituted in phosphate-buffered saline and assayed in duplicate using the SGT-1 antiserum, tritiated tracer and a standard curve of 0.5–1000 pg/tube with an incubation of 16 h at 4 °C and charcoal separation (Singh *et al.* 1995).

Microdissection

Prostates were excised and weighed after which one lobe of VP was used for microdissection and the other lobe snap frozen in liquid nitrogen for subsequent studies. Prior to microdissection, prostatic lobes were dissected free of surrounding fat in calcium and magnesium-free (CMF) Hanks solution and then incubated in Hanks CMF

solution containing 0.5% collagenase for 10 min at room temperature (Sugimura *et al.* 1986). The number of ductal tips were determined after individual ducts were teased apart on a glass slide under a dissecting microscope (Zeiss, Germany) using fine forceps and needles. The whole-mount two-dimensional array of ducts was photographed on an inverted microscope equipped with a Polaroid camera. Ductal tips were counted by a single observer with between-day reproducibility coefficient of variation for both (CV) of 3.4% and between-observer CV of 4.5% ($n=8$).

Histology

Whole body perfusion was performed for four mice/group under anesthesia. Briefly, 20 ml physiologic saline containing 10 IU/ml heparin were pumped manually through the left ventricle, while the right ventricle was incised to allow efflux of perfusate. This was followed by 20 ml fixative consisting of 2% glutaraldehyde, 2% paraformaldehyde and 0.1% picric acid buffered in 0.2 M sodium phosphate (pH 7.4). After perfusion, well-perfused lobes of the VP were excised, further fixed for 2–3 h, rinsed in 0.2 M phosphate buffer (pH 7.4), dehydrated in graded concentrations of ethanol and embedded in Spurr's resin. One-micron semi-thin sections were cut by an ultramicrotome (Reichert-Jung, Austria), stained with 1% toluidine blue and studied under a light microscope (Zeiss).

Stereology

All stereologic estimations were done on histologic sections of the VP using an image analyzer (Kontron Elektronik Imaging System KS-400, Munchen, Germany). Images were captured live on to the screen from sections under a light microscope (Zeiss) with an affixed video camera (Sony, DXC 3000P, Japan) and observed at $\times 20$ objective. Calibration of images was done using a stage micrometer.

Binary images, for measurement, were generated by color thresholding (for stroma) or by interactive contour drawing (for glandular epithelium and lumen). The final stage of analysis was the extraction of quantitative information from images. The following parameters were evaluated on the basis of area measurement-volume densities (percentage of tissue volume occupied by the defined tissue compartment) (Weibel *et al.* 1966, Weibel 1974, Bartsch *et al.* 1979, Rohr & Bartsch 1980, Marks *et al.* 1994) of glandular epithelium, stroma and lumen. The absolute values for each prostatic compartment were obtained as the product of its volume density by the prostatic weight. The diameters of glands, their lumen and epithelial height were estimated by measuring the diameter of their circle with equivalent area.

Statistical analysis

Results are presented as means $\pm$ S.E.M. The presence of statistically significant differences among the various

treatment groups was determined using one way analysis of variance with suitable *post-hoc* linear contrasts. Analysis of stereologic data was done using JMP statistical software (SAS Institute Inc., Cary, NC, USA). A P value of <0.05 was taken to indicate statistical significance.

Results

Body and organ weights

Body weights in mice were significantly increased with EB and EB+TAM ($P<0.05$) while other groups were non-significantly different from controls (data not shown).

Intact SV weights decreased by 53 and 29% with GnRH-A and DES respectively ($P<0.05$), increased 26% with EB ($P<0.05$) and were unaffected by other treatments compared with controls.

SV (empty) weights remained unchanged with DES or ICI, increased by 27% (DES+TAM, EB), 33% (EB+TAM) ($P<0.05$) and decreased by 54% with GnRH-A treatment versus controls ($P<0.0002$, Fig. 1).

DES decreased, whereas EB increased SV fluid compared with controls. TAM or ICI alone or with estrogens had no significant effect on fluid secretion compared with controls while GnRH-A significantly reduced ($P<0.005$) fluid secretion. Epididymal weights were reduced by 41% with GnRH-A compared with controls but remained unchanged with other treatments (Fig.1).

Testosterone levels T levels in control mice were 10.9 ± 7.4 nmol/l. T levels (nmol/l) in various groups were 11.5 ± 6.8 (DES), 9.8 ± 3 (DES+TAM), 14.2 ± 5.7 (EB), 15.8 ± 8.9 (EB+TAM), 14.5 ± 10.4 (TAM), 15.6 ± 8.1 (GnRH-A), 9.7 ± 5.7 (DES+ICI), 2.64 ± 1.4 (EB+ICI), 3.62 ± 1.3 (ICI).

Ventral prostate

Weights VP weights decreased by 41.6% (GnRH-A), 21% (DES), 38% (DES+ICI), 29% (TAM), 36% (EB+ICI, ICI) ($P<0.05$) whereas other groups showed non-significant reductions compared with controls (Fig. 2).

Branching morphogenesis A single neonatal dose of DES or EB with or without anti-estrogens significantly reduced branch tip numbers compared with controls (Fig. 2) ($P<0.0001$). DES reduced ductal tips by 37% (vs controls) and by 25% (vs EB & DES+TAM) and 19% (vs TAM) treatments. Other treatment groups (DES+TAM, EB, EB+TAM, TAM, ICI, DES+ICI, EB+ICI) demonstrated a significant decline in ductal branching (18, 16, 24, 22, 29, 44 and 22% respectively, $P<0.05$) in comparison with controls. GnRH-A treatment demonstrated a significant decline (43%, $P<0.0001$) in ductal tip numbers compared with controls.

Figure 1 Weights of SV with (top left) and without (top right) secretions (mg/g body weight), SV fluid (mg) (lower left) and epididymis (lower right) with various neonatal hormonal treatments. Error bars represent S.E.M. * $P<0.05$ vs control.

Prostate morphometry

Qualitative The normal prostate gland was organized into an extensive irregular epithelial ductal network embedded in connective tissue stroma. This ductal system comprised of columnar epithelium and wide lumen. The epithelium that rests upon a basement membrane had centrally or basally placed rounded or irregular nuclei possessing between one and two nucleoli. The stroma consisted of rings of tissue surrounding the epithelium as thin sleeves and also in the extracellular matrix which also exhibits blood vessels, smooth muscle cells and

macrophages (Fig. 3). Treatment with estrogens exhibited prominent reduction in the size of glandular lumen and epithelial hyperplasia. These abnormal effects were more pronounced with DES which induced formation of multilayered epithelial cells, increased stromal cell density and clusters of intraluminal cells. In addition, the nuclei lost their basal orientation following DES exposure. Combined treatment with DES and TAM increased the thickness of the smooth muscle layer surrounding the epithelium and exhibited highly dense stroma with increased cellularity (Fig. 3). In GnRH-A treated mice the

Figure 2 Number of ductal tips per single lobe of VP (left panel) and weights of VP (right panel; mg/g body weight) after neonatal hormonal treatments. Error bars depict S.E.M., * $P<0.05$ vs control.

Figure 3 Light micrographs of cross-sections of VP (1 μ m) in mice following neonatal exposure to (A) DES, (B) DES+TAM, (C) EB, (D) EB+TAM, (E) TAM, (F) GnRH-A and (G) vehicle.

epithelium depicted many enucleate cells while treatment with TAM alone induced localized detachment of smooth muscle layer from epithelium.

Quantitative Diameters of VP were non-significantly decreased by all treatments with the exception of TAM, which reduced diameters by 43% compared with controls ($P < 0.05$, Fig. 4).

Lumen diameters declined by 39.6% (EB), $\approx$ 49% (DES, EB+TAM) and 65% (TAM), 58% EB+ICI, vs controls ($P < 0.001$) whereas other groups were non-significantly different from controls.

Epithelial height increased significantly ($P < 0.001$) with DES, DES+ICI, EB+ICI EB+TAM treatments (Fig. 4) whereas other groups demonstrated non-significant increases over control group.

Figure 4 Bar graphs showing diameters (μm) of VP glandular duct (top), glandular lumen (middle panel) and bar chart depicting epithelial height of glandular duct of VP in μm (bottom panel) in mice administered neonatal hormones. Error bars represent S.E.M., * $P < 0.05$ vs control.

Volume density of epithelium in the VP was increased ($P < 0.01$) with all treatments in comparison with controls and there were no significant differences between treatment groups (Fig. 5). However, in terms of absolute values (mg/g body weight), epithelium was increased 30, 50 and 40% with EB, EB+TAM, DES+TAM respectively, ($P < 0.001$, Fig. 5).

Lumen (both in volumetric and in absolute terms) was significantly reduced with all treatments compared with controls ($P < 0.001$, Fig. 5).

Volume density of stroma was increased by 61% (EB) and 57% (DES+TAM and DES) over controls and

declined by 27% with GnRH-A treatment (Fig. 5). In absolute terms, the stromal component of VP was increased with DES, DES+TAM, EB and was 37% of controls with GnRH-A (Fig. 5).

Discussion

In mammals, the perinatal period of development is sensitive to hormonal disturbance and the importance of prenatal influences on events and pathology of late-life have been increasingly recognized. For example, Barker's fetal origins hypothesis (1995) suggests that many degenerative diseases of late-life including cardiovascular disease and diabetes have their basis in fetal growth and nutrition. Prenatal influences on human reproductive function have also been identified with prenatal exposure to DES resulting in developmental disorders of the female (Herbst *et al.* 1971) and male reproductive tract (Wilcox *et al.* 1995). Similarly, in rodents, developmental events in fetal life appear to influence late-life functions and morphology, like induction of testicular (Newbold *et al.* 1987) or uterine tumors (McLachlan *et al.* 1980) and differentiation defects in SVs of mice exposed to DES prenatally (Newbold *et al.* 1989). In addition, exposure to low sex steroid concentrations due to fetal position *in utero*, may modify structure and function of the mature prostate (vom Saal 1981, 1989). Developmental exposure to DES evokes long-term effects in a wide range of tissues such as disruption of reproductive function in adult rats (Khan *et al.* 1998), induction of long-range changes in the uterine target cells (Newbold *et al.* 1990) and alteration of bone density in female mice (Migliaccio *et al.* 1992, 1995). Neonatal estrogenization also increases prostatic proto-oncogene expression (Salo *et al.* 1997) and decreases cytosolic estrogen (Turner *et al.* 1989) and prolactin (Edery *et al.* 1990) receptor levels in rodent VP. Based on these experimental findings, this study was undertaken to identify long range effects of neonatal estrogen imprinting on the morphology of the mature mouse prostate. The structural modifications in the prostate were studied in terms of quantitative changes of its tissue components.

A single neonatal dose of estrogens demonstrated prominent effects on the glandular architecture and anatomic composition of the mature prostate. Both DES as well as EB demonstrated regressive effects on branching morphogenesis in the presence or absence of anti-estrogens. This reduction in branching resulted in a corresponding decline in VP sizes and caused persistent changes in the volumetric composition of the prostate. Although ductal tips with DES were significantly reduced compared with EB treatment, VP weight was unaffected owing partially due to increased epithelial width or fibromuscular stroma with DES treatment. Similar reductions in prostate size and alterations in the prostatic composition have also been reported by perinatal exposure

Figure 5 Bar graphs showing % volume occupied by epithelium (top left), lumen (mid-left) and stroma (lower left) in the VP of treated mice (* $P < 0.05$ vs control). Bar graphs representing absolute weights (mg/g body weight) of epithelium (top right), luminal fluid (mid-right) and stroma (lower right) in VP of treated mice. Error bars represent S.E.M.

to DES or estradiol in rodents in earlier studies (Chung & MacFadden 1980, Higgins *et al.* 1981, Naslund & Coffey 1986, Pylkkanen *et al.* 1991). These long-term alterations might be due to differentiation defects induced in prostatic cells as a result of neonatal estrogen exposure that results in their abnormal pattern of growth later in life (Weinberg 1991).

Neonatal estrogens decreased luminal widths suggesting reduced secretory output and also induced epithelial changes as evidenced by cellular hypertrophy indicating their dysplastic potential. Estrogens elicited a marked stimulation of fibromuscular stroma both in proportional and absolute terms, confirming it as being the primary site for estrogen action and implicate complex mesenchymal-epithelial interactions to induce changes at the epithelial level. Similar effects were reported by Pylkkanen *et al.* (1991) who showed localized hyperplasia and dysplasia at distinct sites of the mouse urethro-prostatic complex and increased the expression of the proto-oncogene, *c-myc* in the prostate after neonatal estrogenization. This increase is believed to be associated with increased prostatic dysplasia in mice (Pylkkanen *et al.* 1993) which is the most common possible pre-malignant lesion of the prostate and may represent the initial transformation event in the majority of prostate cancers (McNeal & Bostwick 1986, McNeal 1991).

The greater disruptive potency of DES (compared with EB) on VP histology cannot be readily explained by its estrogenic action alone. In addition to its interaction with the estrogen receptor, DES might mediate some of its effects through another receptor or protein. However, no response to DES was observed in the uteri of estrogen receptor knock-out (ERKO) mice (Korach & Couse 1996) indicating that the estrogen receptor may be the main signaling pathway for the hormonal agonist activity of DES. A recent study of DES and estradiol effects in male hamsters described severe disruption of reproductive function with neonatal DES (Khan *et al.* 1998) which was attributed to lasting damage to the target tissue resulting in aberrant response to normal endocrine signals. Alternatively, DES effects may possibly be mediated by other mechanisms in addition to its interaction with estrogen receptors, like production of toxic metabolites (Korach *et al.* 1978).

GnRH-A administration decreased organ weights, ductal branching, VP size and had no effects on body weights, thereby providing an effective control for indirect effects of estrogen mediated by gonadotropin suppression. However, the significant reduction in epididymal weight with GnRH-A treatment indicates its development as being androgen-dependent. This decline in epididymal weights may be due to elimination of transport of androgen binding protein (ABP) from testis to epididymis as a result of androgen withdrawal (Jean-Faucher *et al.* 1985, Danzo 1995). While estrogens increased SV size and fluid, they did not appear to antagonize epididymal developmental

signal suggesting tissues with common embryologic origin may not share a common critical development period or development signal (Higgins *et al.* 1981).

Both TAM and ICI exhibited significant agonistic effects in this study as evidenced by their failure to inhibit estrogen-induced prostate regression. When administered on their own, they decreased the ductal and luminal size both in relative and absolute terms. TAM is the most widely used estrogen antagonist (Jordan & Murphy 1990) and its biologic activity ranges from full estrogen antagonism to partial agonism in different tissues and varies according to the prevailing estrogenic environment (Baker & Jaffe 1995). For example, in a rat prostate cancer model, TAM reduced prostate weights and induced significant atrophy of the gland (Miyata *et al.* 1997). Estrogen-like partial agonist effects of TAM include development of endometrial carcinoma (Barakat 1995) and slower loss of bone density in women being treated for breast cancer (Love *et al.* 1991) or in ovariectomized rats (Moon *et al.* 1991). On the other hand, ICI has been shown to be a more potent anti-estrogen with no intrinsic agonist activity (Wakeling *et al.* 1991). In animal models with human breast cancer xenografts, ICI provided more effective and durable regression than with TAM (Osborne *et al.* 1995). In addition, TAM manifested mixed effects while ICI antagonized estradiol effects on the CaBP9k gene expression in myometrial cells and intact rat uterus (Blin *et al.* 1995). However, in the present study, ICI was unable to exert estrogen blocking effects. While most synthetic anti-estrogens demonstrate agonistic or antagonistic activity depending upon the tissue and endogenous estrogen milieu, the results here support equally agonistic properties of both TAM and ICI on VP morphology in this strain of mice. However, there is a possibility of TAM acting as an anti-androgen, which might explain its effects in the present study.

The findings suggest that estrogens produce long-term effects on the structural organization of the mature mouse prostate and have both direct (stimulatory) as well as indirect (inhibitory) effects on the VP. In addition, both TAM and ICI act as partial agonists to the estrogen receptor in the setting of the immature male animal with its low background concentrations of endogenous estrogens.

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