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·Original Article·

Gonadotrophin-releasing hormone-I and -II stimulate steroidogenesis in prepubertal murine Leydig cells *in vitro*

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Abstract

Aim: To study the effect and mechanism of gonadotrophin-releasing hormone (GnRH) on murine Leydig cell steroidogenesis. **Methods:** Purified murine Leydig cells were treated with GnRH-I and -II agonists, and testosterone production and steroidogenic enzyme expressions were determined. **Results:** GnRH-I and -II agonists significantly stimulated murine Leydig cell steroidogenesis 60%–80% in a dose- and time-dependent manner ($P < 0.05$). The mRNA expressions of steroidogenic acute regulatory (StAR) protein, P450scc, 3β-hydroxysteroid dehydrogenase (HSD), but not 17α-hydroxylase or 17β-HSD, were significantly stimulated by both GnRH agonists with a 1.5- to 3-fold increase ($P < 0.05$). However, only 3β-HSD protein expression was induced by both GnRH agonists, with a 1.6- to 2-fold increase ($P < 0.05$). **Conclusion:** GnRH directly stimulated murine Leydig cell steroidogenesis by activating 3β-HSD enzyme expression. *(Asian J Androl 2008 Nov; 10: 929–936)*

Keywords: gonadotrophin-releasing hormone; Leydig cells; murine; steroidogenesis; stimulation

1 Introduction

Gonadotrophin-releasing hormone (GnRH), a decapeptide in the hypothalamus, plays a pivotal role in regulating reproduction by stimulating the biosynthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary [1]. Several GnRH molecules have been found in vertebrates and invertebrates as multiple forms [2]. Up to now, two forms of GnRH (GnRH-I and GnRH-II) have been demonstrated with distinct functions among various species and tissues [3]. Studies have illustrated the presence of GnRH or GnRH-like material in cultured rat Sertoli cells and testis [4, 5] and the existence of GnRH-R in rat interstitial cells and testis [5–7]. It has also been well demonstrated that GnRH-R is expressed in Leydig cells [1], and the binding activity is present in rat Leydig cells [8]. Thus far, no direct evidence of the existence of GnRH-I and GnRH-II in the murine testis has been demonstrated. However, in humans, expression of GnRH-I in Sertoli cells and GnRH-R in Leydig cells has been shown [3], indicating that GnRH may function in testicular steroidogenesis.

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It is known that short-term *in vitro* treatment of GnRH stimulates testosterone production by adult rat Leydig cells [9], whereas long-term incubation decreases the response to human chorionic gonadotrophin (hCG) [10]. In addition, GnRH or its agonist induced paradoxical effects on testosterone secretion in adult hypophysectomized rats [11] by inhibiting basal and LH-dependent steroidogenesis in rat fetuses *in vivo* [12]. Some studies from various frog species reveal that GnRH agonists or GnRH-like materials have positive effects on testosterone production [13]. These observations suggest that GnRH has direct effects on Leydig cells, and these effects might be species-specific.

Up to now the cellular responses to the GnRH within Leydig cells are yet to be determined, and few studies are available concerning the biological functions of testicular GnRH in the mouse. Although some reports have demonstrated the absence of a direct effect and binding activity of GnRH or GnRH agonists on murine testis and Leydig cells [14], we now demonstrate that GnRH-I and GnRH-II agonists significantly stimulate purified murine Leydig cell testosterone production dose- and time-dependently. Furthermore, the present study attempts to determine the mechanism of how GnRH could activate murine Leydig cell steroidogenesis.

2 Materials and methods

2.1 Animals

Male B6 (C57BL/6NCrj) mice, 5–7 weeks old, were purchased from National Cheng Kung University Animal Center (Tainan, Taiwan, China). Juvenile B6 mice should be less than 8 weeks old for the experiment to exclude the exposure of Leydig cells to endogenous LH. All animals were housed in groups of four in 29 × 18 × 13 cm polyethylene cages. The animal room was maintained at 22–24ºC under a constant 12 h:12 h light : dark cycle. Purina mouse chow (Ralston-Purina, St. Louis, MO, USA) and water were always available. The procedure for sacrificing animals was approved by the counselors of the National Cheng Kung University Animal Center.

2.2 Leydig cell isolation

Testes were removed from cervically dislocated mice and decapsulated in M199 (Gibco/BRL, Gaithersburg, MD, USA) (1 L containing 4 mmol/L $NaHCO_3$, 25 mmol/L HEPES, 0.06 g penicillin, 0.05 g streptomycin and 0.2% [w/v] bovine serum albumin [BSA], pH 7.35). After decapsulation, the testes were incubated in a shaking water-bath (120 cycles/min) at 37ºC in M199 containing 1% [w/v] BSA and 100 U/mL collagenase for 15 min. After incubation, cold M199 was added to stop the action of collagenase. Seminiferous tubules were separated from interstitial cells by gravity sedimentation. Cells were then collected by centrifugation (300 × *g* for 6 min) and resuspended in 2 mL M199 containing 0.1% [w/v] BSA. This suspension, which comprised interstitial cells only, contained 20%–30% Leydig cells. This interstitial cell preparation was layered onto a Percoll gradient and then centrifuged at 800 × *g* at 4ºC for 20 min. The gradient, which was performed by centrifugation at 25 000 × *g* for 40 min, contained 10 mL isotonic Percoll solution (40%) and 15 mL M199 plus 0.1% [w/v] BSA and 25 mmol/L HEPES. A 1 mL fraction of gradient was collected from the top. Murine Leydig cells were mainly distributed in fractions 23–25. The total number of cells and the percentage of 3β-hydroxysteroid dehydrogenase-positive cells were determined in this Leydig cell preparation [15]. The purity of the Leydig cells was 85%–90%.

2.3 Cell culture

Cells were maintained at 37ºC in a humidified environment containing 95% air and 5% CO_2 for all the following experiments. In total, 5×10^4 cells/100 µL medium (for radioimmunoassay [RIA]), 1×10^6 cells/mL medium (for reverse transcriptase-polymerase chain reaction [RT-PCR]) or 2.5×10^5 cells/mL medium (for Western blot) were plated into 96 well plates or 3.5 cm dish (Techno Plastic Products AG, Trasadingen, Switzerland), respectively.

For the dose- and time-course effects regarding testosterone production, cells were treated with various concentrations of GnRH-I agonist (0.01 pmol/L, 10 pmol/L, 1 nmol/L, 100 nmol/L) or GnRH-II agonist (100 pmol/L, 1 nmol/L, 10 nmol/L, 100 nmol/L) for 24 h, or cells were treated with or without GnRH-I agonist (1 nmol/L) or GnRH-II agonist (10 nmol/L) for various times (0, 3, 6, 12, 24 and 36 h). At the end of incubation, media were withdrawn and testosterone levels were determined by RIA.

In RT-PCR and Western blot experiments, cells were treated with 1 nmol/L GnRH-I agonist, (D-Trp6)-GnRH (Sigma-Aldrich, St. Louis, MO, USA) or 10 nmol/L GnRH-II analog, D-Arg(6)-Azagly(10)-NH2 (Peninsula Laboratories, Belmont, CA, USA), respectively, for 12 or 24 h. At the end of incubation, the expression of

mRNA (steroidogenic acute regulatory [StAR], P450scc, 17α-hydroxylase, 3β-hydroxysteroid dehydrogenase [HSD] and 17β-HSD) and protein (StAR, P450scc and 3β-HSD) were determined.

2.4 RIA

Media from cultures with different treatments were collected and diluted with medium to fall within the standard curves of the respective assays. Twenty μL of diluted sample was withdrawn into a glass tube and 100 μL each of testosterone antiserum (a generous gift from Dr Paulus S. Wang, National Yang Ming University, Taipei, Taiwan, China) and ^{3}H-testosterone (70 Ci/mmol) were added. An equilibrium reaction occurred at room temperature for 2 h, which was stopped by putting the tubes in ice. Charcoal was added and incubated for 15 min at 4ºC and then centrifuged at 12 000 × *g* for 10 min to centrifuge the charcoal bound with free ^{3}H-testosterone [16]. The supernatant was poured into 2 mL of scintillation fluid and samples were counted in a β-counter (Beckman Coulter Instruments, Fullerton, CA, USA) for 2 min.

2.5 Isolation of RNA and RT-PCR

Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. In brief, culture media was discarded and cells were lyzed in 1 mL Trizol. Then 200 mL chloroform was added to the sample to separate it into an aqueous phase (RNA), interphase (DNA) and organic phase (protein). The aqueous phase was transferred to a new vial to avoid contamination with the DNA and protein fractions. The RNA was then precipitated by adding 500 mL isopropyl alcohol. After centrifugation at 12 000 × *g* for 10 min, the supernatant was removed and the RNA pellet was washed with 75% (v/v) ethanol. Finally, the RNA was dissolved in 10 μL diethylpyrocarbonate (DEPC)-treated double distilled water and stored at –80ºC until used. The concentration of the RNA was determined by absorbance using 260 nm (A260).

Reverse transcription (RT) was performed in a mixture containing 5 μmol/L random primer (primer sequence and corresponding sequence of specific genes are listed in Table 1), 200 mol/L deoxyribonucleotide triphosphate (dNTP), 2U/μL moloney murine leukemia virus (MMLV) RT together with 5 μL RNA (3 ng) as the template. The corresponding buffer was prepared at 42ºC for 90 min and then at 95ºC for 10 min. PCR was performed in a mixture containing 2 μL 10 × PCR buffer, 0.4 μL 10 mmol/L dNTP, 0.4 μL 20 mmol/L specific forward and reverse primers, 14.7 μL ddH$_2$O, 0.1 μL 0.5 U Taq with 2 μL RT product as template for each reaction. Thermo-controlling program consisted of 95ºC for 30 s, 55ºC for 30 s (annealing), 72ºC for 30 s and another 5 min of elongation at 72ºC. The whole mixture was subjected to 30 cycles for amplification of L19 (internal control), StAR, P450scc, 3β-HSD, 17α-hydroxylase and 17β-HSD. The PCR product was then separated on a 1.5% w/v agarose gel at 120 V for 30 min in 1 × TBE buffer (0.09 mol/L Tris, 0.09 mol/L boric acid, 0.001 mol/L ethylenediaminetetraacetic acid [EDTA], pH 8.0). The gel was then stained with ethidium bromide (10%) for 10 min

Table 1. Sequence of primers used for reverse transcription-polymerase chain reaction (RT-PCR). L19, internal control; StAR, steroidogenic acute regulatory; HSD, hydroxysteroid dehydrogenase.

	Sequence (5' to 3')	PCR size (bp)
L19	F GAA ATC GCC AAT GCC AAC TC	405
	R TCT TAG ACC TGC GAG CCT CA	
StAR	F ATG TTC CTC GCT ACG TTC AA	451
	R TGA CAT TTG GGT TTC ACT CT	
P450scc	F CGC TCA GTG CTG GTC AAA G	714
	R GGT TGA GCA TGG GGA CAC T	
Cyp17	F CTT GTG GGT CTC TTG CTG CTC AT	655
	R TCT TCA ACC ACG GGA ATA TGT CC	
3β-HSD	F ATG TTG GTG CAG GAG AAA GAA CT	595
	R TGG AGA ATT TGC CAG TAA CAC AC	
17β-HSD	F ACA ACG TTG GAA TGC TCC CCA GC	492
	R GGA ATC GTT GAG CGG TGC TGC TA	

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and destained with double-distilled water. The mRNA of interest in the gel was captured and quantified by using Labwork imager system (Digital CCD Camera, Hamamtsu Photonics system, Bridgewater, NJ, USA). The amount of L19 (405 bp) in each lane was also detected as a control to correct the expression of StAR (451 bp), P450scc714 bp), 3β-HSD (655 bp), 17α-hydroxylase (595 bp) and 17β-HSD (492 bp) proteins.

2.6 Immunoblot analysis

Two hundred and fifty thousand cells were cultured in a 3.5-cm dish. After treatment, cells were rinsed with cold phosphate buffered saline (PBS) and harvested by addition of 30 μL lysis buffer (50 mmol/L Tris-base, 150 mmol/L NaCl, 1% [w/v] NP40, 0.1% [w/v] sodium dodecyl sulfate [SDS], 0.5% [v/v] deoxycholic acid and 1 mmol/L phenylmethanesulfonylfluoride [PMSF]). The cell lysate was subjected to centrifugation at 12 000 × g for 20 min at 4ºC. The supernatant, which contained cell protein, was collected and stored at –20ºC until use. The protein concentration was determined by the Lowry method [17]. Immunoblot analysis was performed as previously described [18]. Antibody against β-actin was purchased from Cell Signaling (Beverly, MA, USA). An antibody generated against residues 89–99 of human and mouse StAR was a gift from Dr Jerome Strauss, III (University of Pennsylvania, Philadelphia, PA, USA) [19]. Antisera against CYP11A1 and 3β-HSD enzymes were generously provided by Dr Bon-Chu Chung (Academia Sinica, Taipei, Taiwan, China) [20]. In brief, 20 μg proteins was solubilized in 1 × SDS sample buffer and loaded on a 12.5% (w/v) SDS-polyacrylamide gel electrophoresis (PAGE) minigel (Mini-Protein II system, Bio-Rad, Richmond, CA, USA). Electrophoresis was performed at 100 V for 100 min using standard SDS-PAGE running buffer. The proteins were transferred to polyvinylidene difluoride membranes (PVDF) (Bio-Rad) at 80 mA for 1 h in transfer buffer. The PVDF membrane with transferred protein was incubated in blocking buffer at room temperature for 1 h, and then incubated in fresh blocking buffer containing the primary antibody for 16–18 h at 4ºC. After washing three times with PBS containing 0.5% [v/v] Tween-20 for 30 min, the signal was detected with a 1 : 4 000 dilution of horseradish peroxidase-conjugated secondary antibody (Amersham, Piscataway, NJ, USA), and visualized with Renaissance chemiluminescence reagent as described by the manufacturer (NEN, DuPont, Boston, MA, USA). Proteins of interest were quantified by a computer-assisted image analysis system (Quantity One, Huntington Station, NY, USA). The amount of β-actin (43 kDa) in each lane was also detected as a control to correct the expression of StAR (30 kDa), P450scc (51 kDa) and 3β-HSD (42 kDa) proteins.

2.7 Data analysis

All data are expressed as mean ± SEM of at least three independent experiments. Statistically significant differences between control and treatments were determined by one-way analysis of variance (ANOVA) and then the least significance difference (LSD). Statistical significance was set at $P < 0.05$.

3 Results

3.1 Dose- and time-dependent effects of GnRH agonists on testosterone production in murine Leydig cells

To determine whether GnRH can modulate testosterone production, purified murine Leydig cells were treated with various concentrations of GnRH-I and -II agonists (0.01 pmol/L to 100 nmol/L) for 24 h and testosterone production was determined by RIA. As shown in Figure 1A and 1B, significant effects of GnRH agonists on testosterone production were observed at 1 nmol/L, causing a ~60% increase ($P < 0.05$). There was no significant difference in testosterone production by either GnRH agonists at concentrations less than 1 nmol/L ($P > 0.05$).

GnRH-I agonist at 1 nmol/L and GnRH-II agonist at 10 nmol/L were used to determine the temporal effect. Testosterone production was significantly increased by 186% and 160% by GnRH-I and GnRH-II agonist, respectively, after 24-h treatment (Figure 1C and 1D) ($P < 0.05$).

These results illustrate that GnRH-I and -II agonists significantly stimulated murine Leydig cell steroidogenesis in a dose- and time-dependent manner.

3.2 Effects of GnRH agonists on steroidogenic enzyme mRNA expressions in murine Leydig cells

Steroidogenic protein and/or enzymes associated with steroidogenesis were investigated by measuring transcript levels of mRNAs coding for StAR, P450scc, 17α-hydroxylase, 3β-HSD and 17β-HSD. Figure 2A illustrates RT-PCR results that GnRH-I agonist at 1 nmol/L and GnRH-II at 10 nmol/L induced StAR, P450scc and 3β-HSD mRNA expression after 12-h treatment. However, the 17α-hydroxylase and 17β-HSD mRNA

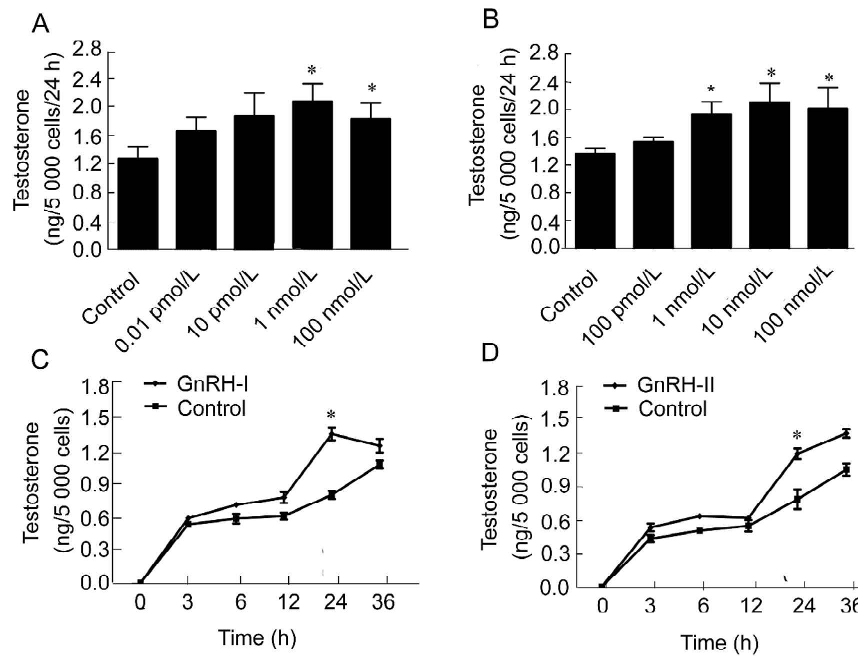

Figure 1. Dose and temporal effects of gonadotrophin-releasing hormone (GnRH) on testosterone production in murine Leydig cells. Cells were treated with or without various concentrations of GnRH-I agonist (A) or GnRH-II agonist (B) for 24 h, or cells were treated with or without 1 nmol/L of GnRH-I agonist (C) or 10 nmol/L GnRH-II agonist (D) for various times. Media were collected and assayed for testosterone production by radioimmunoassay (RIA). Each data point in the figure represents the mean ± SEM of testosterone production of three separate experiments performed in triplicate among each treatment. $^*P < 0.05$, compared with the control.

levels were not affected by either GnRH agonist after 12- and 24-h treatments.

Figure 2B illustrates the integrated optical density (IOD) of StAR, P450scc, 17α-hydroxylase, 3β-HSD and 17β-HSD mRNA expressions from RT-PCR results of Figure 2A after normalization for L19 mRNA expression. The normalized results show that GnRH-I agonist (1 nmol/L for 12 h) significantly induced 2.3-, 1.5- and 1.8-fold increases of StAR, P450scc and 3β-HSD mRNA expression, respectively ($P < 0.05$); and GnRH-II (10 nmol/L for 12 h) significantly induced 2.8-, 1.4- and 2.2-fold increases of StAR, P450scc and 3β-HSD mRNA expressions, respectively ($P < 0.05$).

3.3 Effects of GnRH agonists on StAR, P450scc and 3β-HSD protein expressions in murine Leydig cells

Since GnRH-I and -II agonists significantly stimulated StAR, P450scc and 3β-HSD mRNA expressions in murine Leydig cells, Western blotting assays were further used to define the effects of GnRH-I and GnRH-II agonists on protein expression of StAR, P450scc and 3β-HSD.

Figure 3A illustrates Western blotting results that StAR and P450scc protein levels were not affected by GnRH-I at 1 nmol/L and GnRH-II at 10 nmol/L after 12- and 24-h treatments. In contrast, 3β-HSD protein expression was induced by both GnRH agonists after 24-h treatment, but not 12-h treatment (Figure 3A).

Figure 3B illustrates the integrated optical density (IOD) of StAR, P450scc and 3β-HSD protein expressions from Western blotting results of Figure 3A after normalization with β-actin protein expression. The normalized results show that GnRH-I agonist (1 nmol/L for 24 h) significantly induced 2.0-fold increase of 3β-HSD protein expression ($P < 0.05$) and GnRH-II (10 nmol/L for 24 h) significantly induced 1.7-fold increase of 3β-HSD protein expression ($P < 0.05$).

These results illustrate that GnRH-I and -II agonists only significantly stimulated 3β-HSD, but not StAR and P450scc, protein expression in murine Leydig cells.

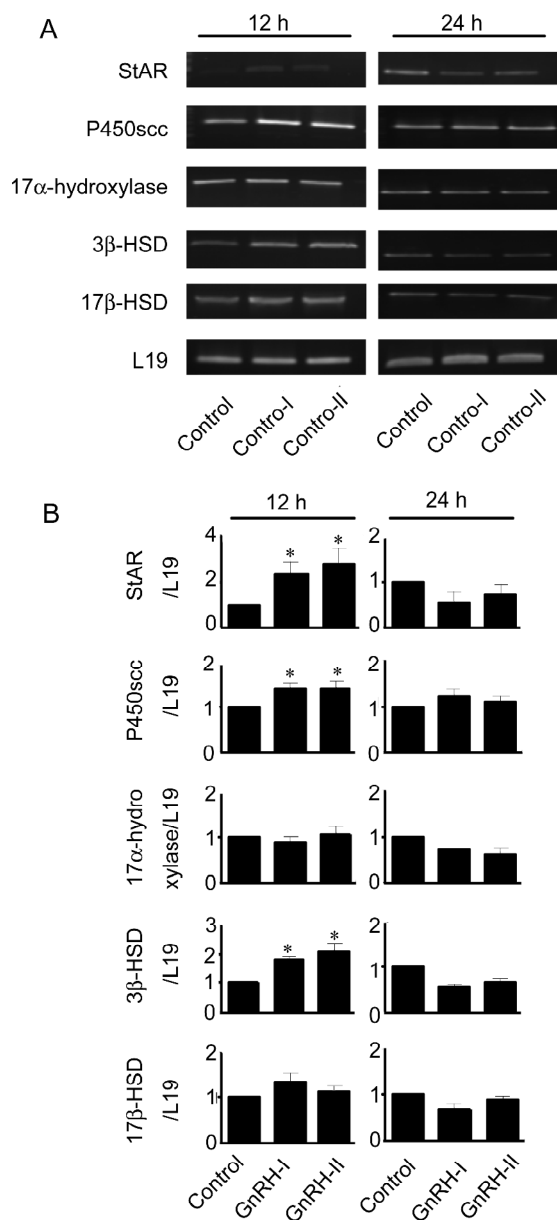

Figure 2. Effects of gonadotrophin-releasing hormone (GnRH) agonists on steroidogenic enzyme mRNA expressions in murine Leydig cells. Cells were treated with 1 nmol/L GnRH-I agonist or 10 nmol/L GnRH-II agonist for 12 and 24 h, and the expression of mRNA coding for steroidogenic acute regulatory (StAR), P450scc, 17α-hydroxylase, 3β-hydroxysteroid dehydrogenase (HSD) and 17β-HSD was determined by reverse transcriptase–polymerase chain reaction (RT-PCR). (A): A representative RT-PCR product of StAR, P450scc, 17α-hydroxylase, 3β-HSD and 17β-HSD mRNA expression in response to GnRH-I and GnRH-II agonists for 12 and 24 h. L19 is the housekeeping gene used as an internal control. (B): Integrated absorbance of StAR, P450scc, 17α-hydroxylase, 3β-HSD and 17β-HSD mRNA expression after normalization with L19. Each data point in the figure represents the mean ± SEM of three separate experiments. $^{*}P < 0.05$, compared with the control.

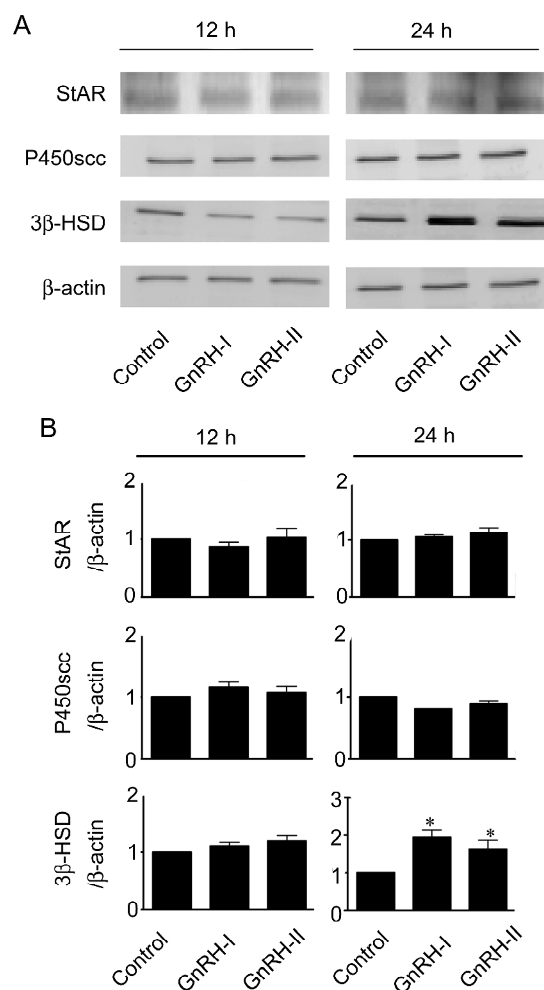

Figure 3. Effects of gonadotrophin-releasing hormone (GnRH) agonists on steroidogenic acute regulatory (StAR), P450scc and 3β-hydroxysteroid dehydrogenase (HSD) protein expressions in murine Leydig cells. Cells were treated with 1 nmol/L GnRH-I agonist or 10 nmol/L GnRH-II agonist for 12 and 24 h, and the protein expression of StAR, P450scc, and 3β-HSD was determined by western blotting. (A): A representative western blot of StAR, P450scc and 3β-HSD protein expression in response to GnRH-I and GnRH-II agonists for 12 and 24 h. β-actin is used as an internal control. (B): Integrated absorbance of StAR, P450scc and 3β-HSD expression after normalization with β-actin. Each data point in the figure represents the mean ± SEM of three separate experiments. $^{*}P < 0.05$, compared with the control.

4 Discussion

In the present study, GnRH-I and GnRH-II agonists significantly activated the expression of 3β-HSD enzyme to stimulate murine Leydig cell testosterone production.

These results differ from previous reports proclaiming no direct effect of GnRH on murine Leydig cell steroidogenesis [14]. In fact, 5–7-week-old male B6 (C57BL/6NCrj) mice were used in the present study whereas adult CD-1, BALB/c or NSC mice of different age and strains were used in previous studies. Furthermore, it has been shown that the postnatal development of Leydig cells can be divided into progenitor Leydig cells, immature Leydig cells and adult Leydig cells with different testosterone-producing activity, associated with changes in the expression levels of several different clusters of genes, including steroidogenic enzymes [21]. In fact, Leydig cells from immature mice possess higher steroidogenic response to trophic ligands than cells from mature mice [21]. Thus, it is possible in the present study that GnRH/GnRH-like receptor and 3β-HSD enzyme exist in immature murine Leydig cells, and GnRH agonists could associate with the receptor to activate 3β-HSD enzyme and then stimulate steroidogenesis.

Many studies have demonstrated that factors from other cell types in testis can mediate Leydig cell functions [22, 23]. Thus, the purity of isolated Leydig cells would influence the effect of GnRH on steroidogenesis in mouse. The purity of isolated Leydig cells in the present study was 85%–90%. In previous studies testicular interstitial cells (approximately 10%–5% purity) or Leydig cells of undefined purity were used in which other cell types may exert paracrine interaction on Leydig cells reducing the GnRH stimulatory effect. This might explain the reports proclaiming no effect of GnRH on murine Leydig cell steroidogenesis [14].

It is well established that steroidogenesis in Leydig cells is regulated by LH to activate the G-proteins > adenylate cyclase > protein kinase A signal transduction pathway that phosphorylates or activates proteins, such as StAR protein or steroidogenic enzymes. The function of StAR protein is to transfer free cholesterol from the cytoplasm into the inner compartment of mitochondria, where P450scc enzyme converts cholesterol to pregnenolone [19]. Pregnenolone will then be transported to the smooth endoplasmic reticulum for further synthesis to testosterone by 17α-hydroxylase, 3β-HSD and 17β-HSD enzymes [24]. The function of StAR protein and the enzymatic activity of 17α-hydroxylase have been proposed to be the critical steps for steroidogenesis [25]. Furthermore, the expression and activity of P450scc, 17α-hydroxylase, 3β-HSD and 17β-HSD enzymes can be regulated by various factors to influence steroidogenesis [25]. In the present study, the mRNA transcript levels of StAR, P450scc and 3β-HSD were significantly increased by both GnRH-I and GnRH-II agonists in murine Leydig cells after 12-h treatment. Interestingly, only 3β-HSD protein expression was further induced by both GnRH-I and GnRH-II agonists after 24-h treatment, indicating that StAR and P450scc were not the major proteins regulating GnRH-induced testosterone production in mouse Leydig cells. Indeed, the temporal trend of 3β-HSD mRNA expression at 12 h and then protein expression at 24 h activated by both GnRH agonists are reasonable and consistent with other studies in which the expression of steroidogenic enzyme takes about 12–24 h or longer to be fully expressed [26].

Mammalian spermatogenesis and steroidogenesis are primarily controlled by the hypothalamus and pituitary. Additionally, various local mediators (paracrine and autocrine factors) modulate the hormone actions both in somatic and germ cells [24, 27]. These cell-to-cell interactions have been shown to play important roles in the control of testicular functions at different phases of testicular development [24]. Increasing evidences show that many growth factors, cytokines and secreted proteins or peptides are involved in interactions between Sertoli cells and Leydig cells. These Sertoli cell factors, such as activin, inhibin, IGF-1, IGF-2, FGF, TGF-α, TGF-β and oestradiol, might regulate the Leydig cell functions via either stimulatory or inhibitory effects on testosterone production [28–31]. Given that GnRH are produced from Sertoli cells, GnRH-receptors are present on Leydig cells [6, 7] and both forms of GnRH are able to stimulate 3β-HSD expression and testosterone production, it is reasonable to believe that GnRH-I and GnRH-II should be Sertoli cell paracrine factors that mediate the cross-compartment communication between seminiferous tubules and Leydig cells.

Taken together, GnRH directly activated murine Leydig cells to stimulate 3β-HSD expression and testosterone production, implying a role of locally produced GnRH in the control of murine Leydig cellsteroidogen esis.

References

1. Cheng CK, Leung PC. Molecular biology of gonadotropins-releasing hormone (GnRH)-I, GnRH-II and their receptors in humans. Endocr Rev 2005; 26: 283–306.
2. Pierantoni R, Cobellis G, Meccariello R, Fasano S. Evolutionary aspects of cellular communication in the vertebrate hypothalamo–hypophysio–gonadal axis. Int Rev Cytol 2002;

218: 69–41.
3. Bahk JY, Hyun JS, Chung SH, Lee H, Kim MO, Lee BH, *et al.* Stage specific identification of the expression of GnRH mRNA and localization of the GnRH receptor in mature rat and adult human testis. J Urol 1995; 154: 1958–61.
4. Paull WK, Turkelson CM, Thomas CR, Arimura A. Immunohistochemical demonstration of a testicular substance related to luteinizing hormone-releasing hormone. Science 1981; 213: 1263–4.
5. Ramakrishnappa N, Rajamahendran R, Lin YM, Leung PC. GnRH in non-hypothalamic reproductive tissues. Anim Reprod Sci 2005; 88: 95–113.
6. Bourne GA, Regiani S, Payne AH, Marshall JC. Testicular GnRH receptors: characterization and localization on interstitial tissue. J Clin Endocr Metab 1980; 51: 407–9.
7. Clayton RN, Katikineni M, Chan V, Dufau ML, Catt KJ. Direct inhibition of testicular function by gonadotropin-releasing hormone: mediation by specific gonadotropin-releasing hormone receptors in interstitial cell. P Natl Acad Sci USA 1980; 77: 4459–63.
8. Khan MA, Ferro VA, Stimson WH. Use of a highly specific monoclonal antibody against the central variable amino acid sequence of mammalian gonadotropin releasing hormone to evaluate GnRH-I tissue distribution compared with GnRH-I binding sites in adult male rats. Am J Reprod Immunol 2003; 49: 239–48.
9. Molcho J, Zakut H, Naor Z. Stimulation of prostaglandin E and testosterone production in rat interstitial cells by a gonadotropin-releasing hormone agonist. Endocrinology 1984; 114: 2382–7.
10. Browning JY, D'Agata R, Steinberger A, Grotjan Jr. HE, Steinberger E. Biphasic effect of gonadotropin-releasing hormone and its agonist analog (HOE766) on *in vitro* testosterone production by purified rat Leydig cells. Endocrinology 1983; 113: 985–91.
11. Kerr JB, Sharpe RW. Effects and interactions of LH and LHRH agonists on testicular morphology and function in hypophysectomized rats. J Reprod Fertil 1986; 76: 175–92.
12. Habert R. Effect of decapitation and chronic *in-vivo* treatment with a gonadotrophin-releasing hormone agonist on testicular steroidogenesis in the rat fetus. J Endocrinol 1992; 133: 245–51.
13. Gobbetti A, Zerani M. Mammalian GnRH involvement in prostaglandin F2 alpha and sex steroid hormones testicular release in two amphibian species: the anuran water frog, Rana esculenta, and the urodele crested newt, Triturus carnifex. Gen Comp Endocrinol 1992; 87: 240–8.
14. Wang NG, Sundaram K, Pavlou S, Rivier J, Vale W, Bardin CW. Mice are insensitive to the antitesticular effects of luteinizing hormone-releasing hormone agonists. Endocrinology 1983; 112: 331–5.
15. Huang BM, Stocco DM, Norman RL. The cellular mechanisms of corticotropin-releasing hormone (CRH)-stimulated steroidogenesis in mouse Leydig cells are similar to those for LH. J Androl 1997; 18: 528–34.
16. Poon SL, Leu SF, Hsu HK, Liu MY, Huang BM. Regulatory mechanism of Toona sinensis on mouse Leydig cell steroidogenesis. Life Sci 2005; 76: 1473–87.
17. Lowry OH, Rosenborough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol regent. J Biol Chem 1951; 193: 265–75.
18. Chen YJ, Huang YL, Huang BM. Cordyceps sinensis mycelium activates PKA and PKC signal pathways to stimulate steroidogenesis in MA-10 mouse Leydig tumor cells. Int J Biochem Cell Biol 2005; 37: 214–23.
19. Stocco DM, Clark BJ. Regulation of the acute production of steroids in steroidogenic cells. Endocr Rev 1996; 17: 221–44.
20. Guo IC, Wu LS, Lin JH, Chung BC. Differential inhibition of progesterone synthesis in bovine luteal cells by estrogens and androgens. Life Sci 2001; 68: 1851–65.
21. Ge RS, Dong Q, Sottas CM, Chen H, Zirkin BR, Hardy MP. Gene expression in rat Leydig cells during development from the progenitor to adult stage: a cluster analysis. Biol Reprod 2005; 72: 1405–15.
22. Chen L, Xin ZC, Li X, Tian L, Yuan YM, Liu G, *et al.* Cox7a2 mediates steroidogenesis in TM3 mouse Leydig cells. Asian J Androl 2006; 8: 589–94.
23. Sultana T, Svechnikov KV, Gustafsson K, Wahlgren A, Tham E, Weber G, *et al.* Molecular identity, expression and functional analysis of interleukin-1alpha and its isoforms in rat testis. Asian J Androl 2004; 6: 149–53.
24. Saez JM. Leydig cells: endocrine, paracrine, and autocrine regulation. Endocr Rev 1994; 15: 574–626.
25. Payne AH, Hales DB. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr Rev 2004; 25: 947–70.
26. Payne AH, Youngblood GL. Regulation of expression of steroidogenic enzymes in Leydig cells. Biol Reprod 1995; 52: 217–25.
27. Reddy PR. Hormonal contraception for human males: prospects. Asian J Androl 2000; 2: 46–50.
28. Calkins JH, Sigel MM, Nankin HR, Lin T. Interleukin-1 inhibits Leydig cell steroidogenesis in primary culture. Endocrinology 1988; 123: 1605–10.
29. Lin T, Haskell J, Vinson N, Terracio L. Direct stimulatory effects of insulin-like growth factor-I on Leydig cell steroidogenesis in primary culture. Biochem. Bioph Res Co 1986; 137: 950–6.
30. Lin T, Blaisdell J, Haskell JF. Transforming growth factor-beta inhibits Leydig cell steroidogenesis in primary culture. Biochem. Bioph Res Co 1987; 146: 387–94.
31. Lin T, Calkins JK, Morris PL, Vale W, Bardin CW. Regulation of Leydig cell function in primary culture by inhibin and activin. Endocrinology 1989; 125: 2134–40.

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