Altered Gene Expression during Rat Wolffian Duct Development in Response to in Utero Exposure to the Antiandrogen Linuron

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Linuron is an herbicide with weak androgen receptor (AR) antagonist activity. Exposure to linuron from gestation days (GD) 12 to 21 perturbs androgen-dependent male reproductive development. In utero exposure to 50-mg/kg/day linuron induces malformations of the epididymis and the vas deferens. The objective of this study was to identify alterations in gene expression within the testis and epididymis associated with abnormal Wolffian duct development and to correlate changes in gene expression with the gross morphology of the affected epididymides. Pregnant Sprague-Dawley rats were administered either corn oil vehicle or linuron (50 mg/kg/day) by gavage from GD 12 to 21 (n = 3–6 controls, n = 5–10 linuron-treated dams per time point). Changes in gene expression were evaluated in testes on GD 21 and in epididymides on GD 21 and postnatal day (PND) 7, using cDNA microarrays and confirmed by real-time reverse transcriptase polymerase chain reaction (RT-PCR) analyses. RNA was isolated from intact epididymides with reduced or no ductal coiling from the linuron groups, and epididymides with noncontiguous ducts were excluded. In the fetal testis, exposure to linuron did not result in reduced mRNA expression of the AR or that of several steroidogenic enzymes, supporting the hypothesis that linuron does not reduce fetal testosterone production. Linuron induced a significant decrease in AR mRNA expression in GD 21 epididymides. Significant changes in mRNA expression in GD 21 and PND 7 epididymides were also identified in the epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), bone morphogenetic protein (BMP), fibroblast growth factor (FGF), and Notch signaling pathways. These pathways are involved in tissue morphogenesis. Changes in the expression of AR and IGF-1 receptors were detected by immunostaining in malformed epididymides from linuron-exposed rats. Linuron induced changes in epididymal gene expression suggestive of altered paracrine interactions between the mesenchyme and epithelial cells during development. The EGF, Notch, IGF-1, BMP4, and FGF signaling pathways may be involved in normal testosterone-mediated development of the Wolffian duct.

Key Words: linuron; androgen receptor antagonist; Wolffian duct development; epididymis.

Linuron is a commonly used pre- and postemergence herbicide applied to suppress broadleaf and grassy weeds (U.S. EPA, 1995). Studies have demonstrated that linuron is a weak competitive androgen receptor (AR) antagonist in vitro and that prenatal exposure induces dose-dependent alterations in androgen-mediated reproductive development in male rats (McIntyre et al., 2000). Furthermore, in utero exposure to 50 mg/kg/day linuron and higher dose levels induces epididymal and testicular malformations (Lambright et al., 2000; McIntyre et al., 2000, 2002a). Thus demonstrating that linuron preferentially impairs testosterone-mediated, rather than dihydrotestosterone (DHT)-mediated, reproductive development. The primary targets of linuron-induced toxicity appear to be the epididymis and vas deferens (McIntyre et al., 2002a). Linuron-induced epididymal malformations caused obstruction of testicular fluid outflow, which resulted in testicular atrophy (McIntyre et al., 2002a). On postnatal day (PND) 7, approximately 21% of the male offspring exposed to 50 mg/kg/day linuron in utero displayed epididymides that were malformed as evidenced by decreased number of ductules or absence of the head, body, or tail (McIntyre et al., 2002b). In contrast, no testicular lesions were observed during late gestation and early postnatal life. Intratesticular and serum testosterone levels were not decreased in the linuron-exposed male fetuses on gestation days (GD) 17, 19, and 21 (McIntyre et al., 2002b). Since linuron is a weak AR antagonist but does not alter fetal testosterone production, we hypothesize that linuron disrupts the development of the Wolffian duct by inhibiting AR signaling.

Testosterone, rather than DHT, is required for stabilization and differentiation of the Wolffian duct (George and Wilson, 1994). Fetal rat testes begin synthesizing testosterone around GD 15.5, and production becomes markedly elevated around GD 19.5, which coincides with the time when testosterone is required for stabilization of the Wolffian ducts (Warren et al., 1994).
growth factor expression by androgens mesenchyme to the epithelium. However, direct regulation of epidemic and fetal rat have shown that during normal development of the Wolffian duct, the AR is initially expressed in the mesenchyme cells surrounding the developing Wolffian duct, with subsequent induction of expression within the epithelial cells around GD 18 (Bentvelsen et al., 1995). Elegant tissue recombination experiments have demonstrated that it is the mesenchyme that determines the morphologic and functional fate of the overlying epithelium (Cunha et al., 1992). For example, recombination of seminal vesicle mesenchyme with embryonic upper Wolffian duct reprograms differentiation of the duct to become a seminal vesicle rather than an epididymis (Higgins et al., 1989). Growth factors and components of the extracellular matrix have been proposed to mediate signaling from the mesenchyme to the epithelium. However, direct regulation of growth factor expression by androgens in vivo has yet to be demonstrated unequivocally.

The objective of this study was to identify alterations in gene expression within the testis and epididymis associated with abnormal Wolffian duct development and to correlate changes in gene expression with the gross morphology of the affected epididymides. Since linuron is an AR antagonist, we hypothesize that epididymal malformations are the result of linuron-induced attenuation of AR signaling. To date, 17 types of signal transduction pathways have been identified in animals. These pathways are used repeatedly in various combinations and at different times and locations in the developing embryo and fetus (Gerhart, 1999). Signaling pathways are of critical importance for all aspects of development since they regulate many cellular responses including gene expression, protein secretion, cell proliferation, differentiation, migration, and apoptosis (Gerhart, 1999). These pathways have been conserved through evolution and are potential targets for disruption by toxicants; therefore we were especially interested in determining whether linuron exposure altered the expression of components of cell signaling pathways. Pregnant rats were treated with linuron (50 mg/kg/day) from GD 12 to 21, since this has been shown in previous studies to maximize the incidence of epididymal abnormalities with minimal maternal toxicity (McIntyre et al., 2000; 2002a,b). Gene expression was analyzed in testes and epididymides obtained from fetuses on GD 21 and from epididymides removed from pups on PND 7 using microarrays and real-time reverse transcriptase polymerase chain reaction (RT-PCR) analyses. Immunohistochemistry was used to evaluate whether alterations in AR and insulin-like growth factor-1 receptor (IGF-1R) mRNA expression correlated with changes in a distribution or intensity of expression of the protein. The changes in epididymal gene expression were linked to abnormal differentiation of the upper Wolffian duct.

**MATERIALS AND METHODS**

**Animals.** This study was conducted in accordance with Federal guidelines for the care and use of laboratory animals (National Research Council, 1996) and was approved by the Institutional Animal Care and Use Committee of the CIIT Centers for Health Research (CIIT). Time-mated, 8- to 10-week-old, nulliparous Crl:CD(SD)BR rats were obtained from Charles River Laboratories Inc. (Raleigh, NC) on GD 0, defined as the day sperm was found in the vagina of the mated female. Animal allocation to treatment groups was done by body-weight (bw) randomization. Animals were housed in a HEPA-filtered, mass-air-displacement room with a 12-h light-dark cycle at 18–26°C and relative humidity of 30–70% in the CIIT animal care facility, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. Animals had access to deionized water and rodent chow ad libitum (NIH-07, Zeigler Brothers, Gardner, PA). Individual dams and offspring were housed in polycarbonate cages on ALPHA-dri bedding (Shepherd Specialty Papers, Kalamazoo, MI).

**Treatment.** For each treatment block, dams were gavaged daily (between 0800 and 0900 a.m.) from GD 12 to 21 with either corn oil (Sigma Chemical Co. St. Louis, MO) or linuron (Chem Service, Inc., West Chester, PA) in corn oil at 50 mg/kg/day at a volume of 2 ml/kg/day. This dose level of linuron was selected based on previous studies in which 50 mg/kg/day linuron administered to pregnant rats from GD 12 to 21 resulted in limited maternal toxicity (decreased weight gain during treatment without alteration of organ weights or any litter parameters) but induced epididymal abnormalities with an approximate incidence of 25% (McIntyre et al., 2002). Dams were examined daily for clinical signs of toxicity.

**Study design.** The tissues analyzed in the present study were obtained from three separate blocks of animals. In the first block, six control dams were gavaged with corn oil, and six dams were treated with 50 mg/kg/day linuron. All the dams were euthanized on GD 21 by CO₂ asphyxiation and exsanguination, and the fetuses were removed. The epididymides and testes were removed from the male fetuses and inspected under a dissecting microscope with transillumination. The gross morphology of the testes and epididymides from these fetuses was recorded. These tissues were then separated and snap-frozen in liquid nitrogen for use in the molecular analyses. A second block of pregnant rats, four control and five linuron-treated dams, was treated so that macroscopic images of the testes and epididymides on GD 21 could be captured and the morphology recorded. These tissues were left intact and fixed in neutral-buffered formalin for 7 h for immunohistochemistry. The molecular analyses and immunohistochemistry performed on epididymides removed from pups on PND 7 utilized tissue collected in a previous study (McIntyre et al., 2002b). This block included three control dams and 10 dams treated with linuron. The right testes and epididymides were separated and snap-frozen in liquid nitrogen. The left testes and epididymides were fixed individually in 10% neutral-buffered formalin and then processed and embedded in paraffin for immunohistochemistry.

**DNA array analysis.** Total RNA was isolated from testes on GD 21 and epididymides on GD 21 and PND 7 using STAT-60™ (Tel-Test, Inc., Friendswood, Texas). Epididymides were pooled from 3–4 pups from the same litter to make separate pools of epididymides that showed decreased versus no coiling. Epididymides with noncontiguous ducts were excluded from the
molecular analyses, as the absence of tissue would have complicated interpretation of the biological relevance of decreases in gene expression. Tests were pooled from the same pups as the epididymides. The quality of the RNA was evaluated using a spectrophotometer and gel electrophoresis. The total RNA was DNase-treated with RNase-free DNase (Roche, Indianapolis, IN) at 37°C for 60 min to ensure the absence of genomic DNA. Reverse-transcription (RT) reactions were performed using 3.5 μg of total RNA, [32P]-dATP, and SuperScript-II MMLV reverse transcriptase (Gibco BRL, Gaithersburg, MD) for 60 min at 50°C. Following purification, probes were added to each Clontech (Palo Alto, CA) Atlas Rat Toxicology 1.2 cDNA expression array (1279 genes), with hybridization and washing performed according to the manufacturer’s instructions. Arrays were placed on a phosphor screen for 2–4 days and visualized using a PhosphorImager™ SI (Molecular Dynamics, Amersham Biosciences Corp, Piscataway, NJ). These images were imported into AtlasImage software (Clontech) for quantification. These data were then imported into GeneSpring (Silicon Genetics, Redwood, CA) for analysis. Array analysis was performed on three to four samples (representing at least three individual litters) per treatment group. During analysis of the arrays within GeneSpring the raw data was normalized by gene and by array, and genes with a signal below 10 within the control group were excluded from the analysis to prevent inclusion of data close to the background signal of the arrays. To obtain lists of genes that showed significantly different expression levels between the control and lirunron groups, a Welch t-test (variances not assumed equal) was performed without a multiple testing correction. Lists were also generated showing genes that were twofold up- or downregulated by linuron exposure.

**Real-time RT-PCR.** Changes in gene expression were confirmed using real-time RT-PCR on the GeneAmp® 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). Total RNA (1 μg) from each tissue pool was aliquoted in quadruplicate for RT using TaqMan® RT Reagents (Applied Biosystems) and SuperScript-II reverse transcriptase (Gibco BRL), with one aliquot designated to receive no enzyme. Quality of the RT reactions was confirmed by comparison of triplicate RT versus no enzyme control for each RNA sample, using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer set according to manufacturer’s protocols (Applied Biosystems). PCR was performed using SYBR® Green PCR Core Reagents (Applied Biosystems) according to manufacturer’s instructions, using a reaction volume of 25 μL. Primer sets were selected by Primer Express software. Production of a single PCR product was confirmed using gel electrophoresis, and primer efficiency was determined according to manufacturer’s recommended protocol (Applied Biosystems). Sequences of gene primer sets are given in Table 1. Quantitation of P450 side chain cleavage (P450ccc) and 17αhydroxylase, c17,20 lyase (17αOH) were performed using TaqMan® probes in conjunction with the primers listed in Table 1, according to the manufacturer’s instructions (Applied Biosystems). The probe sequences for GAPDH, P450ccc, and 17αOH (5’ to 3’) were CCGCCTGGAGAAACCTGCAAATTG, CCAGCGGTTCATC-GACACGCCG, and CCTACCTGATCTGATTCTCCCTT, respectively. A standard curve was generated by making appropriate dilutions of a sample of cDNA, synthesized from each individual RNA from control fetuses. The samples were quantitated using values calculated from the standard curves for the gene- and the GAPDH-specific primers. Real-time RT-PCR and Taqman® analyses were performed in triplicate on at least 4 samples per treatment group, representing a minimum of three individual litters per time point. PCR reactions for the mRNA of interest were performed in parallel with reactions using GAPDH specific primers to allow standardization across the samples for cDNA input. Relative quantitation of gene expression was performed according to User Bulletin #2 supplied by Applied Biosystems.

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*Note. Te, testis; Epi, epididymis (including both GD 21 and PND 7 samples).*
**RESULTS**

**Effect of Linuron on Dams and Reproductive Performance**

Dams treated with linuron showed some signs of toxicity based on decreases in body weight on GD 21 and in body weight gain over the 10-day dosing period (GD 12 to 21) when compared to vehicle-treated control animals (Table 2). The blocks were analyzed individually since the total number of pups born to each dam was not recorded for block 1 and thus could not be used as a covariate. In addition, there were significant differences in the control-dam body weight on GD 21 among the blocks. Body weight on GD 21 was decreased by 10% in the three blocks of linuron-treated dams relative to the corresponding control group. However, the difference in body weight on GD 21 between the control and linuron-treated dams reached statistical significance only in the third block of dams. Body weight gain was significantly decreased by 35% in the dams in blocks 1 and 3, relative to the corresponding control group. Body weight gain in the second block of linuron-treated dams was decreased by 25% but did not attain statistical significance. Gestational exposure did not delay parturition or alter litter size and sex ratio (data not shown) in agreement with previous studies performed within our group (McIntyre et al., 2000, 2002a,b).

Male fetuses exposed to linuron from GD 12 to 21 exhibited a small decrease in fetal weight on GD 21 (Table 2). This
reached statistical significance only in the fetuses from the dams in block 2. Testes and epididymides from these fetuses were fixed for immunohistochemistry and not utilized in the molecular analyses. Pup weights on PND 7 were also decreased by approximately 6% in the linuron-exposed group, but did not reach statistical significance (Table 2).

**Effect of Linuron on the Gross Morphology of the Developing Epididymis**

The morphology of epididymides was noted during dissection of the fetal and postnatal male offspring. Representative images of testes and epididymides on GD 21 from a control fetus and from three linuron-exposed fetuses (three different litters) are shown to illustrate the different morphologies that were observed using a dissection microscope with transillumination (Fig. 1). Approximately half the linuron-exposed fetuses possessed developing epididymides that showed no apparent differences in morphology in comparison with the controls (Figs. 1A and 1B; Table 2). However, prenatal exposure to linuron resulted in 16–30% of fetuses with epididymides that displayed reduced ductal coiling in comparison with controls (Fig. 1C; Table 2). In addition, linuron exposure induced a 20% incidence of epididymides that had an incomplete duct or the duct showed no visible coiling on GD 21 (Fig. 1D; Table 2). This incidence of epididymal malformations is consistent with that noted on PND 7 (Table 2) and in previous studies where necropsies were performed on PND 14, 35, 56, and 100 following *in utero* exposure to 50 mg/kg/day linuron (McIntyre et al., 2000; 2002a,b).

**Analysis of Gene Expression**

Microarray analysis was performed utilizing GeneSpring software. Gene lists showing genes with significantly different expression levels between the control and linuron groups were generated by performing a Welch *t*-test with no multiple testing correction within the GeneSpring analysis software. Gene lists with corresponding information on the fold change and direction of change are shown in the supplemental data associated with this article. Since the analysis only made use of three to four arrays per treatment group at each time point, the statistical analysis of the gene expression data was not considered sufficiently quantitative to provide a definitive assessment of alterations in gene expression. However, the arrays provided a useful guide for the selection of other mRNAs to quantify, besides those indicated by the literature on the role of androgens in the development of the male reproductive tract. Thus, the real-time RT-PCR data discussed in the subsequent sections of the results is considered to be the most accurate representation of the alterations in gene expression induced by exposure to linuron. All the mRNAs that were quantified during this investigation are listed in Table 1. This table shows whether the mRNAs were selected for quantification based on the array data or from information in the literature, and also whether real-time RT-PCR was performed on GD 21 testes.
and/or GD 21 and PND 7 epididymides. It should be noted that if an mRNA was selected for quantification at one of the epididymal time points it was automatically quantified at the other time point. Thus, it was possible to assess whether a change in expression at GD 21 was maintained through to PND 7 or occurred following the cessation of treatment with linuron. In addition, when a change in mRNA expression was identified by real-time RT-PCR, primers were designed to the corresponding ligand or receptor to further substantiate a potential change in the signaling pathway.

**Linuron Exposure and Testicular mRNA Expression**

Changes in testicular mRNA expression were suggested for a few genes (24/1176) by the array data (see supplemental material for a list of the genes). The following mRNAs were selected from the gene list for confirmation by real-time RT-PCR (Table 1): follicle-stimulating hormone receptor (FSHR), the transcription factor GATA-4, insulin-like growth factor binding protein 6 (IGFBP6), and bone morphogenetic protein 4 (BMP4); however, real-time RT-PCR analysis was unable to confirm any alterations in gene expression between control and linuron samples (data not shown). It was not possible to design primers that could accurately quantify the following mRNAs that also showed changes in expression based on the array analysis: activin receptor, inhibinβA, thyroid stimulating hormone receptor, and the 5-hydroxytryptamine receptor 1B. Since linuron is a weak AR antagonist, AR mRNA expression was quantified. EGFR and IGF-1R were selected as they represented growth factor signaling pathways relevant to the development of the male reproductive tract. The steroidogenic enzymes 3β-hydroxysteroid dehydrogenase (3βHSD), P450 side-chain cleavage (P450scc), or 17α-hydroxylase/C17,20 lyase (17αOH) were quantified because of suggestions that linuron might inhibit testosterone synthesis (Lambright et al., 2000). Prenatal linuron exposure did not alter mRNA expression of AR, EGFR, and IGF-1R or of the steroidogenic enzymes 3βHSD, P450scc, or 17αOH within the GD 21 testes (data not shown). Thus, real-time RT-PCR was unable to identify any alterations in the expression of 10 mRNAs (Table 1) that had the potential to be modulated by androgens.

**Effect of Linuron on Androgen Receptor and Epidermal Growth Factor Receptor mRNA Expression in Epididymides**

In contrast to the fetal testis, quantitation of AR expression by real-time RT-PCR demonstrated that this mRNA was significantly reduced by 45% in the epididymides of fetuses exposed to linuron in comparison with the control group (Fig. 2A). This decrease in expression was not observed in the epididymides removed from pups on PND 7 that were exposed to linuron in utero. Quantitation of EGFR expression showed that the steady-state levels of this mRNA were significantly decreased in epididymides on GD 21 by 36% (Fig. 2A). On PND 7, EGFR mRNA expression was decreased by 22% and approached statistical significance (p = 0.068; Fig. 2B). Com-

![FIG. 2. Effect of prenatal linuron exposure from gestation day (GD) 12 to 21 on androgen receptor (AR) and epidermal growth factor receptor (EGFR) epididymal mRNA expression on GD 21 (A) and PND 7 (B). Total RNA from fetal and postnatal epididymides was subjected to real-time RT-PCR analysis. The results shown represent the relative change in expression compared to the control group where the mean expression is assumed to be 100%. The data presented are % means ± SE, where n = 4 control samples (4 litters) and n = 7 linuron-exposed samples (6 litters) on GD 21 and n = 5 control samples (3 litters) and n = 6 linuron-exposed samples (6 litters) on PND 7; *p < 0.05 was significantly different from the corresponding control groups.]
parison of the GD 21 and PND 7 arrays hybridized with epididymal samples did not indicate that AR and EGFR were altered in expression between the control and linuron groups. Several attempts were made to design primers that could accurately quantitate the expression of EGF but these proved unsuccessful.

**Effect of Linuron on mRNA Expression of Delta Like and the Notch2 Receptor in Epididymides**

Comparison of the arrays hybridized with epididymal samples from GD 21 fetuses indicated that the mRNA for a gene known as delta like (Dlk) was markedly upregulated in the linuron group. The Notch2 receptor, a potential target for Dlk, showed a small decrease in expression on the linuron arrays. The array results were confirmed by real-time RT-PCR such that Dlk mRNA expression was significantly increased, relative to the control group, by 253%, and Notch2 was decreased by 40% (Fig. 3A). No difference in their expression was apparent on the PND 7 arrays when compared to controls, and was supported by the PCR results (Fig. 3B).

**Effect of Linuron on Insulin-Like Growth Factor-1 Receptor mRNA Expression in Epididymides**

Array analysis suggested that mRNA expression of IGF-1 was increased in the epididymides from linuron-exposed rats on GD 21 and that IGF-1R mRNA expression was increased on PND 7. A change in IGF-1R expression was confirmed by real-time RT-PCR, although the direction of the change in expression was found to be opposite to that indicated by the arrays. IGF-1R mRNA expression was significantly decreased relative to the control group on GD 21 and PND 7 by 42% and 32%, respectively (Fig. 4). However, IGF-1 mRNA expression was not significantly altered at either time point (Fig. 4). IGFBP5 mRNA expression was significantly reduced in the linuron group by 38% in comparison to the controls (Fig. 4B). No change in IGFBP5 expression was detected by the arrays or by PCR on GD 21, where as the expression of IGFBP5 appeared to be increased relative to the control samples on the PND 7 arrays.

**Effect of Linuron on mRNA Expression of Bone Morphogenetic Protein 4 and One of Its Receptors in Epididymides**

Comparison of the arrays hybridized with GD 21 epididymal samples indicated that the mRNA expression of BMP2 and BMP4 were decreased in the linuron group. Real-time RT-PCR confirmed that steady-state levels of these two mRNAs were significantly decreased relative to the control group by 45 and 34%, respectively (Fig. 5A). There was no indication from the array analysis that BMP2 and BMP4 mRNA expression was altered on PND 7. However, real-time PCR showed that BMP4 mRNA expression was significantly reduced by 43% in epididymides in the linuron group (Fig. 5B). Since BMP4 mRNA expression was altered at both GD 21 and PND 7, primers were
designed to quantitate the expression of the two receptors that bind BMP4, BMPR-1A (also known as ALK3), and BMPR-1B (also known as ALK6) (ten Dijke et al., 1994). Real-time PCR showed that expression of BMPR-1B was significantly decreased relative to the controls on both GD 21 and PND 7 by 70 and 39%, respectively (Fig. 5). BMPR-1A mRNA expression was not altered at either time point.

FIG. 4. Effect of prenatal linuron exposure from gestation day (GD) 12 to 21 on the epididymal mRNA expression of insulin-like growth factor-1 receptor (IGF-1R), and insulin-like growth factor–1 (IGF-1), and insulin-like growth factor binding protein 5 (IGFBP5) on GD 21 (A) and PND 7 (B). Total RNA from fetal and postnatal epididymides was subjected to real-time RT-PCR analysis. The results shown represent the relative change in expression compared to the control group where the mean expression is assumed to be 100%. The data presented are % means ± SE, where n = 4 control samples (4 litters) and n = 7 linuron-exposed samples (6 litters) on GD 21 and n = 5 control samples (3 litters) and n = 6 linuron-exposed samples (6 litters) on PND 7; *p < 0.05 was significantly different from the corresponding control group.

FIG. 5. Effect of prenatal linuron exposure from gestation day (GD) 12 to 21 on the epididymal mRNA expression of bone morphogenetic protein 2 (BMP2), BMP4, the BMP-1A receptor (BMPR-1A), and the BMP-1B receptor (BMPR-1B) on GD 21 (A) and PND 7 (B). Total RNA from fetal and postnatal epididymides was subjected to real-time RT-PCR analysis. The results shown represent the relative change in expression compared to the control group where the mean expression is assumed to be 100%. The data presented are % mean ± SE, where n = 4 control samples (4 litters) and n = 7 linuron-exposed samples (6 litters) on GD 21, and n = 5 control samples (3 litters) and n = 6 linuron-exposed samples (6 litters) on PND 7; *p < 0.05 was significantly different from the corresponding control group.
Effect of Linuron on mRNA Expression of Fibroblast Growth Factor Receptor 2, Glypican, and Tissue Inhibitor of Metalloproteinases 3 in Epididymides

To evaluate whether mRNA expression of members of the fibroblast growth factor (FGF) signaling pathway were altered during perturbation of epididymal development by linuron, attempts were made to design primers to FGFR2, as well as FGFs 2, 7, and 10. Suitable primers were designed to allow quantitation of FGFR2 and FGF10. The primers to FGFR2 recognized the iiib variant, which is expressed in epithelial cells, and the iiic variant, which is expressed in mesenchyme cells (Ornitz et al., 1996). Real-time RT-PCR demonstrated that FGFR2 mRNA expression was significantly reduced by 34% in epididymides in the linuron group on PND 7 but not on GD 21 (Fig. 6). No alteration in FGF10 mRNA expression was detected at either time point (data not shown). FGFR2 was not present on the arrays, and the expression of FGFs 2, 7, and 10 was variable between arrays from the same treatment group.

The PND 7 array comparisons indicated that glypican1, a heparan sulfate glycoprotein expressed on the cell surface, was upregulated in epididymides from the linuron group. Real-time RT-PCR showed that glypican mRNA expression was significantly decreased in the epididymides from the pups exposed to linuron in utero on GD 21 and PND 7 by 32 and 27%, respectively (Fig. 6).

Array analysis on PND 7 showed that mRNA expression of tissue inhibitor of metalloproteinases 3 (TIMP3) and matrix-metalloproteinase 16 (MMP16) were upregulated in the linuron group. Real-time RT-PCR demonstrated a significant reduction (33%) in TIMP3 mRNA, whereas expression of MMP16 showed no change on PND 7 (data not shown, Fig. 6B). No alterations in TIMP3 and MMP16 expression were observed on GD 21 by real-time RT-PCR. Although no change in TIMP3 expression was indicated by the arrays at this time point, the arrays did suggest that MMP16 was decreased relative to the control group.

Since many of the receptors analyzed above are expressed in epithelial cells, it was important to determine whether there were any changes in the relative amounts of the epithelial and mesenchymal tissue compartments as a result of exposure to linuron. Cytokeratin 8 mRNA was selected as a marker of the epididymal epithelium since it is expressed within the epithelial cells of the Wolffian duct (Sainio et al., 1997). Fibronectin mRNA expression was also quantitated since fibronectin is a component of the basement membrane that is synthesized by stromal cells; changes in fibronectin levels are associated with epithelial proliferation (Woodward et al., 2001). The epididymal expression of these two mRNAs in the linuron group was not altered relative to the controls at either time point, suggesting that the changes in steady-state mRNA levels described above are not the result of a marked change in the ratio of epithelial to mesenchymal cells (data not shown).

Immunolocalization of Androgen Receptor and IGF-1 Receptor

Immunohistochemical localization of AR within epididymides from control fetuses confirmed previous reports that the

FIG. 6. Effect of prenatal linuron exposure from gestation day (GD) 12 to 21 on the epididymal mRNA expression of fibroblast growth factor receptor 2 (FGFR2), the heparan sulfate proteoglycan, glypican, and tissue inhibitor of metalloproteinases 3 (TIMP3), on GD 21 (A) and PND 7 (B). Total RNA from fetal and postnatal epididymides was subjected to real-time RT-PCR analysis. The results shown represent the relative change in expression compared to the control group where the mean expression is assumed to be 100%. The data presented are % means ± SE, where n = 4 control samples (4 litters) and n = 7 linuron-exposed samples (6 litters) on GD 21, and n = 5 control samples (3 litters) and n = 6 linuron-exposed samples (6 litters) on PND 7; *p < 0.05 was significantly different from the corresponding control groups.
receptor is expressed in the nuclei of both mesenchymal and epithelial cells on GD 21 (Fig. 7A). On GD 21, AR immunostaining in controls was more abundant in the mesenchyme when compared with the epithelium. This pattern of AR immunostaining was altered in malformed epididymides from linuron-exposed fetuses such that AR immunostaining in the ductal epithelial cells was decreased (Fig. 7B). In some fetuses with epididymides showing decreased coiling, the number of AR-positive mesenchymal cells appeared reduced (data not shown); however, the response was variable. On PND 7, immunostaining for the AR in control epididymides was more intense in the ductal epithelial cells in comparison with that seen on GD 21 (data not shown). In addition, the density of mesenchymal cells surrounding the ducts and expressing AR was greatly reduced in comparison to GD 21 epididymides. This pattern of immunostaining was not altered by prenatal exposure to linuron (data not shown). AR immunostaining was absent in sections incubated with antisera preabsorbed with peptide.

On GD 21 and PND 7, immunostaining for the IGF-1R was detected in the cytoplasm of both the ductal epithelial cells and the mesenchymal cells immediately surrounding the ducts (Fig. 7C, E). Immunexpression of IGF-1R was slightly decreased in the mesenchymal cells of malformed epididymides on GD 21 (Fig. 7C, D). This reduction in immunexpression of IGF-1R within the mesenchymal cells was also apparent on PND 7 in the epididymides from the linuron group (Fig. 7E, F). Specificity of the antiserum was confirmed by the absence of immunostaining in sections incubated with antisera preabsorbed with peptide (Fig. 7E, inset).

**DISCUSSION**

The objective of the present study was to identify alterations in gene expression associated with linuron-induced malformations of the epididymis. Since linuron is an AR antagonist, we hypothesized that changes in gene expression would be a result of inhibition of AR signaling. Real-time RT-PCR identified...
changes in mRNA expression in a variety of growth factor signaling pathways that were consistent with a disturbance of paracrine interactions between the mesenchymal and epithelial cells within the Wolffian ducts of linuron-exposed fetuses. Further investigation is required to determine whether these changes in the pattern of gene expression are the direct result of repression of AR-mediated gene transactivation or are secondary events downstream of the AR.

Several studies have demonstrated that prenatal exposure to linuron induces epididymal malformations; however, the incidence was variable such that only 20–40% of male offspring displayed lesions and not all offspring from within the same litter were affected (McIntyre et al., 2000, 2002a,b). This was also observed in the present study such that 20–30% of the male fetuses had epididymal malformations on GD 21 and only half of the litters had affected fetuses. Epididymal malformations have never been observed in control Sprague-Dawley rats during any of the studies that have been performed by our laboratory. Lambright et al. (2000) have shown that administration of a higher dose of linuron (100 mg/kg/day) from GD 14 to 18 resulted in a greater than 50% incidence in epididymal lesions in the adult offspring. This issue of variability in response was not addressed in the present study; however, a study investigating the distribution and metabolism of linuron within the pregnant dam and her male offspring would help to understand this phenomenon. In a previous study performed by our laboratory, no apparent differences in Wolffian duct morphology were observed on GD 17, 19, and 21 in fetuses that had been exposed to 50 mg/kg/day linuron (McIntyre et al., 2002b). Alterations in gross morphology were not observed until PND 7. In the present study, alterations in the morphology of the Wolffian duct indicative of abnormal development were apparent on GD 21. A potential explanation for this discrepancy in incidence of abnormalities is the use of dissection microscopes that employ transillumination, which improves the ability of the dissector to evaluate whether the Wolffian duct is intact and exhibits normal ductal coiling. The variability in incidence of epididymal lesions that has been observed may also contribute to the discrepancy. Androgens are required for stabilization and induction of coiling of the Wolffian duct during GD 17 to 21 in the rat (George and Wilson, 1994). Thus, morphological differences should be apparent by GD 21 if androgen action is inhibited during fetal development. The incidence of epididymal abnormalities observed on GD 21 appeared to be greater than that observed on PND 7. A significant number of epididymides looked underdeveloped (intact with decreased coiling) and it is possible that with the cessation of linuron-exposure that these epididymides may recover during postnatal life. Whereas epididymides displaying no coiling or with noncontiguous ducts will result in the permanent malformations observed in adulthood. However, it is difficult to draw definite conclusions about the ability of underdeveloped epididymides to undergo complete recovery when there is such a variable incidence in epididymal lesions from study to study.

Microarray and real-time RT-PCR analysis of testes from the fetuses with epididymides that displayed decreased or no ductal coiling did not identify any consistent alterations in gene expression in comparison with the control group. This supported the observations of McIntyre et al. (2002b), who demonstrated that fetal testosterone concentration was not reduced by exposure to the 50-mg/kg/day linuron dose from GD 12. Moreover, there was no evidence of testicular pathology in testes examined during late gestation and early postnatal life (McIntyre et al., 2002b). Fetal testicular mRNA expression was altered by prenatal exposure to 50 mg/kg/day flutamide, a more potent AR antagonist than linuron, which downregulated the expression of several genes involved in cholesterol synthesis and enhanced expression of P450scC and 17αOH during late gestation (Shultz et al., 2001). These gene changes occurred in the absence of any alteration in testosterone concentration. Evaluation of the expression of 10 testicular mRNAs by real-time RT-PCR was unable to identify any alterations in expression between the control and linuron samples. Based on the limited data available linuron did not appear to significantly alter testicular gene expression on GD 21, which is in agreement with the hypothesis that the epididymis is the primary target of linuron-induced developmental toxicity (McIntyre et al., 2002a,b).

Linuron exposure disrupts stabilization and differentiation of the upper Wolffian duct into the epididymis, and the most plausible mode of action appears to be attenuation of AR signaling within the Wolffian duct. An alteration in AR signaling during development of the Wolffian duct was supported by the observation that AR mRNA expression was decreased in epididymides on GD 21 following exposure to linuron. Furthermore, AR immunoexpression was decreased in the ductal epithelial cells of severely malformed epididymides and was reduced in the mesenchymal cells of epididymides showing reduced ductal coiling. Epididymal AR immunoexpression has been shown to be modulated by AR antagonists or inhibition of testosterone levels during fetal life (Bentvelsen et al., 1995; Mylchreest et al., 2002). Treatment of pregnant rats with 100 mg/kg/day flutamide from GD 11 to 20 resulted in a complete absence of AR immunostaining in the mesenchyme surrounding Wolffian ducts removed from fetuses on GD 21 (Bentvelsen et al., 1995). Mylchreest et al. (2002) reported that AR immunoexpression was decreased in mesenchymal and epithelial cells of fetal epididymides following flutamide treatment. Gestational exposure to 500 mg/kg/day DBP, an inhibitor of fetal testosterone production, resulted in similar effects on AR immunoexpression as seen with linuron exposure, such that AR immunostaining was absent from the ductal epithelial cells in severely malformed epididymides (Mylchreest et al., 2002). This dose level of DBP did not induce maternal toxicity or any changes in litter parameters.
Several studies have proposed that EGF may be involved in the development of the male reproductive system; thus, the decrease in epididymal EGF mRNAs expression observed in the present study, following linuron exposure, was of note. Cain et al. (1994a,b) demonstrated that epididymal abnormalities induced by flutamide treatment of either pregnant rats or mice could be reversed by the supplementation of EGF during gestation. In vitro studies utilizing mouse genital tract explants have shown that EGF mediated partial masculinization of the Wolffian duct, and that the presence of antisera to either EGF or its receptor inhibits development of the Wolffian duct, even in the presence of testosterone (Gupta, 1996; Gupta et al., 1991). EGF protein has been localized to the epithelial cells of the mouse Wolffian duct and both protein and mRNA expression are reduced by prenatal exposure to flutamide (Gupta, 1997; Gupta and Singh, 1996). The receptors for EGF are also expressed in the epithelium of the Wolffian duct (Bossett et al., 1990). EGF can stimulate androgen-binding activity and enhance androgen-induced transcriptional activity in cells of the mouse reproductive tract in vitro (Gupta, 1999). It has been proposed that activation of the mitogen-activated protein kinase signal transduction cascade by EGF may induce phosphorylation of the AR; this phosphorylation is essential for AR-mediated transcriptional activity (Gupta, 1999).

Alterations in Dlk and Notch2 mRNA expression in fetal epididymides on GD 21 were of interest, since the proteins encoded by these genes are known to be members of a highly conserved signaling pathway that operates between adjacent cells and is important for determining cell fate decisions during development (Artavanis-Tsakonas et al., 1999). Dlk is known to be expressed in embryonic tissues, but its presence in the male reproductive tract has not been reported previously. The expression of Dlk in several tissues correlates with cellular maturation such that differentiation is accompanied by a down-regulation of Dlk expression (Laborda, 2000). The increased steady-state levels of Dlk mRNA expression in epididymides, following linuron treatment, may indicate a delay or block in cell differentiation. The role of Notch2 in mammalian development is not well defined, but Notch2 signaling appears to be essential for normal morphogenesis of several epithelial tissues in Drosophila (for references, see McCright et al., 2002).

Real-time RT-PCR analysis confirmed that components of the IGF-1 signaling pathway in fetal epididymides appeared to be decreased in expression following linuron exposure. The IGF system is known to be essential for normal embryonic and fetal growth (Stewart and Rotwein, 1996). Mice homozygous for a null mutation in the IGF-1 gene were infertile dwarfs and all androgen-dependent organs were decreased in size, which was consistent with an 80% reduction in testosterone production in these mice (Baker et al., 1996). However, the reduction in weight of the epididymis was not uniform for the entire length of the duct, such that the corpus and cauda regions were more severely affected than the caput, and the cauda showed decreased ductal coiling. The pattern of expression of IGF-1 and IGF-1R has been evaluated in rodent epididymides only during postnatal life. IGF-1 mRNA expression has been localized to the myofibroblast cells surrounding the epididymal duct but not the epithelial cells (Baker et al., 1996), while the protein is present in the myofibroblast cells and in the apical cytoplasm of the epithelial cells (Leheup and Grignon, 1993). The mRNA and protein for IGF-1R are expressed in epithelial cells (Antich et al., 1995; Baker et al., 1996). There were no previous reports of androgen regulation of IGF-1R expression at either the mRNA or protein level within the epididymis, however, testosterone has been shown to increase the mRNA expression of IGF-1 and IGF-1R in growing follicles of the primate ovary (Vendola et al., 1999). IGFBP5 levels can also be modulated by androgens. In vitro studies utilizing foetal fibroblasts have shown that coexposure to testosterone and IGFBP5 has increased the abundance of IGFBP5 bound within the extracellular matrix (Yoshizawa and Clemmons, 2000). Thus, IGFBP5 is ideally localized to regulate the availability of IGF-1 to its receptor by providing a local storage depot of the growth factor.

Bone morphogenetic proteins are members of the TGF-β superfamily and are involved in the development of many organs and tissues (Hogan, 1996). No reports were found in the literature demonstrating androgen regulation of either BMP4 or BMPR-1B mRNA expression. BMP4 is usually expressed in regions of developing organs where inductive paracrine interactions occur between the mesenchyme and the adjacent epithelium (Hogan, 1996). Consistent with this pattern of localization, BMP4 has been shown to regulate ductal budding and elongation into the surrounding mesenchyme during branching morphogenesis in the developing prostate (Lamm et al., 2001), kidney (Miyazaki et al., 2000), and lung (Weaver et al., 2000). Expression of the BMP4 receptors has been localized within the Wolffian duct and showed contrasting patterns of expression such that BMPR-1B (ALK6) mRNA expression was confined to the epithelium of the Wolffian ducts (Clarke et al., 2001), while BMPR-1A (ALK3) mRNA was expressed in mesenchymal cells in fetal mice (Jamin et al., 2002). Developing epididymides do not display ductal branching, but instead the duct undergoes complex coiling to give rise to morphologically distinct regions. BMP4 may conceivably play some role in defining how the duct coils in normal epididymal development.

The FGFs comprise a large family of proteins that are highly conserved in vertebrate species and have many diverse roles during embryonic development that include the regulation of cell proliferation, migration and differentiation (Goldfarb, 1996; Ornitz and Itoh, 2001). Similar to BMP4, FGFs and their receptors are also expressed at sites of mesenchymal-epithelial cell interactions and are involved in the regulation of branching morphogenesis in the prostate (Thomson, 2001), kidney (Qiao et al., 2001), and lung (Metzger and Krasnow, 1999). FGFs
have been proposed as mediators of androgen-induced development of the male reproductive tract (Cunha et al., 1992; Thomson, 2001). Studies have shown that FGF10 mimics androgen-stimulated growth of the ventral prostate and seminal vesicles by eliciting growth and ductal branching (Thomson, 2001). FGF10 is a ligand for the iiib variant of the type 2 FGF receptor (FGFR2). In the present study, epididymal FGFR2 mRNA expression was decreased postnatally following linuron exposure. The primers used did not distinguish between the epithelial (FGFR2iiib) and mesenchymal (FGFR2iiic) isoforms of the receptor (Orritz et al., 1996). Thus, it was not possible to implicate specific ligands that may be affected. However, an alteration in FGF signaling within epididymides was supported by the observation that steady-state levels of glypican1 mRNA expression were decreased in fetal and postnatal epididymides from rats exposed to linuron. Glypican belongs to a family of glycosylphosphatidylinositol anchored proteoglycans, which are attached to the cell surface and possess heparan sulfate chains (Litwack et al., 1998). FGFs have a high affinity for heparan sulfate proteoglycans and require heparan for effective biological activity (Orritz and Itoh, 2001). Glypicans have been shown to modulate the activity of FGFs in vitro (Bonneh-Barkay et al., 1997), and show marked changes in expression during fetal and postnatal development suggestive of their involvement in morphogenesis through the regulation of growth factor signaling pathways (Litwack et al., 1998; Song and Filmus, 2002).

Growth factors are the main determinants of cell fate. However, the extracellular matrix, as well as providing mechanical support to cells, can also modulate cell proliferation and differentiation by regulating the availability of growth factors reaching the cell surface (Streuli, 1999). Tissue morphogenesis is associated with reorganization of the components of the extracellular matrix and this process is regulated by matrix metalloproteinases (MMPs) and their inhibitors (TIMPs; Streuli, 1999). In addition to observing decreases in mRNA expression of IGFBP5 and glypican, the expression of TIMP3 was decreased in postnatal epididymides from rats exposed to linuron in utero. TIMP3 is the only member of the MMP inhibitor family that is bound to the extracellular matrix and this would imply that TIMP3 provides local restriction of MMP activity (Yeow et al., 2002). TIMP3 may be involved in regulating extracellular matrix reorganization during the folding and branching of ductal epithelia, since TIMP3 is specifically localized to the epithelia of ductal organs in the developing mouse embryo (Apte et al., 1994). In support of a role for TIMP3 in ductal branching, mRNA expression of MMPs decreased while TIMP3 showed a marked increase in expression as the structural complexity of the branching tubules increased during branching morphogenesis of the ureteric bud in vitro (Pohl et al., 2000). In the present study, disruption of epididymal development was associated with a decrease in TIMP3 mRNA expression, which may be a reflection of the decreased ductal coiling that was observed. Further work is required to address whether the MMP to TIMP ratio is altered, which would be indicative of a disruption in the normal turnover of the extracellular matrix.

In summary, we demonstrate that exposure to linuron in utero results in altered expression of genes within the epididymis known to be involved in growth factor signaling and tissue morphogenesis. Immunohistochemical evaluation of the cellular distribution of protein expression for AR and IGF-1R was supportive of the mRNA changes observed. The specific functions of many of these genes during the development and differentiation of the epididymis have not been established. However, the changes observed were consistent with a disruption of paracrine interactions between mesenchymal and epithelial cells. The fundamental importance of these growth-factor signaling pathways is highlighted by the fact that they have been conserved during evolution (Gerhart, 1999). Further investigation is required to determine whether these changes in gene expression can be considered as biomarkers of abnormal development of the epididymis or represent alterations in cell communication as a consequence of attenuation of AR signaling. Evaluation of the gene expression profiles in linuron-exposed epididymides that did not display any visible abnormalities would aid correlation of the changes in gene expression with pathogenesis. Additional studies are necessary to examine the time course of alterations in gene expression in relation to protein levels and Wolffian duct morphology on GD 17 through to GD 21, since this represents the most critical period for testosterone-mediated stabilization and differentiation of the Wolffian duct.

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