The Effects of Low-level Pb on Steroidogenic Acute Regulatory Protein (StAR) in the Prepubertal Rat Ovary


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Estradiol (E$_2$) is suppressed in prepubertal females exposed maternally to lead (Pb); thus, we assessed effects of Pb on ovarian steroidogenic acute regulatory protein (StAR) as a potential mechanism for this action. Adult Fisher 344 females were dosed with 12 mg of lead acetate per ml of Pb acetate (PbAc) or sodium acetate (NaAc; control), beginning 30 days prior to breeding and continuing until their pups were weaned. For the first part of this study, animals from both groups were killed when 31 days old, at 0800 h, for assessment of basal ovarian StAR gene expression. Results indicated Pb decreased ($p < 0.01$) both StAR transcripts. In the second part of the study, pregnant mare serum gonadotropin (PMSG) was administered to half of the Pb-treated and control animals at 0800 h. These animals, and animals from both groups that did not receive PMSG, were killed and ovaries and blood collected at 1600 h to assess ovarian StAR protein and E$_2$ responsiveness to gonadotropin stimulation. Pb decreased ($p < 0.0001$) basal StAR protein expression and lowered ($p < 0.001$) E$_2$ levels in animals that did not receive PMSG. PMSG induced ($p < 0.0001$) StAR protein in both the Pb-treated and control animals, an action associated with increased ($p < 0.001$) serum levels of E$_2$. These results are the first to show that Pb alters basal StAR synthesis, but does not alter gonadotropin-stimulated StAR synthesis, hence, suggesting the primary action of Pb to suppress E$_2$ is through its known action to suppress the serum levels of luteinizing hormone and not due to decreased responsiveness of StAR synthesizing machinery.

Key Words: lead (Pb) toxicity; steroidogenic acute regulatory protein; pubertal development; Fisher 344 rats; luteinizing hormone; estradiol.

The steroidogenic acute regulatory protein (StAR) is a 30-kDa protein responsible for the transfer of cholesterol from the outer to the inner mitochondrial membrane (Lin et al., 1995; Orly and Stocco, 1999; Sugawara et al., 1995). Cholesterol is then converted by the enzyme cytochrome P-450 side-chain cleavage to pregnenolone (Luo et al., 1998; Miller, 1988; Stocco and Clark, 1997) initiating a cascade of enzymatic reactions resulting in the formation of E$_2$ (Miller, 1988). StAR protein, present in both rodent (Clark et al., 1995; Hilderbrand 1973; Sandhoff and McLean, 1996) and human ovaries (Mushak, 1992), is the rate-limiting step essential in maintaining hormone-stimulated steroidogenesis (Chung et al., 1998; Clark et al., 1995; Luo et al., 1998; Pescador et al., 1997; Sandhoff and McLean, 1996). Specific effects of Pb on ovarian function have been observed in rats and monkeys (Franks et al., 1989, Hilderbrand et al., 1973; Stowe and Goyer, 1971). Additionally, low Pb levels have been associated with decreased ovarian weight, fewer corpora lutea, and abnormal estrous cycles in maternally exposed offspring (McGivern et al., 1991). Recently, it has been shown that prepubertal females exposed maternally to low levels of lead (Pb) exhibited suppressed circulating levels of both luteinizing hormone (LH) and estradiol (E$_2$) (Dearth et al., 2002; Ronis et al., 1996, 1998). Because StAR gene expression is controlled by gonadotropins (Chung et al., 1998; Clark et al., 1995; Luo et al., 1998; Pescador et al., 1997; Sandhoff and McLean, 1996), it is expected that ovarian E$_2$ synthesis would be altered by decreased ovarian trophic support due to depressed LH. It is not known, however, whether there is also an action of Pb directly within the ovary. Therefore, the aim of the present study was to determine if there were any intra-ovarian actions of Pb.

MATERIALS AND METHODS

Animals. As described previously (Dearth et al., 2002), adult Fisher 344 female rats purchased from Harlan Laboratories, Houston, TX, were housed within the Laboratory Animal and Resources Facility at Texas A&M University and kept under controlled conditions of temperature (23°C), lights (on: 0600 h; off: 1800 h) and had ad libitum access to food (Harland Teklad Diet, Madison, WI) and tap water.

Experimental protocol. Starting 30 days prior to breeding, animals were gavaged daily with a 1.0-ml solution of lead acetate (PbAc) containing 12 mg of Pb/ml until their pups were weaned 21 days after birth. Additional animals served as controls and received a daily 1.0 ml dose of an equimolar sodium acetate (NaAc) solution by gavage. Following the 30-day dosing period, both Pb-treated and control females were bred to non-Pb-exposed proven males and the morning that vaginal plugs were observed was considered day-one of gestation. Dams dosed with Pb were bled via tail tip on days 0, 15, and 30 prebreeding, day 10 of gestation, and days 1, 10, and 21 of lactation. NaAc-treated rats were bled on day 0 and at the end of the experiment, in order to verify that they were not exposed to Pb throughout the study. Rats were dosed at approximately 1:00 P.M. daily to help ensure uniform absorption.
All animals were weaned at 21 days, and females were housed, 4 per cage, until they were thirty days old. The following morning, both the Pb-exposed and NaAc-treated control animals were divided such that the study would have two parts. The first consisted of 31-day-old Pb-treated and NaAc-treated control animals that were killed by decapitation at 0800 h, and their ovaries, which were removed and frozen at −80°C for subsequent determination of basal StAR gene expression. Animals for the second part of the study were used for the determination of StAR protein and serum E2 levels, following gonadotropin stimulation. For this, both groups were subdivided such that half of the Pb-exposed animals and half of the NaAc control animals received subcutaneous injections of 15 IU of pregnant mare serum gonadotropin (PMSG; purchased from Pituitary Hormones and Antisera Center, Harbor-UCLA Medical Center, Torrance, CA) at 0800 h. The other half of the animals from each group were not injected with PMSG. At 1600 h, all of the animals were killed by decapitation and trunk blood was collected. For each sample, an aliquot was removed for measurement of Pb concentrations, and the rest of the sample was allowed to clot, then was centrifuged and stored at −80°C until assayed to determine the serum level of E2. Ovaries were collected and frozen at −80°C for subsequent Western-blot analysis for StAR protein. Pb concentrations in both blood and ovary were determined by atomic absorption spectrophotometry.

**Northern-blot analysis.** Total RNA was prepared from ovaries, using RNA Zol B followed by precipitation with isopropanol and ethanol washes, according to the manufacturer’s instructions (Tele-test, Friendswood, TX). Northern-blot analysis was done as described previously (Srivastava et al., 2001). Total RNA (30 μg) was denatured in the presence of 6% formaldehyde and 50% formamide at 65°C for 15 min, and run on 1% agarose gels containing 2 M formaldehyde. Following size fractionation, RNA was transferred onto a nylon membrane (NEN Life Sciences Products, Inc., Boston, MA), followed by UV cross-linking of RNA (0.12 J/cm<sup>2</sup>) (Stratalinker Crosslinker, Stratagene, La Jolla, CA). The membranes were prehybridized at 45°C for 4 h in a mixture containing 5 × SSC, 5 × Denhardt’s solution: 50% formamide, 20 mM sodium phosphate (pH 6.8), 2.5 mM EDTA, and 0.1 mg/ml denatured salmon testis DNA. Hybridization was carried out in the same solution at 45°C for 16 h with 10% dextran sulfate and 2 × 10<sup>6</sup> cpm StAR cDNA probe labeled with [α-<sup>32</sup>P] dCTP (3000 Ci/mmmol; NEN) by using Random Primers DNA-labeling System (Gibco-BRL, Rockville, MD). Unincorporated nucleotides were separated from radiolabeled DNA probes by using miniQuick spin oligo columns (Roche Molecular Biochemicals, Indianapolis, IN). After hybridization, the blots were washed three times at room temperature (10 min) in 1 × SSC/1% SDS and three times under high-stringency conditions at room temperature (10 min) in 0.1 × SSC/0.1% SDS. After washing, the membranes were exposed to XAR-5 film for 24 h with an intensifying screen at −70°C. Following, Northern-blot analysis with StAR cDNA, blots were stripped and rehybridized with a mouse β-actin cDNA (Ambion, Inc., Austin, TX) labeled with Random Primers DNA-labeling kit (Gibco-BRL, Rockville, MD). The autoradiograms were quantified using scanning densitometry. The integrity of the RNA was confirmed by ethidium-bromide staining of the gel, which showed the intact ribosomal RNAs, i.e., 18S and 28S subunits, and whether equal amounts of RNA had been loaded in each lane. The RNA transcript size was determined by comparison with an RNA molecular weight marker on a 12% SDS–PAGE under reducing conditions, along with prestained mw markers (Amer sham Pharmacia Biotech, Piscataway, NJ). The separated proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes using a mini trans-blot apparatus (Bio-Rad, Hercules, CA). The transfer was performed at a constant voltage of 100V for 1.5 h in a buffer consisting of 48 mM Tris, 39 mM glycine, pH 8.3, 0.037% SDS, and 20% methanol. Following transfer, membranes were blocked overnight at 4°C in the presence of 5% nonfat dry milk in phosphate buffered saline (PBS) and subsequently incubated at room temperature for 3 h with a rabbit antiserum polyclonal antibody to the StAR protein (diluted 1: 1000 in PBS containing 0.01% T-20), which was kindly provided by Dr. Douglass Stocco, Texas Tech University, Lubbock, TX). After washing, the membranes were incubated with horseradish peroxidase-labeled donkey antirabbit IgG at a dilution of 1: 14,000 for 2 h at room temperature. After another wash, the immunoreactive 30 kDa StAR protein was detected by the enhanced chemiluminescence method (Western-blot Chemiluminescence Reagent Plus, NEN). The integrated optical density of the bands was quantified using scanning densitometry.

**Dosing solutions.** A certified standard (Lot no. 8240–11), made with Pb acetate-trihydrate and containing 100 mg Pb/ml in 2% nitric acid, was purchased from Scientific Equipment Company, Aston, PA and served as the stock Pb solution. Using the method described previously (Dearth et al., 2002), a dosing solution of 12 mg/ml of Pb was made fresh daily, immediately before dosing, in a 50-ml centrifuge tube. The pH of the NaAc dose given to the control animals was adjusted to 5.5 with ultrapure nitric acid, to match that of the Pb-dosing solution.

**Blood and tissue Pb measurements.** All Pb levels were measured by a Perkin Elmer SIMAA 6000 simultaneous multi-element atomic absorption spectrophotometer (AA) with a transversely heated graphite furnace, Zeeman background correction system, AS-72 autosampler, and an AA Winlab applications package (Perkin Elmer Corporation, Norwalk, CT). Blood and ovary Pb levels were processed and analyzed using methods described previously (Dearth et al., 2002).

**Radioimmunoassay and statistical analysis.** Serum estradiol (E<sub>2</sub>) was measured by an RIA kit purchased from Diagnostic Products Corp. (Los Angeles, CA). The assay sensitivity was 8 pg/ml and intra- and inter-assay coefficients of variation were <10%. Differences between NaAc- and Pb-treated animals were analyzed using an unpaired Student t-test, assuming random sampling, Gaussian distribution, and independent observations. Two-sided probability (p) values were used, and those less than 0.05 were considered to be significantly different. The IBM PC software programs, INSTAT and PRISM (GraphPad, San Diego, CA, USA), were used to calculate and graph the results.

**RESULTS**

At the times of breeding, through weaning on day 21, the blood Pb (B Pb) levels of the dams averaged 39.3 ± 3.5 SEM μg/dl. This resulted in Pb levels averaging 2.9 ± 0.28 SEM μg/dl and ovary Pb levels averaging 0.0161 ± 0.005 SEM ppm in their 31-day-old offspring (Fig. 1). The Pb levels in these pups were similar to those known to cause delayed female puberty (Dearth et al., 2002). Note, the Pb levels in both the NaAc control dams and their offspring were all <1.0 μg/dl. Additionally, there were no differences in body weights, litter sizes, fetal resorptions/litter, and pup deaths/litter between control and Pb-exposed dams throughout the study.

The initial portion of this study was designed to determine the effect of maternal Pb exposure on StAR gene expression in prepubertal ovaries. Northern-blot analysis showed two major StAR-specific transcripts at 3.8 and 1.7 kb in the ovary, as
depicted in Figure 2A by the composite autoradiogram. Figure 2B illustrates the densitometric values normalized with β-actin mRNA by determining the ratio of the density of StAR mRNA to that of β-actin mRNA. These data clearly show that maternal Pb exposure resulted in a greater than 50% decrease (\(p < 0.01\)) in both the 3.8 and 1.7 ovarian StAR transcripts, compared to the NaAc-exposed controls.

A second portion of this study was conducted to determine the effect of maternal Pb exposure on prepubertal ovarian StAR protein and E2 responsiveness to gonadotropin stimulation. Figure 3A illustrates the representative Western-blot analysis of StAR protein, detected at 30 kDa, in ovaries from Pb-treated and control animals with or without PMSG stimulation. Figure 3B is a composite of all animals, which depicts the ability of Pb to decrease basal ovarian StAR protein expression by 27% (\(p < 0.0001\)) compared to NaAc-exposed controls, a result that paralleled the decrease in mRNA expression shown in Figure 2. Importantly, PMSG stimulated a 216% increase (\(p < 0.0001\)) in StAR protein expression in NaAc control and a 400% increase (\(p < 0.0001\)) in Pb-treated ovaries compared with the respective non-PMSG stimulated ovaries; indicating that the StAR synthesizing machinery was functional in both groups. Furthermore, serum E2 levels were measured in these animals to confirm that the observed changes in StAR protein expression were associated with changes in steroid secretion. Figure 4 depicts a 46% decrease (\(p < 0.001\))

**FIG. 1.** Blood and ovary lead (Pb) levels from 31-day-old Pb-exposed female rats: The bars represent the mean (±SEM) of \(n = 10\) in each group.

**FIG. 2.** Effect of maternal Pb exposure on basal ovarian sodium acetate steroidogenic acute regulatory protein (StAR) gene expression in 31-day-old rats by Northern-blot analysis: (A) Representative autoradiogram showing the expression of StAR mRNA in total ovarian RNA (30 μg) isolated from animals exposed to NaAc (control, lanes 1–3), or lead acetate (PbAc) (lanes 4–6). The autoradiogram represents a 24-h exposure at –70°C. Note that the probe showed two StAR-specific transcripts of 3.8 and 1.7 kb. The transcript for the internal control, β-actin, at 2.1 kb was detected by hybridization with a mouse β-actin cDNA. β-Actin was exposed to autoradiographic film for 4 h at –70°C. (B) Composite drawings showing the densitometric quantitation of the bands from two blots corresponding to the ovarian StAR transcripts. These data were normalized to the internal control β-actin mRNA; the densitometric units represent the StAR/β-actin (mRNA) ratios. Note the expression of mRNA was decreased (\(p < 0.01\)) for both StAR transcripts in animals that were exposed to Pb. Each bar is the mean (±SEM) and depicts \(n = 6\) lanes, with each lane representing the pair of ovaries from one animal; **\(p < 0.01\) vs. NaAc control.
in basal serum E2 levels from 14.3 ± 1.2 pg/ml in the controls to 7.7 ± 1.4 pg/ml in the Pb-exposed animals, coinciding with the Pb-induced decreases observed in basal StAR gene and protein expression shown in Figures 2 and 3, respectively. The NaAc controls and Pb-treated animals that were administered PMSG showed E2 levels of 23.1 ± 2.4 pg/ml and 24.9 ± 1.8 pg/ml, respectively. These levels depict 61% (p < 0.001) and 223% (p < 0.0001) increases over the basal E2 levels from the control and Pb-treated rats that did not receive the PMSG stimulation, indicating E2 secretion was functional in both groups.

**DISCUSSION**

As puberty approaches, the ovary is responsible for releasing an increasing amount of E2 necessary for the maturation of the hypothalamic LHRH neuronal circuitry responsible for driving the pubertal process (Ojeda et al., 1996a,b). The release of LHRH/LH subsequently stimulates ovarian steroid production (Ojeda et al., 1996a). Previous studies have shown that offspring exposed to Pb have suppressed levels of LH and E2 (Dearth et al., 2002; McGivern et al., 1991; Ronis et al., 1998). However, less is known regarding the effect of Pb on the developing ovary; specifically, whether the metal can act directly within the gland to alter steroid production. It has become clear that StAR, the 30-kDa mitochondrial protein, is the rate-limiting step in gonadotropin-stimulated steroid production (Luo et al., 1998; Stocco and Clark, 1997). It is well known that in response to hormone stimulation, the StAR 37-kDa precursor protein is synthesized in the cytosol and is directed to the mitochondria. This is where cleavage of the mitochondrial targeting sequence occurs, yielding first to a 32-kDa form and then to the mature 30-kDa StAR protein (Epstein and Orme-Johnson, 1991; King et al., 1995; Stocco and Sodeman, 1991). At some point during the processing of these proteins, the facilitation of cholesterol transfer occurs from the outer to the inner mitochondrial membrane where the
that Pb decreased both LH and FSH testicular receptor expression and inhibited hCG-stimulated progesterone release in cultured Leydig cells caused a decrease in StAR protein expression and inhibited hCG-stimulated progesterone release (Huang et al., 2002, 1997), suggesting Pb may act directly to disrupt the StAR-synthesizing machinery necessary for ovarian steroidogenesis.

The present study is the first to show altered intraovarian StAR gene and protein expression and a subsequent decrease in E$_2$ production by ovaries from late juvenile offspring that were maternally exposed to Pb at levels equal to those of human concern (CDC, 1997, 1991). Specifically, 31-day-old offspring exposed to Pb throughout gestation and lactation showed a significant decrease in both basal StAR gene and protein expression. These results coincided directly with decreased serum E$_2$ levels. As shown, both BPb and ovary Pb levels in these females were very low, having almost returned to preexposure levels; hence, suggesting an effect on the ovary that is long-lasting.

During the late juvenile phase of pubertal development, the release of LH from the pituitary plays an important role in ovarian maturation by increasing gonadotropin receptor numbers and facilitating production of E$_2$ (Ojeda et al., 1986a). This trophic stimulation of receptors on the ovary causes, through several signaling pathways, activation of the StAR gene, thus producing an increase in StAR protein expression and, subsequently, ovarian steroids (Chung et al., 1998; Clark et al., 1995; Pescador et al., 1997; Sandhoff and McLean, 1996). Previous studies in prepubertal and adult rats showed that Pb decreased both LH and FSH testicular receptor concentrations (Kempinas et al., 1994; Wiebe et al., 1982). Additionally, Pb has been shown to alter ovarian LH receptor concentrations in prepubertal females (Wiebe et al., 1988); however, it is important to note that the animals in those studies were exposed to Pb levels higher than those relative to human concerns. In the current study, prepubertal ovarian receptor responsiveness was not hindered by low-level Pb exposure, as shown by the ability of PMSG to stimulate StAR protein and E$_2$ secretion. Importantly, the exposure levels of Pb in this study were markedly lower than those previously associated with altered LH receptor populations (Wiebe et al., 1988). Although the present study did not assess the possibility that more subtle changes in LH receptor populations could have occurred with the low-level Pb exposure used, the responsiveness of the receptors to a dose of PMSG used routinely to assess ovarian function was not altered. The stimulatory response PMSG has on E$_2$ release replicates the ability of both FSH and LH to induce ovarian steroidogenesis (Dees et al., 1990; Mizutani et al., 1997). Maternal Pb exposure, however, only alters LH, but not FSH secretion (Dearth et al., 2002; unpublished observation). Apparently FSH had a limited ability to influence ovarian function, because basal E$_2$ levels were present in Pb exposed females; however, these E$_2$ levels were significantly decreased compared to controls. Thus, because LH facilitates the gradual increase in serum E$_2$ necessary for a normal progression through puberty (Ojeda et al., 1996a,b), we suggest that Pb exposure does not affect the ovarian StAR synthesizing machinery, and that the Pb-induced decrease in basal E$_2$ levels observed were likely due to the suppressed trophic support of LH to the prepubertal ovary. In support of this, we have observed previously that serum LH levels in 30-day-old Pb-exposed pups were 0.25 ng/ml, compared to 0.7 ng/ml from their age-matched controls (Dearth et al., 2002).

In conclusion, these results showed a decrease in both basal ovarian StAR gene and protein expressions in late prepubertal female offspring from Pb-exposed dams. This Pb-induced effect coincided with a decrease in E$_2$ production. Low ovarian Pb levels and the ability of PMSG to stimulate StAR protein expression and subsequent E$_2$ release in Pb-exposed females strongly suggests that the metal does not directly affect ovarian responsiveness to gonadotropin stimulation. Since it is known that Pb causes a decrease in serum LH and E$_2$ levels in maternally exposed female offspring (Dearth et al., 2002; Ronis et al., 1998, 1996), we suggest that Pb acts at the hypothalamic-pituitary level to disrupt the peripubertal gonadotrophic support contributed by LH, necessary to drive StAR production and subsequent E$_2$ synthesis.

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