

Insulin-Like Growth Factor I of Peripheral Origin Acts Centrally to Accelerate the Initiation of Female Puberty*

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ABSTRACT

In several species, including humans, circulating insulin-like growth factor I (IGF-I) levels increase during the onset of puberty, suggesting that this peptide contributes to attaining sexual maturity. Because IGF-I elicits LHRH release from the median eminence (ME) of immature female rats *in vitro*, we hypothesized that it may represent one of the peripheral signals suspected to link somatic development to the LHRH-releasing system at puberty. We now present evidence in support of this concept. Quantitation of IGF-I messenger RNA (mRNA) levels by ribonuclease protection assay revealed that expression of the IGF-I gene did not change in the medial basal hypothalamus or preoptic area of female rats during peripubertal development. In contrast, the contents of both IGF-Ia and IGF-Ib mRNA, the two alternatively spliced forms of the IGF-I gene, increased significantly in the liver during the early proestrous phase of

puberty. This change was followed by an elevation in serum IGF-I levels during the late proestrous phase of puberty along with a concomitant increase in serum gonadotropin levels. The proestrous change in serum IGF-I levels was accompanied by a selective increase in IGF-I receptor (IGF-IR) mRNA in the ME. Small doses of IGF-I (2–200 ng), administered intraventricularly, effectively induced LH release in both juvenile and peripubertal female rats, an increase prevented by prior immunoneutralization of LHRH actions. Importantly, intraventricular injections of IGF-I (20 ng), administered twice daily in the afternoon to immature animals, significantly advanced puberty. Thus, these results suggest that IGF-I of peripheral origin contributes to the initiation of female puberty by stimulating LHRH release from the hypothalamus, an effect that appears to be amplified by the increased synthesis of IGF-I receptors in the ME during first proestrus. (*Endocrinology* 137: 3717–3728, 1996)

THE INITIATION of mammalian puberty depends on a complex series of events that occurs within the central nervous system and appears to require the interactive participation of neuronal circuitries and glial networks (for review, see Ref. 1). In addition, it has been hypothesized that the initiation and progression of the pubertal process may be influenced by metabolic signals of peripheral origin (for review, see Ref. 2). Identification of such signals has been difficult because of the large number of peripherally originated substances that can act centrally to modify neuronal function. Among these substances, insulin-like growth factor I (IGF-I) is a bioactive molecule whose circulating levels increase strikingly during puberty in rodents (3, 4), ruminants (5, 6), and primates (7, 8), including humans (9). Otherwise, unfavorable metabolic conditions can lower IGF-I and LH secretion and, thus, may contribute to delayed puberty (10–14). The fact that IGF-I can act directly on the hypothalamus of immature animals to affect LHRH neuronal activity was first shown by *in vitro* experiments in which IGF-I stimulated LHRH release from the rat median eminence (ME) (15), one of the brain regions with the highest concentration of IGF-I receptors (IGF-IR) (16–20). Other *in vitro* experiments, using hypothalamic explants, demonstrated that IGF-I can also have an inhibitory effect on LHRH

release (21). Whether IGF-I plays an inhibitory or stimulatory role on LHRH secretion in the intact peripubertal animal is not known. Equally unresolved is the question of the capacity of the hypothalamus to synthesize IGF-I at the time of puberty. The present study addresses these issues. Specifically, we show that IGF-I synthesis increases in the liver, but not in the hypothalamus, at the time of puberty, and that the corresponding increase in circulating IGF-I levels is accompanied by increased synthesis of IGF-I receptors in the ME region of the hypothalamus. We also show that central administration of small amounts of IGF-I to intact immature animals increases plasma LH levels via activation of LHRH release and advances the onset of puberty. A partial report of these results has appeared (22).

Materials and Methods

Animals

All rats used in the present study were of the Sprague-Dawley line and were purchased from Sasco Laboratories (Omaha, NE). Pregnant rats arrived on day 18 of gestation and were allowed to deliver their pups normally. The pups were weaned at 21 days of age, and females of the same litter were housed together (four per cage) for subsequent use in the following experiments. All rats were maintained under controlled conditions of light (lights on, 0600 h; lights off, 1800 h) and temperature (23 C), with *ad libitum* access to food and water.

Exp 1: developmental patterns of IGF-I and IGF-IR messenger RNA (mRNA) levels at puberty; association with serum IGF-I and reproductive hormones

Female rats ranging in age from 30–37 days were killed by decapitation between 0900–1100 h, and trunk blood was collected. The liver

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and three areas of brain [preoptic area (POA), medial basal hypothalamus (MBH), and ME] were removed and frozen on dry ice. Based on previously defined criteria (23), the tissues were grouped according to the respective phase of pubertal development of each animal (*i.e.* immature or juvenile, early proestrous, late proestrous, first estrous, and first diestrous). Tissues were analyzed for changes in IGF-I and IGF-IR gene expression using specific ribonuclease (RNase) protection assays (see below). Trunk blood was centrifuged at 4 C, the serum was stored at -70 C until assayed for IGF-I, estradiol (E₂), LH, and FSH.

Exp 2a: IGF-I-induced LH secretion during juvenile development

Twenty-six-day-old female rats were stereotaxically implanted (24) with a stainless steel cannula (23 gauge) in the third ventricle (3V) of the brain and allowed 7 days for recovery. Twenty-four hours before the experiment, SILASTIC brand cannulas (Dow Corning, Midland, MI) were inserted into the right external jugular vein of each rat according to a previously described method (25). On the day of the experiment, an extension of tubing was attached to each cannula, then flushed with heparinized saline (100 IU/ml). Four basal blood samples were drawn from each freely moving animal at 10-min intervals, immediately followed by a 3V injection of IGF-I [1, 2, 20, or 200 ng/3 μ l; human recombinant IGF-I, Mallinkrodt (St. Louis, MO) or Eli Lilly Co. (Indianapolis, IN)] or an equal volume of saline. The range of doses used in this experiment was selected based on our previous *in vitro* study using nanogram quantities of the peptide (15). After the respective injection, four more samples were taken for a total of eight samples. All blood samples were taken between 0900–1100 h. After the experiment, the brains were inspected to verify proper placement of the cannulas, and animals were confirmed to be in the juvenile phase of development. Blood samples were centrifuged at 4 C, and serum was stored at -70 C until assayed for LH.

Exp 2b: immunoneutralization of LHRH during juvenile development

Twenty-six-day-old female rats were treated in the same manner as in the above experiment except for the following modifications. After jugular surgery, the rats received a 3V injection of either anti-LHRH serum (3 μ l Kelch no. 13, provided by Dr. R. P. Kelch, University of Iowa, Iowa City, IA) or the same volume of normal rabbit serum (NRS). The next day, two basal blood samples were taken at 10-min intervals, then the animals were injected again with either anti-LHRH or NRS. After 60 min, two more samples were taken, then all of the animals received a 3V injection of IGF-I (200 ng/3 μ l) before four final blood samples were drawn. After the experiment, cannula placement and the phase of pubertal development were confirmed. Blood samples were processed as described above in Exp 2a.

Exp 3: IGF-I-induced LH secretion during peripubertal development

Twenty-eight-day-old female rats were implanted with 3V and jugular cannulas and allowed to recover as described above for Exp 2a. To study the LH response to IGF-I during proestrous, estrous, and diestrous phases of puberty, animals received 6 IU PMSG (Sigma) 48 h (proestrous) and 72 h (estrous and diestrous) before the administration of IGF-I. Twenty-four hours before the experiment, jugular cannulas were implanted as described above. Thereafter, four basal blood samples were drawn at 10-min intervals, immediately followed by a 3V injection of IGF-I (200 ng/3 μ l) or an equal volume of saline. After the respective injection, four more samples were taken for a total of eight samples. For this study, all samples were collected between 1300–1500 h. After the experiment, cannula placement and each animal's respective phase of puberty were confirmed. Blood samples were processed as described above for Exp 2a.

Exp 4: effect of daily IGF-I 3V administration on the onset of puberty

Twenty-four-day-old female rats were stereotaxically implanted with 3V cannulas and allowed 4 days for recovery. On day 27, the animals

were weighed and divided into two groups. Starting on day 28, group 1 received a 3V injection of IGF-I (20 ng/3 μ l saline) at 1500 h and again at 1700 h. Group 2 served as the controls and thus received 3V injections of sterile saline in place of IGF-I. The animals were weighed and checked daily for vaginal opening (VO) before their respective afternoon injections. When VO occurred, the animals were killed, classified as to the phase of pubertal development, and checked for proper placement of the 3V cannula.

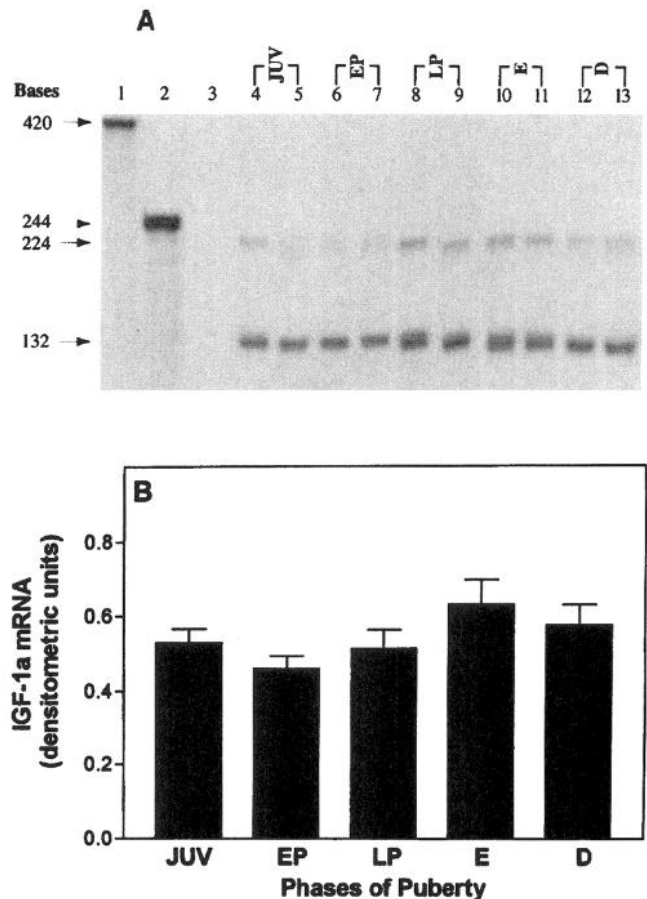


FIG. 1. Detection of IGF-Ia mRNA in the POA during peripubertal development. A, Composite autoradiogram of three RNase protection assays. Lanes 1 and 2 illustrate the undigested full-length IGF-I (420 bases) and cyclophilin (244 bases) RNA probes, respectively. The protected bands of IGF-Ia and cyclophilin are 224 and 132 bases, respectively. Experimental lanes (designated 4–13) depict samples from juvenile (JUV), early proestrous (EP), late proestrous (LP), first estrous (E), and first diestrous (D) animals. Lane 3 shows that no hybridization occurred when only yeast tRNA was used in the presence of RNase-1 as a negative control. Each experimental lane was loaded with 30 μ g total POA RNA. The composite represents three autoradiograms obtained after a 24-h exposure at -70 C. B, Composite graph representing the densitometric quantitation of the band corresponding to IGF-Ia mRNA in the POA from three experiments. Note that the levels of IGF-I mRNA in the POA did not change during the peripubertal period. Each autoradiogram scanned contained samples from all groups, and each sample (lane) represents two pieces of POA tissue, hence the total of five lanes for JUV, five for LP, four for E, and four for D. These data were normalized to cyclophilin mRNA, and the values represent the mean \pm SEM. Differences between groups were analyzed using ANOVA with *post-hoc* testing using Student-Newman-Keuls multiple range test.

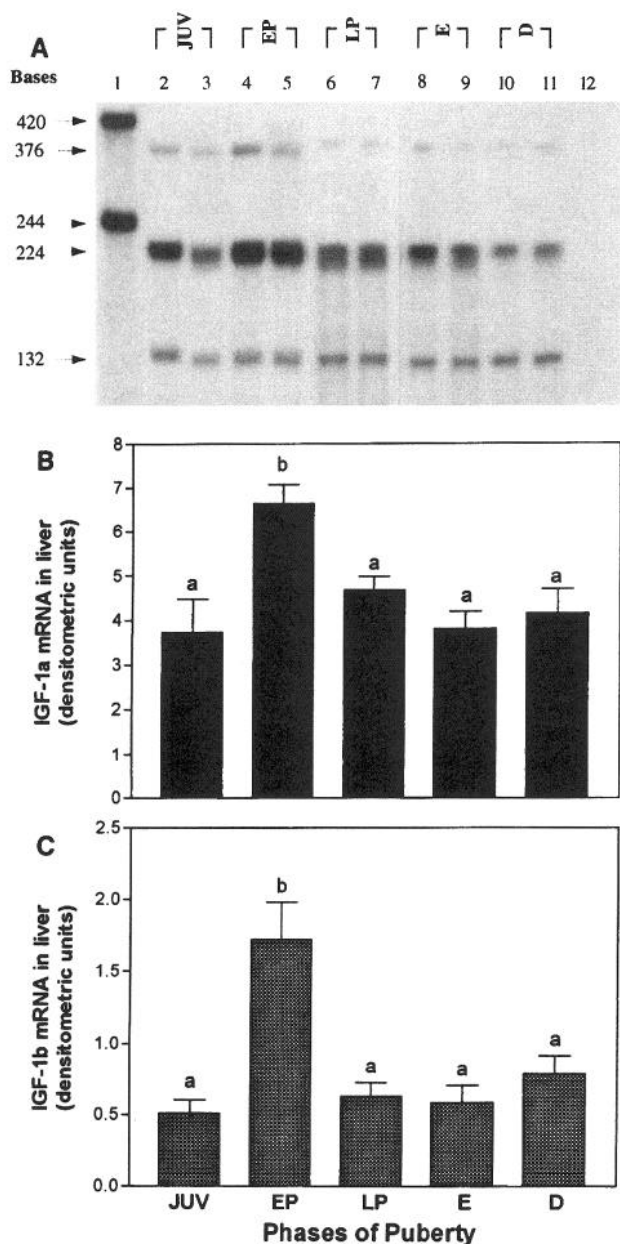


FIG. 2. Changes in IGF-I mRNA levels in the liver during peripubertal development. A, Composite autoradiogram of three RNase protection assays. Lane 1 illustrates the undigested full-length IGF-I (420 bases) and cyclophilin (244 bases) RNA probes, respectively. In the liver, the IGF-I probe detects the two alternatively spliced forms of IGF-I mRNA, *i.e.* IGF-Ia (224 bases) and IGF-Ib (376 bases). The band protected by cyclophilin mRNA is 132 bases in length. Experimental lanes (designated 2–11) depict samples from juvenile (JUV), early proestrous (EP), late proestrous (LP), first estrous (E), and first diestrous (D) animals. Lane 12 shows that no hybridization occurred when only yeast tRNA was used in the presence of RNase-1 as a negative control. Each experimental lane was loaded with 2.5 μ g total liver RNA. The composite represents autoradiograms obtained after a 24-h exposure at -70°C . B and C, Composite graphs representing the densitometric quantitation of the bands corresponding to liver IGF-Ia and IGF-Ib mRNAs from two or three experiments; there was a total of four lanes for JUV, six for EP, six for LP, four for E, and four for D. Each autoradiogram scanned contained samples from all groups, and each sample (lane) analyzed represents one piece of liver tissue. Note that the level of expression of both IGF-Ia and IGF-Ib mRNA increased significantly during early proestrus, then declined. These data were normalized to the internal control cyclophilin

Assays and statistics

Solution hybridization/RNase protection assay. RNA preparation: Total RNA was isolated from brain and liver tissues of juvenile and peripubertal female rats, using RNA Zol B followed by precipitation with isopropanol and ethanol washes according to the manufacturer's instructions (Tele-Test, Friendswood, TX). The integrity of the RNA was checked by visualization of the ethidium bromide-stained 28S and 18S ribosomal RNA bands, and quantitation was performed by measuring its absorbance at 260 nm.

Riboprobe synthesis: The antisense IGF-I complementary RNA (cRNA) was obtained by *in vitro* transcription of a 376-bp fragment of a rat IGF-I complementary DNA (cDNA; kindly provided by Dr. C. T. Roberts, Oregon Health Sciences University, Portland, OR) (26). This fragment was cloned into the pGEM-3 vector, linearized with *Hind*III, and transcribed with T7 RNA polymerase in the presence of [α - ^{32}P]CTP using a RNA transcription kit (Promega Biotec, Madison, WI). A 265-bp fragment of a rat IGF-IR cDNA (27) (also provided by Dr. C. T. Roberts), cloned into pGEM-3 vector and linearized with *Eco*RI, was used to synthesize a [α - ^{32}P]CTP-labeled antisense RNA with SP6 RNA polymerase. A plasmid containing a 132-bp cDNA fragment complementary to cyclophilin mRNA, cloned at the *Nco*I site of pGEM5ZF(-), was used to synthesize a cRNA probe to detect cyclophilin mRNA. As cyclophilin is constitutively expressed in various tissues, including brain and liver (28), quantitation of its mRNA levels can be used to normalize the values obtained for the mRNAs of interest. For generation of the antisense probe, this construct was linearized with *Apa*I and transcribed with SP6 RNA polymerase. All riboprobes were gel purified on a 5% polyacrylamide-7 M urea denaturing gel.

Protection assays were performed using the methods of Ausubell and Bent (29) with minor modifications (14). Total RNA from liver (2.5 μ g), MBH (15 μ g), and POA (30 μ g) was hybridized overnight at 45 C with 2.5×10^5 cpm ^{32}P -labeled antisense IGF-I cRNA in a buffer containing 80% deionized formamide, 40 mM P; perazine-N,N'-bis[2-ethanesulfonic acid] (PIPES) (pH 6.4), 0.4 M NaCl, and 1 mM EDTA. Total RNA from the ME (8 μ g), POA (30 μ g), and MBH without the ME (15 μ g) was hybridized to equal counts of ^{32}P -labeled antisense IGF-I receptor probe. All tissue samples were also simultaneously hybridized to 20,000 cpm ^{32}P -labeled cyclophilin cRNA for liver and 4,000 cpm for other tissues. After hybridization, the samples were digested with 2 U RNase-1 (Promega) in RNase digestion buffer containing 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 200 mM sodium acetate at 37 C for 1 h. The digestion reaction was terminated by the addition of 10 μ l 20% SDS, and the protected hybrids were precipitated in the presence of 15 μ g transfer RNA (tRNA) and 2–3 vol ethanol at -20°C for 1 h. After precipitation, the pellets were collected by centrifugation, dried in a vacuum, and resuspended in a formamide loading buffer containing 80% deionized formamide, 10 mM EDTA (pH 8.0), 0.1% bromophenol blue, 0.1% xylene cyanol, and 0.1% SDS. Samples were denatured at 85 C for 5 min, chilled on ice, and loaded on 5% polyacrylamide-7 M urea denaturing gel. The sizes of the bands were determined by simultaneously loading a pGEM-3 DNA marker that was end labeled with T4 polynucleotide kinase and [γ - ^{32}P]ATP. The autoradiographic exposures from each gel were quantified by scanning densitometry.

IGF-I RIA. Serum samples and pooled serum from 30-day-old juvenile rats were extracted using the acid-ethanol extraction method of Daughaday *et al.* (30) with the following modifications. An acid-ethanol mixture (87.5% ethanol-12.5% 2 N HCl) was added at a ratio of 4 parts solvent to 1 part serum. The mixture was vortexed and incubated in a water bath at 37 C overnight. After centrifugation, the supernatant (0.5 ml) was removed, neutralized with 0.2 ml 0.855 M Tris base, then incubated for 1 h at -20°C . The extracts were centrifuged, and the supernatant was removed and diluted to 1:20 with assay buffer [0.03 M sodium phosphate buffer (pH 7.4) containing 0.25% BSA (RIA grade, Sigma Chemical Co.)] before being added to the assay tubes.

IGF-I levels were measured by a modification of the RIA method of Crawford *et al.* (31). IGF-I standards, the extracted rat standard serum

mRNA and represent the mean \pm SEM. B: a vs. b, $P < 0.01$; C: a vs. b, $P < 0.001$. Differences between groups were analyzed using ANOVA with *post-hoc* testing using the Student-Newman-Keuls multiple range test.

pool, and samples were incubated for 24 h at 4 C with anti-IGF-I (UB3-189 from Drs. Louis Underwood and Judson J. Van Wyk, National Hormone and Pituitary Program, Ogden Bioservices Corp., Rockville, MD) at a final dilution of 1:10,000. After this incubation, (3-[¹²⁵I]iodotyrosyl)IGF-I (Amersham Corp., Arlington Heights, IL) was added, and incubation proceeded for 24 h at 4 C. Antibody-bound radioactivity was precipitated by the addition of goat antirabbit IgG (1:75), normal rabbit serum (1:200), and 6% polyethylene glycol. Samples were compared to a standard curve prepared using human recombinant IGF-I (Bachem, Torrance, CA). The standard rat serum pool curve yielded a displacement curve that paralleled the purified IGF-I standard curve. Values were expressed as nanograms per ml rat serum. The inter- and intraassay variations were both less than 10%.

E₂, LH, and FSH RIAs. Serum E₂ levels were measured using a kit purchased from Diagnostic Products Corp. (Los Angeles, CA). Serum LH and FSH levels were measured by a modification of previously described methods (32, 33). The antisera, rLH-S-11 and rFSH-S-11, as well as the reagents were obtained from the National Hormone and Pituitary Program, Ogden Bioservices Corp. Results are expressed in terms of LH RP-3 and FSH RP-2 reference standards, respectively. The sensitivities of the LH and FSH assays were 0.07 and 0.4 ng/ml, respectively. The inter- and intraassay variations for both assays were less than 10%.

Statistical analysis. Differences between groups were initially analyzed using ANOVA with *post-hoc* testing using the Student-Newman-Keuls multiple range test and, when appropriate, unpaired Student's *t* test. These statistical tests were conducted using INSTAT software for the IBM PC (GraphPad, San Diego, CA). Comparisons with *P* < 0.05 were considered to be significantly different. Areas under the curve of secretion were determined using the trapezoid rule provided by Prism software (GraphPad). These values are expressed as nanograms per unit time interval. LH pulse assessment was also conducted using Prism software.

Results

Exp 1: developmental patterns of IGF-I and IGF-IR mRNA levels at puberty; association with serum IGF-I levels and reproductive hormones

The changes in IGF-I mRNA levels in brain and liver were determined by a RNase protection assay using an IGF-I probe that detects the two alternatively spliced products of the IGF-I gene. As this probe is complementary to a region in IGF-I mRNA that extends from the middle of the A domain through the first 172 bp of the 3'-untranslated region (26) and contains an alternatively spliced 52-bp segment within the E domain, it detects two IGF-I mRNA species, IGF-Ia (224 and 100 bases) and IGF-Ib (376 bases). Only the 224-base protected band was used in the measurements for the IGF-Ia mRNA species. As shown in the composite autoradiogram depicted in Fig. 1, no differences in the levels of IGF-Ia mRNA were observed in the POA of the brain between the juvenile and peripubertal phases of development. Likewise, no differences in IGF-Ia mRNA levels were detected in the MBH (not shown). In both the POA and MBH, IGF-Ib mRNA levels were negligible and could be detected only after a much longer film exposure. In the liver, however, developmental changes in both IGF-Ia and IGF-Ib mRNA abundance were detected. Figure 2A demonstrates a composite autoradiogram illustrating these peripubertal changes in IGF-Ia (224b) and IGF-Ib (376b) mRNA levels. As shown in Fig. 2, the levels of IGF-Ia mRNA (Fig. 2B) and IGF-Ib mRNA (Fig. 2C) increased (*P* < 0.01) in the early proestrous phase compared to those in the juvenile phase. Figure 3A illustrates that these changes in the levels of IGF-I mRNA were followed by

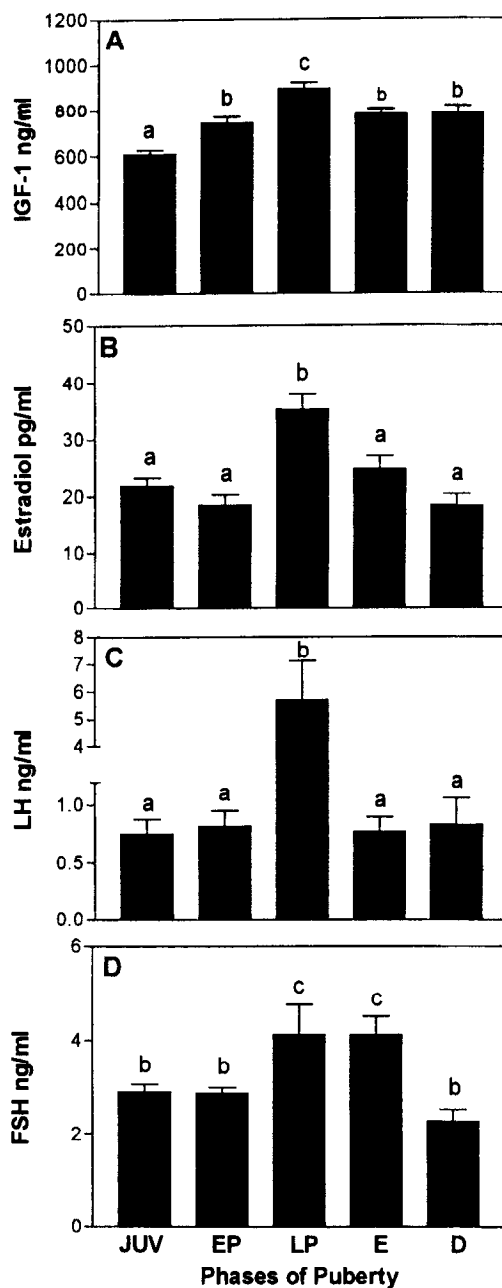


FIG. 3. Serum IGF-I, E₂, and gonadotropin levels during pubertal development. A, Serum IGF-I levels increased significantly during early proestrous (EP), peaked during late proestrous (LP), and remained elevated over juvenile (JUV) levels through first estrus (E) and diestrus (D). B–D, Serum levels of E₂, LH, and FSH, respectively, were also elevated significantly during LP. Note that elevated FSH levels could be seen during E. a vs. b and c, *P* < 0.001; b vs. c, *P* < 0.05. Differences between groups were analyzed using ANOVA with *post-hoc* testing using Student-Newman-Keuls multiple range test. n = 40, JUV; n = 25, all other stages.

an increase in the mean concentration of serum IGF-I, which increased slightly on early proestrous and peaked during late proestrous (*P* < 0.001), then remained elevated (*P* < 0.01) over juvenile levels through the first diestrus. Figure 3, B–D, shows that the increase in serum IGF-I during late proestrous was accompanied by the increases in E₂ (*P* < 0.001), LH (*P* < 0.001), and FSH (*P* < 0.05). As depicted in Fig. 4, A and B, the

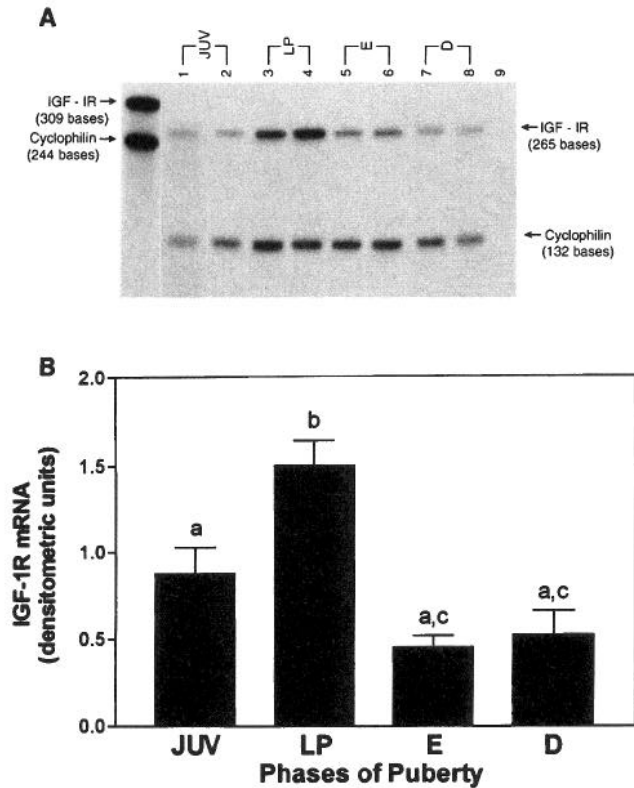


FIG. 4. Detection of peripubertal levels of IGF-I receptor mRNA in the ME by RNase protection assay. A, Composite of autoradiograms from two assays showing detection of protected IGF-IR (265 bases) mRNA by hybridizing 8 μ g total ME RNA with a 32 P-labeled antisense IGF-I receptor mRNA probe. The far left lane shows the undigested full-length RNA probes for IGF-IR (309 bases) and cyclophilin (244 bases). The protected bands for IGF-IR mRNA and cyclophilin are 265 and 132 bases, respectively. Experimental lanes 1–8 depict samples from juvenile (JUV), late proestrous (LP), estrous (E), and diestrous (D) animals. Lane 9 shows that no hybridization occurred when only yeast tRNA was used in the presence of RNase-1 as the negative control. B, Composite graph demonstrating the densitometric quantitation of the bands from different autoradiograms. There were a total of five lanes for JUV, seven for LP, six for E, and five for D. Each autoradiogram scanned contained samples from all groups, and each sample (lane) analyzed represents a pool of eight MEs. Note that the level of expression of the IGF-I receptor mRNA in the ME increased during LP compared to JUV levels (a vs. b, $P < 0.01$). These levels then decreased during E and D (b vs. c, $P < 0.001$). Differences between groups were analyzed using ANOVA with *post-hoc* testing using Student-Newman-Keuls multiple range test.

late proestrous elevation in serum IGF-I was accompanied by a marked increase ($P < 0.001$) in IGF-IR mRNA levels in the ME. This increase in receptor mRNA abundance was followed by a marked decline ($P < 0.001$) during the first estrous and diestrous phases of puberty (Fig. 4). IGF-IR mRNA levels in the ME during early proestrous were not measured in this case because we specifically wanted to determine whether the receptor mRNA increased in association with the peak serum IGF-I and LH levels that occurred during late proestrous. To determine the hypothalamic specificity of the proestrous phase increase in receptor mRNA expression, a comparison was made of hypothalamic fragments (*i.e.* MBH without the ME, and the POA) from juvenile and late proestrous animals only. Figure 5, A and B, demonstrates

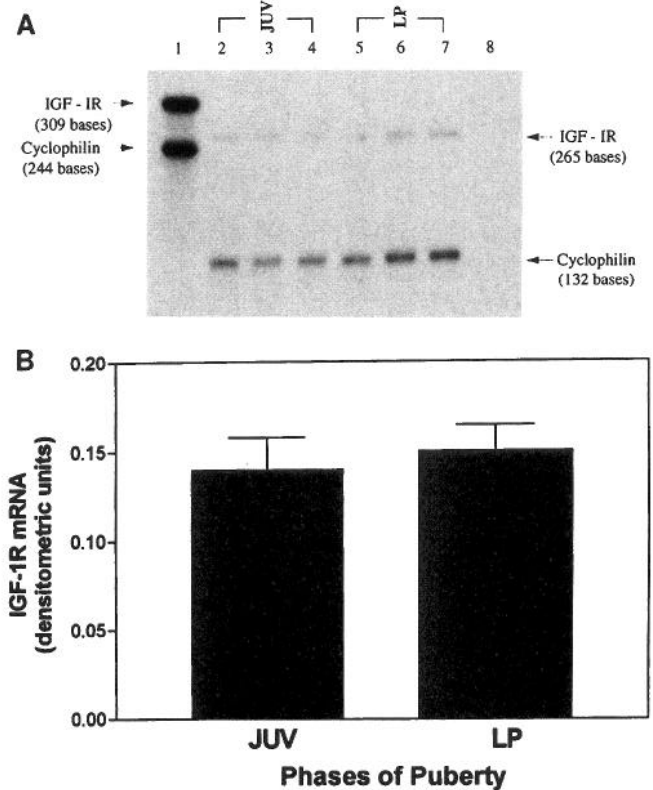


FIG. 5. Detection of juvenile and late proestrous levels of IGF-I receptor mRNA in the MBH (without the ME) by RNase protection assay. A, Autoradiogram showing detection of protected IGF-IR (265 bases) mRNA by hybridizing 15 μ g total MBH RNA with a 32 P-labeled antisense IGF-I receptor mRNA probe. The far left lane shows the undigested full-length RNA probes for IGF-IR (309 bases) and cyclophilin (244 bases). The protected bands for IGF-IR and cyclophilin mRNAs are 265 and 132 bases, respectively. Experimental lanes 1–3 depict samples from juvenile (JUV) animals, and lanes 4–6 depict samples from late proestrous (LP) animals. Lane 7 shows that no hybridization occurred when only yeast tRNA was used in the presence of RNase-1 as the negative control. B, Densitometric quantitation of the autoradiographic bands shown in A. Each sample (lane) analyzed represents a pool of three MBHs and a total of three lanes for JUV and three lanes for LP. Unlike the ME alone (see Fig. 4), no increase in the level of expression of the IGF-IR mRNA occurred in the MBH without the ME between JUV and LP.

that the increase during proestrous was specific to the ME, as it was not detected in the MBH without the ME. Furthermore, no change in IGF-IR mRNA was detected in the POA (not shown).

Exp 2: IGF-I-induced LH secretion during juvenile development

Figure 6 depicts representative LH secretory profiles from juvenile females that received a 3V injection of either saline or IGF-I. Neither saline nor the lowest dose of IGF-I (1 ng; not shown) altered preinjection LH levels, whereas IGF-I doses of 2, 20, and 200 ng were all capable of stimulating LH release over their respective preinjection levels. Figure 7 demonstrates the combined data from each group, showing the arithmetic means of the areas under the LH curves of secretion. As can be seen, the 2-, 20-, and 200-ng doses of IGF-I all caused significant increases in LH levels, with the 2-ng dose

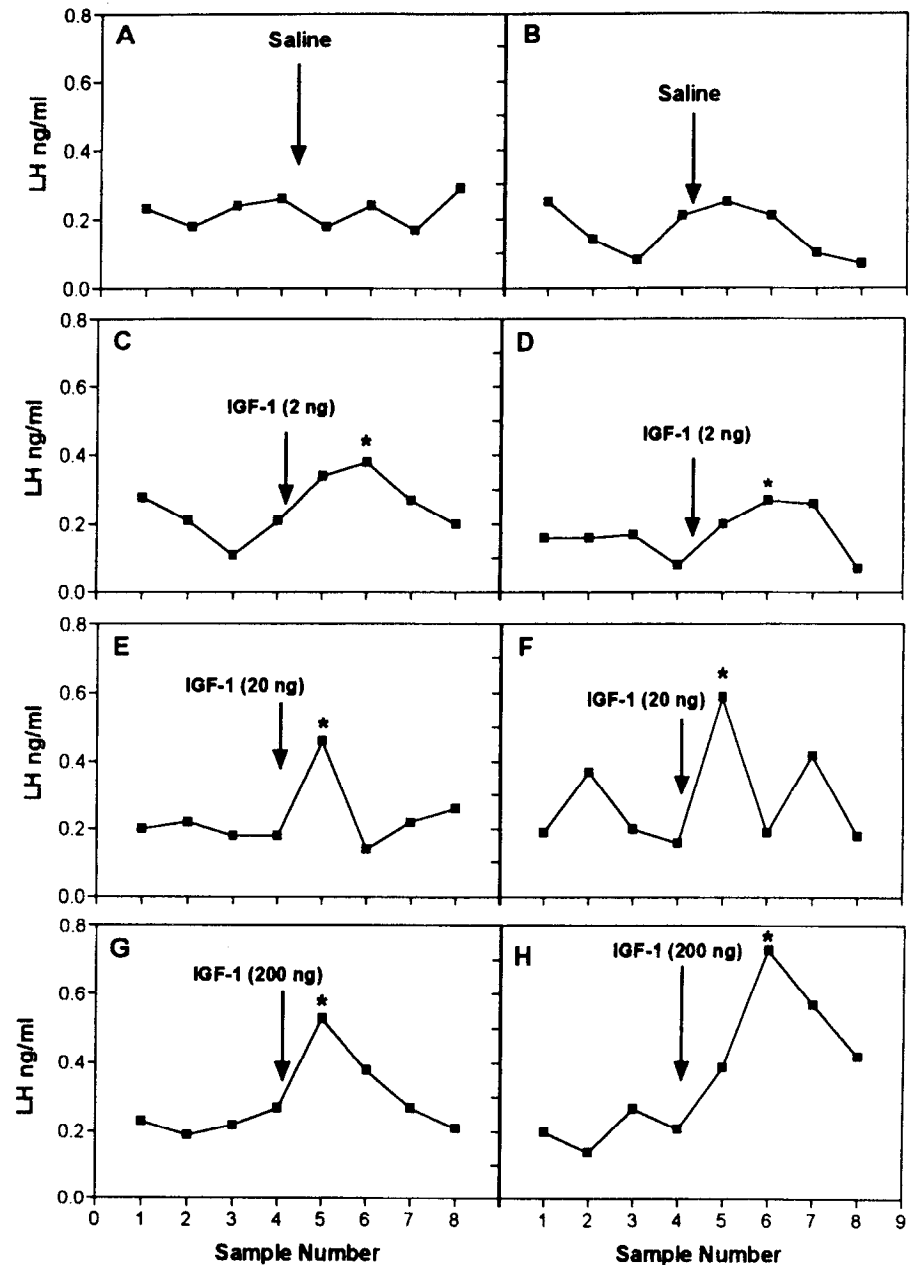


FIG. 6. The effect of 3V administration of IGF-I on LH release from late juvenile female rats. Representative LH secretory profiles from rats before and after receiving saline or one of the designated doses of IGF-I. The animals that received saline (A and B) showed no significant changes in LH secretion compared with basal levels. Conversely, the animals that received 2 ng (C and D), 20 ng (E and F), and 200 ng (G and H) doses of IGF-I showed a marked postinjection increase in LH secretion. *, The maximum LH response determined by Prism software. Arrows denote the injection of either saline or the respective IGF-I dose after the fourth 10 min sample.

being least effective ($P < 0.05$) and the 20- and 200-ng doses both resulting in similar ($P < 0.001$) responses. It is important to note that not all of the animals showed a significant LH response to IGF-I. Specifically, the 2-ng dose of IGF-I only stimulated a 2-fold increase in LH release in 4 of 9 animals, whereas the 20- and 200-ng doses were both able to stimulate from 2- to 4-fold increases in 11 of 15 animals. In all cases of stimulation, however, the LH response was noted between 10–30 min postinjection.

Immunoneutralization of LHRH via 3V administration of LHRH antiserum also indicated a hypothalamic site of action of IGF-I to induce LH release. Figure 8, A–D, depicts representative LH secretory profiles of individual animals that received either NRS plus IGF-I (A and B) or anti-LHRH plus IGF-I (C and D). Figure 8E shows the combined data from all

animals in each group, demonstrating that the IGF-I-induced LH response was blocked by the anti-LHRH serum, but not by NRS.

Exp 3: IGF-I-induced LH secretion during peripubertal development

Figure 9 depicts representative secretory profiles of LH from animals in first proestrus, estrus, and diestrus after the acceleration of puberty by PMSG administration. These results indicate that IGF-I is able to induce LH secretion during each of the phases of puberty. Figure 10 demonstrates composite LH profiles from all animals in each of the three peripubertal groups. The areas under the respective LH curves of secretion during proestrus were similar to those

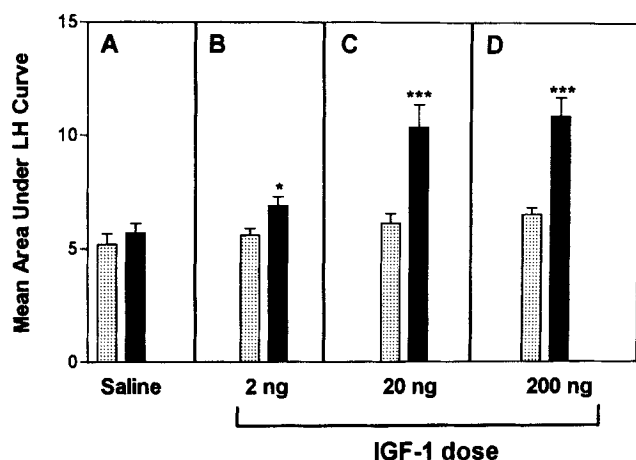


FIG. 7. Composite drawing using all rats per group and showing the effect of IGF-I on the mean areas under the respective LH curves of secretion. For each panel, the *hatched bar* represents the arithmetic mean (\pm SEM) of the area under the basal or preinjection segment (samples 1–4) of the LH curve, and the *solid bar* represents the arithmetic mean (\pm SEM) of the area under the postsaline or IGF-I injection segment (samples 5–8) of the LH curve. Note that the saline-injected animals (A) showed no change in the area under the postinjection segment of the LH curve, whereas the IGF-I-injected animals (B–D) showed postinjection increases in the areas under their respective LH curves. *, $P < 0.05$; ***, $P < 0.001$ (by Student's *t* test). $n = 11$ for saline, and 9, 15, and 15 for the 2-, 20-, and 200-ng doses of IGF-I, respectively.

depicted above in the juvenile animals, and the most prominent response was observed during first estrus, *i.e.* the day of first ovulation. The LH response in diestrus, however, was much lower than that detected at the earlier developmental stages.

Exp 4: daily 3V injections of IGF-I advanced the onset of puberty

To determine the effect of IGF-I on the timing of puberty, a 20-ng dose of IGF-I was administered via the 3V twice daily, beginning on day 28. Figure 11 demonstrates that VO occurred earlier ($P < 0.001$) in the six animals given IGF-I (34.0 ± 0.36 days of age) than in the seven animals given saline (38.9 ± 0.40 days of age). The daily weight gains for the IGF-I-treated and control animals through day 34 (mean age at VO when the IGF-I-treated rats were killed) were not significantly different (not shown). At the time of VO, vaginal smears were taken, the uterus was assessed for presence of fluid and weighed, and the ovaries were inspected for signs of ovulation. Using these data and previously described criteria (23), we were able to assess each animal's phase of pubertal development on their respective day of VO. Of the six animals that received IGF-I, three were in proestrus (*i.e.* their uteri had large amounts of intraluminal fluid and weighed >300 mg, and the ovaries contained numerous follicles), two were in estrus (*i.e.* uteri had no fluid and weighed <200 mg, the vagina was open with a cytology showing cornified cells, and corpora lutea were present on the ovaries), and one was designated as being pseudo-diestrus (*i.e.* atypical vaginal cytology and no corpora lutea present on the ovary). Of the seven animals that received saline, four were in proestrus and three were in estrus.

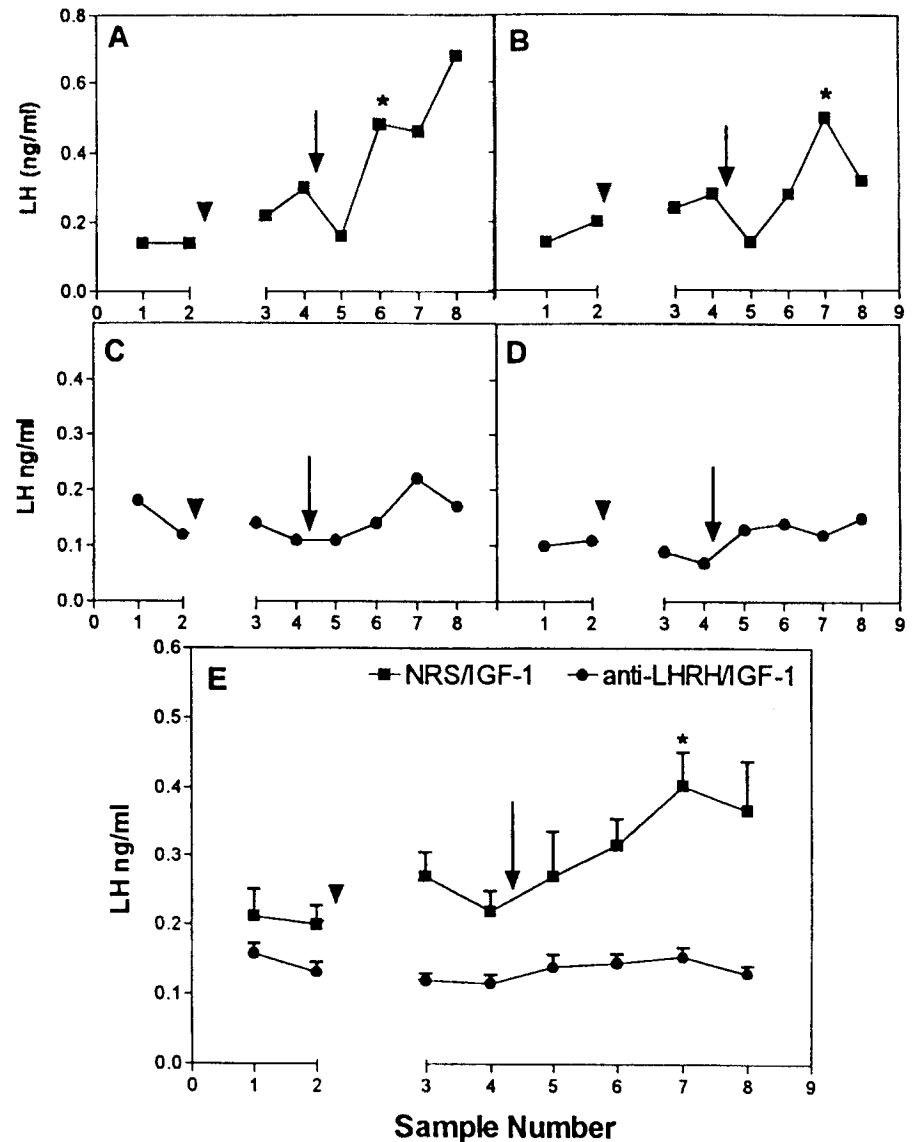
Discussion

It has been hypothesized for many years that somatic development may influence sexual maturation via metabolic signals that can act centrally to modify neuronal function (for review, see Refs. 2 and 34). Results from a previous *in vitro* study (15) lead us to suggest that IGF-I may be one of the signals linking the development of the somatotrophic axis to activation of the LHRH/LH-releasing system during female puberty. In the present *in vivo* study, we show that the initiation of female puberty is associated with an increased expression of the IGF-I gene in liver, but not in hypothalamus, and with elevated levels of IGF-I peptide in the peripheral circulation. We also show that central administration of the peptide elicits LH release, that this increase is prevented by immunoneutralizing LHRH actions, and, more importantly, that the exposure of the juvenile hypothalamus to increased IGF-I levels significantly advances the timing of puberty.

In both the hypothalamus and liver, IGF-Ia mRNA is the predominant mRNA transcript found. In agreement with the study of Lowe, Jr., *et al.* (35), the abundance of IGF-Ib mRNA in the liver was much greater than that in the hypothalamus, a region in which IGF-Ib mRNA levels were negligible. GH stimulation of liver IGF-I synthesis has been shown to coordinately increase the levels of both IGF-Ia and IGF-Ib mRNA species, with a 3-fold greater increase in the abundance of IGF-Ib than IGF-Ia transcript (35). Our results are in keeping with these findings, as the liver contents of both mRNA transcripts increased during the early proestrous phase of puberty, with the greatest fold increase occurring in the levels of IGF-Ib mRNA. As GH levels increase during late juvenile development in the female rat (36), it appears plausible that these peripubertal changes in liver IGF-I gene expression are caused by the elevated serum levels of GH that accompany the pubertal process.

The peripheral increase in liver IGF-I synthesis was closely followed by an elevation of the circulating levels of IGF-I, suggesting that a significant fraction of the increase in serum IGF-I results from an enhanced secretion of the peptide by liver cells. The change in serum IGF-I levels lagged behind the activation of IGF-I gene expression in liver, so that maximal values were detected at the time of first (late) proestrus, coinciding with the augmentation in serum E_2 and gonadotropin levels found at this time. Earlier studies in primates suggested that the peripubertal increase in serum IGF-I is induced by gonadal steroids (7–9). In the rat, however, this change appears to be gonad independent, as it occurs even in the absence of the gonads (3). Whatever the case, the increase in serum IGF-I levels at this time of heightened hypothalamic-pituitary-ovarian activity, particularly on the day of first proestrus, suggests that the peptide contributes to the pubertal process. The ability of IGF-I and its subproducts to inhibit LHRH release from the hypothalamic fragments *in vitro* (21) indicates that such a role might be of an inhibitory nature. Exposure of isolated MEs to IGF-I, however, results in an increase in LHRH secretion (15), suggesting that an increase in IGF-I availability to the hypothalamic region where the nerve terminals of LHRH neurons converge enhances, instead of suppresses, secretion of the neuropep-

FIG. 8. Effect of immunoneutralization of LHRH biological activity on IGF-I-induced LH release. A–D, Representative LH secretory profiles from juvenile rats before and after receiving either NRS (■) plus IGF-I (200 ng; A and B) or anti-LHRH serum (●) plus IGF-I (C and D). E, The combined data from all animals in each group. Each point represents the mean \pm SEM LH values for all animals in each group. For each panel, note that the animals that received NRS showed a significant post-IGF-I increase in LH release, whereas this increase was blocked in the animals that received the anti-LHRH serum. *, The maximum LH response determined by Prism software. Arrowheads denote the 3V injection of NRS or anti-LHRH serum after the second sample. The break between samples 2 and 3 is a 60-min absorption phase. Arrows denote the 3V injection of IGF-I after the fourth sample. *, $P < 0.05$, by Student's *t* test. $n = 8$ animals receiving NRS/IGF-I and $n = 12$ animals receiving anti-LHRH/IGF-I.



tide. The relevancy of this *in vitro* finding to the physiological situation at the time of puberty is suggested in the present study by the marked increase in IGF-IR mRNA levels that occurs in the ME on the day of first proestrus; however, it will no doubt be important to measure changes in IGF-I receptor protein and binding before a more complete assessment of function can be made.

With regard to the ME, we determined that IGF-IR mRNA levels show a transient increase during late proestrus at the same time that serum IGF-I levels peak. Conversely, Bohannon *et al.* (37) showed that IGF-IR binding in the ME was correlated to low levels of serum IGF-I. This difference may be due to the fact that Bohannon and colleagues used adult male rats undergoing food restriction, whereas we used peripubertal females. Also, Pons *et al.* (38) have shown in males that type 1 receptors were highest during fetal life, then declined steadily to reach low adult levels; however, they measured whole hypothalamic samples and not the ME specifically and did not directly assess changes during the female peripubertal period. Additionally, Michels and Saave-

dra (39) measured the binding of IGF-I at the level of the ME in males on the day of birth and subsequently when the rats were 5, 10, 20, 45, and 270 days of age. They found an increase only between days 5 and 10, but they also did not measure binding during the peripubertal phase of development.

As the ME is devoid of a blood-brain barrier, synthesizes high concentrations of IGF-I receptors (16–20), and contains the LHRH nerve terminals, it is likely that this region is a primary site of action for peripherally derived IGF-I to activate LHRH/LH secretion at puberty. The fact that IGF-I is capable of inducing LHRH release directly from the ME has been shown (15) and will be discussed in further detail below. It does not appear that this is a direct action of IGF-I on the LHRH nerve terminals, because immortalized LHRH neurons have been recently shown to be unresponsive to IGF-I (40). It is more likely that IGF-I enhances LHRH release via a glial intermediacy, because the ME does not contain neuronal cell bodies but does have glial elements that express a high concentration of IGF-IR mRNA (18, 20). Additionally, Duenas *et al.* (41) found that during the afternoon of late

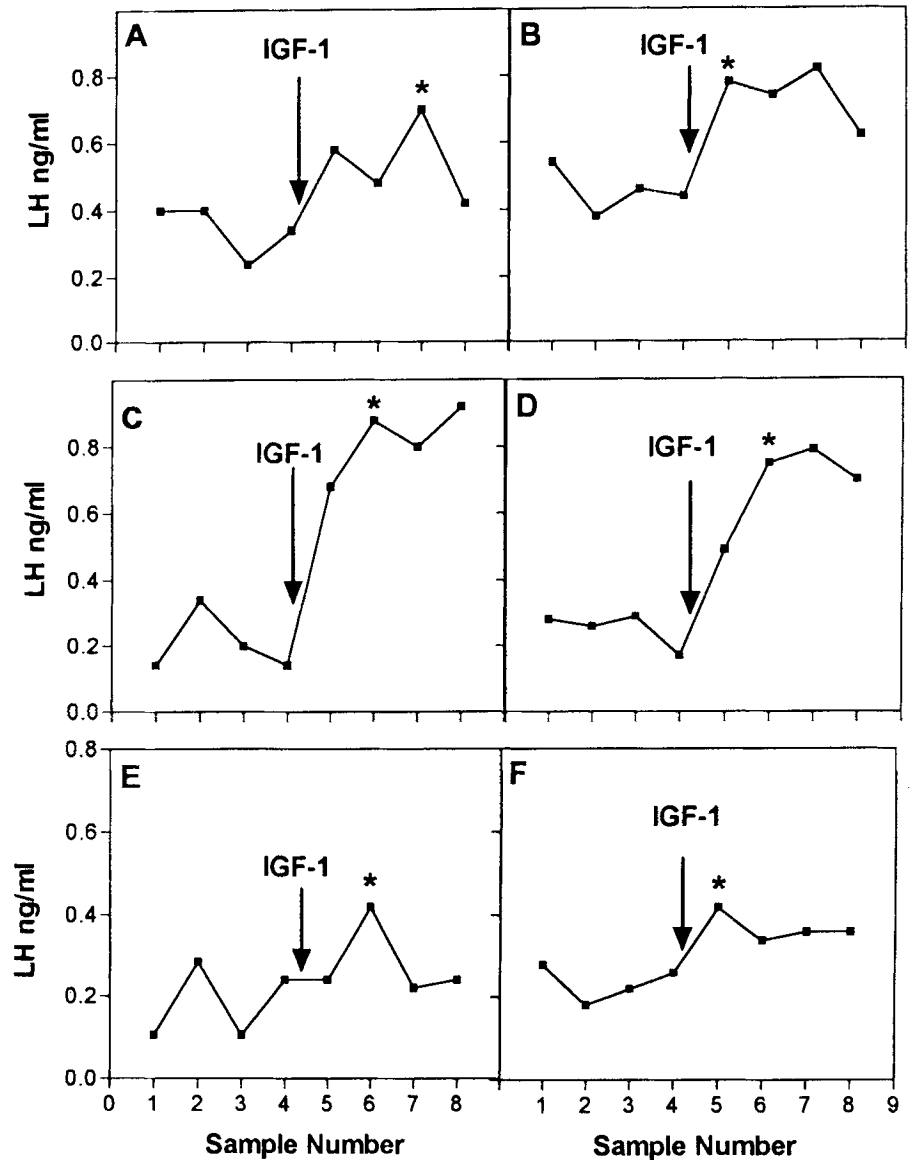


FIG. 9. The effect of 3V administration of IGF-I on LH release during the peripubertal phase of development. Representative LH secretory profiles from rats before and after receiving a 200-ng dose of IGF-I are shown. Note that IGF-I is capable of inducing a marked increase in LH secretion during early proestrus (A) and late proestrus (B) as well as during estrus (C and D) and diestrus (E and F). *, The maximum LH response determined by Prism software. Arrows denote the injection of IGF-I after the fourth sample.

proestrus, a marked increase in the surface density of the IGF-I-like immunoreactivity occurred in the astroglial elements of the ME and suggested that this was due to the uptake of IGF-I from the peripheral circulation. Furthermore, it was shown that this increase in IGF-I-like immunoreactivity is developmentally regulated and increased during sexual development (41). This information along with the results of the present study support the idea that elevated peripheral levels of IGF-I, which occur at the onset of puberty, can act directly at this time on glial elements within the ME to activate the LHRH/LH-releasing system.

Vital to the hypothesis that IGF-I is involved in the hypothalamic control of LH secretion at the time of puberty was our ability to show that intraventricular administration of small amounts of IGF-I was capable of stimulating LH release from both juvenile and peripubertal animals. We previously reported that systemic administration of IGF-I to prepubertal animals can significantly stimulate LH release (22); however, we determined that this route of delivery was not as efficient

or effective as the central route of delivery depicted by the present results. This was most likely due to peripheral competition (of the exogenous IGF-I) with binding proteins and/or endogenous IGF-I. Our present results indicate that centrally administered IGF-I stimulated similar LH responses during both juvenile and proestrous phases. It is possible that even though receptor mRNA levels were elevated during the afternoon of proestrus, the translation to protein or the binding may not have changed significantly (compared to juvenile animals) at that specific time point; hence, it will also be important to determine whether a greater LH response could be seen during the evening of late proestrus. The greatest LH response to IGF-I observed in the present study was during first estrus. This may be the result of an elevation in available receptors after the significant increase in receptor mRNA noted during late proestrus or due to enhanced sensitivity of receptors at a time of attenuated receptor synthesis. More information regarding LH responsiveness to IGF-I at specific times of proestrus and

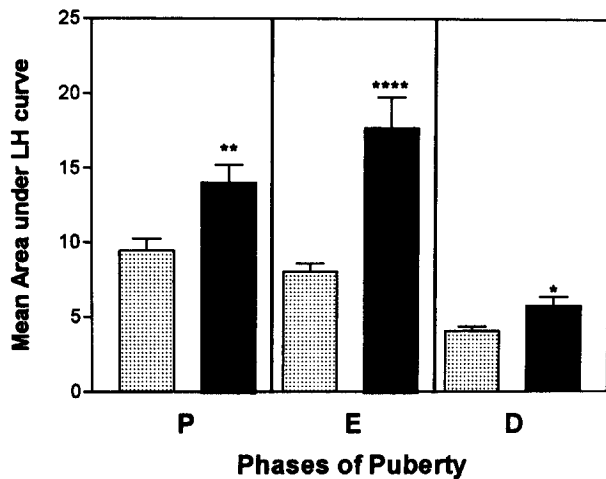


FIG. 10. Composite drawing, using all peripubertal animals per group, showing the effect of IGF-I on the mean areas under the respective LH curves of secretion. For each panel, the *hatched bar* represents the arithmetic mean (\pm SEM) of the area under the basal or preinjection segment (samples 1–4) of the LH curve, and the *solid bar* represents the arithmetic mean (\pm SEM) of the area under the post-IGF-I injection segment (samples 5–8) of the LH curve. Note that during each of the three peripubertal stages of development, IGF-I-induced a postinjection increase in the area under the LH curve of secretion, with the greatest responses being from animals in first proestrus (P; represents combined data from animals in EP and LP, since their responses were similar) and estrus (E), and the lowest response being from animals in first diestrus (D). *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$ (by Student's *t* test). $n = 14, 15,$ and 15 for P, E, and D, respectively.

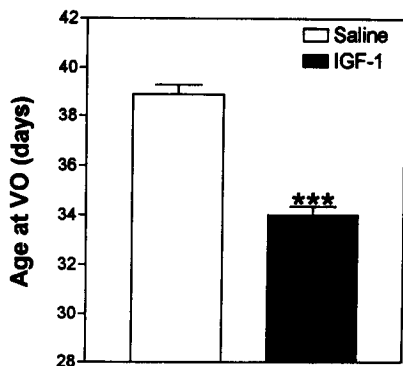


FIG. 11. The effect of daily 3V administration of IGF-I on the onset of female puberty. *Open and solid bars* represent the mean (\pm SEM) age in days at VO for the saline- and IGF-I-treated animals, respectively. Note that VO was advanced by 4.9 days in the IGF-I-treated animals. ***, $P < 0.001$, by Student's *t* test. $n = 6$ animals receiving IGF-I; $n = 7$ animals receiving saline.

estrus is needed, and this is a subject of our current investigation.

As noted in *Results*, not all of the animals responded to the IGF-I injection with LH release. This could be due to differences in bioavailability of the injected peptide caused by dissimilar circulating and/or tissue levels of IGF-binding proteins at the time of the injection or to differences in the rate of peptide transcytosis across endothelial cells of the ME. Production of IGF-I binding protein-3, which binds most endogenous IGF-I, varies greatly among different tissues (42), and IGF-I is known to access the brain by first binding

to receptors located on endothelial cells (43). As blood-borne IGF-I penetrates the blood-brain barrier, reaching hypothalamic regions such as the paraventricular and supraoptic nucleus (43), a lack of LH response to IGF-I may also reflect the activation of inhibitory mechanisms controlling LHRH secretion in hypothalamic regions away from the ME, such as the arcuate nucleus (21).

Kanematsu *et al.* (44) showed that IGF-I can induce LH and FSH release from primary pituitary cultures and enhances the LHRH-induced release of LH. We have now shown that immunoneutralization of hypothalamic LHRH can inhibit the increase in LH released after the 3V injection of IGF-I, thus demonstrating *in vivo* that IGF-I acts, via a centrally mediated mechanism, to stimulate LH release. These data lend support to the findings of our earlier study, which showed that IGF-I can induce LHRH release from ME fragments of immature female rats (15). Our findings differed from the *in vitro* data generated by Bourguignon *et al.* (21), who reported that the entire IGF-I peptide was unable to induce LHRH release from arcuate nucleus/ME fragments of male and female rats, whereas the truncated N-terminal tripeptide, IGF-I-(1–3), inhibited LHRH release. The principal difference between the results of these two studies is attributed to the respective tissue fragment used and, hence, to potentially different anatomical sites of IGF-I action. Data from the present *in vivo* study as well as our *in vitro* study (15) support the hypothesis that peripherally derived IGF-I is capable of acting directly at the level of the ME in an endocrine manner to elicit LHRH secretion during pubertal development. The *in vitro* study by Bourguignon *et al.* (21) suggests that the inhibitory action of the truncated IGF-I on LHRH release is due to either paracrine actions of brain-derived IGF-I or an inhibitory effect of peripherally derived IGF-I exerted on hypothalamic regions such as the arcuate nucleus. Interestingly, we recently administered the truncated peptide *in vivo*, via the 3V, and found that LH was stimulated (unpublished observations) in a similar manner to that achieved with the whole molecule. Furthermore, we reported that during pubertal development, there were no changes in the levels of expression of IGF-I mRNA in the reproductive areas of the brain, which included the arcuate nucleus. Thus, based on the information to date, we suggest that the endocrine action of peripherally derived IGF-I to induce LHRH secretion from the ME may override any inhibitory paracrine or endocrine action of IGF-I during this developmental period.

Previous studies have shown that administration of IGF-I via osmotic minipumps can increase organ and body weights (45, 46), and that suppression of IGF-I production via immunoneutralization of GH-releasing hormone action does not affect the timing of sexual maturation in female rats (47). Our findings are at odds with these observations, as we have now shown that IGF-I is capable of advancing the onset of female puberty. To simulate the enhanced afternoon increase in LH levels that occurs before puberty (48, 49), we administered IGF-I twice daily in the afternoon to juvenile animals. This method of IGF-I administration significantly advanced VO by almost 5 days compared with that in controls. Also, it is important to note that of the six IGF-I-treated animals, five were in either first proestrus or estrus phases of pu-

erty at the time of VO. These results indicate that not only did precocious VO occur, but the animals were showing maturational changes known to be associated (23) with the advent of sexual maturation. Also, our results indicated that the central route of administration of small doses of IGF-I was capable of stimulating the reproductive axis without significantly altering the daily growth patterns of the animals.

In summary, the results from this study support the hypothesis that IGF-I is a metabolic signal capable of activating the LHRH/LH-releasing system at the time of puberty. Specifically, we have shown that hepatic synthesis of the peptide, followed by the secretion of IGF-I into the peripheral circulation markedly increases during puberty, whereas central synthesis does not change. We suggest that the rising circulating levels of IGF-I during late prepubertal development act at the level of the ME, via IGF-I receptors, to facilitate LHRH secretion. Furthermore, our data show that IGF-I stimulates LH secretion during pubertal development via a centrally mediated effect and that recurrent exposure of the hypothalamus to increased IGF-I levels effectively accelerates the timing of pubertal development. A physiological involvement of peripherally produced IGF-I in the central control of female puberty implies that the actions of this growth factor on LHRH release must be tightly linked and coordinated with those facilitatory actions affected by members of the transforming growth factor- α family. Evidence exists that some of these tropic factors are produced by hypothalamic glial cells and can facilitate LHRH secretion at puberty via paracrine effects involving the glial production of neuroactive substances, such as PGE₂ (50, 51).

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