Introduction

Insulin-like peptide 3 (INSL3; formerly, Leydig cell insulin-like factor or relaxin-like factor) was first discovered in 1993. It is a member of the relaxin-insulin family of peptide hormones owing to its structural similarities with the rest of the group members including relaxin, insulin and insulin-like growth factors. INSL3 is highly expressed in the Leydig cells of many mammalian species. Thus, it is considered as a specific product of testicular Leydig cells (LCs) and a major circulating hormone in males. Along with testosterone, circulating INSL3 has emerged as a novel clinical marker of LC function in humans. However, reports regarding INSL3 concentrations in peripheral blood are limited to humans and rodents, and INSL3 dynamics related to sexual development and aging are still unknown for domestic animal species including bulls and male dogs. The circulating concentrations of both INSL3 and testosterone are worth studying in male domestic animals to evaluate Leydig cell function in vivo.

INSL3, in concert with testosterone, play a critical role in the process of testicular descent which is strictly hormonally regulated. Failure in testicular descent, cryptorchidism (CO), is a common male genital anomaly. Among domestic species, dogs suffer high incidence of CO ranging from 1.2 to 10.7%. Peripheral plasma testosterone concentrations were lower in bilateral cryptorchid dogs than in normal dogs, according to limited information available on naturally occurring CO. Despite its importance in the process of testicular descent, plasma INSL3 concentrations have not been investigated in both normal and cryptorchid dogs.

Information is lacking on the regulatory substances of INSL3 secretion and its effect on LCs. In men, INSL3 secretion is regulated by long-term trophic effects of luteinizing hormone (LH),
and at present, it is believed to be constitutively regulated. No in vitro evidences were found in literature regarding the direct regulatory substances of INSL3 secretion from LCs of any species including humans. In rat LCs, however, testosterone-stimulated INSL3 gene expression has been suppressed by phthalates which are readily available estrogenic environmental chemicals. To date, effects of INSL3 on LCs have not been elucidated regardless of the localization of its receptor (RXFP2 or LGR8) in the LCs.

In this study, novel immunoassay systems were developed to quantify bovine and canine INSL3. Establishment of these assays enabled us to conduct a series of in vivo and in vitro studies on bovine and canine INSL3, and to compare those novel findings with a well-elucidated hormone such as testosterone. In chapter 1, dynamics of plasma INSL3 and testosterone concentrations were investigated in normal beef bulls from birth to pubertal age. In chapter 2, the effects of age and CO on changes of plasma INSL3 concentrations were examined in dogs, and were compared with those of plasma testosterone concentration changes. In chapter 3, INSL3 and testosterone secretory capacity in response to various effectors of Leydig cells was investigated in cultured testicular interstitial cells (TICs) of dogs. In chapter 4, the effects of INSL3 on canine and mouse TICs, and on purified mouse LCs were examined by measuring testosterone and cAMP as end-points.

Chapter 1: Dynamics of plasma INSL3 and testosterone from birth to pubertal age in bulls

Concentrations of INSL3 in peripheral blood of bulls have not been previously reported and relationship between bovine INSL3 and testosterone is unknown. In this study, plasma INSL3 dynamics from birth to pubertal age in beef bulls were investigated using newly developed enzyme immunoassay (EIA), and the changes in plasma INSL3 concentrations were compared with those in testosterone. The minimum detection limit of the INSL3 assay was 0.5 ng/ml and the detection range was 0.5–20 ng/ml. Plasma samples were collected from beef bull calves (n=15) at birth (0 months) and at 1, 2, and 3 months after birth. Furthermore, in beef bulls around pubertal age (n=26; age range 3–22 months), plasma samples were collected at 1–4 month intervals. Steer plasma had INSL3 concentrations below the sensitivity of the assay. Plasma INSL3 concentrations increased (P<0.05) from 0 to 1, 1 to 2, and from 2 to 3 months of age. Plasma testosterone concentrations increased (P<0.001) from 0 to 1 month, and from 1 to 2 months, but did not change from 2 to 3 months. For bulls around pubertal age, plasma INSL3 concentrations did not change from the pre-pubertal phase (3–6 months) to the early pubertal phase (6–12 months), but increased (P<0.05) from the early to late pubertal phase (12–18 months), and from the late pubertal to post-pubertal phase (18–22 months). Plasma testosterone concentrations increased from the pre-pubertal to early pubertal phase (P<0.001), but did not change thereafter. These results demonstrated that plasma INSL3 concentrations increased during the first 3 months of life and throughout the pubertal age in beef bulls. Dynamic patterns of circulating INSL3 were similar to that of testosterone during the first 3 months of life, but patterns subsequently diverged in bulls around pubertal ages.

Chapter 2: Age-related plasma INSL3 and testosterone dynamics, and effects of cryptorchidism in dogs

Age-related plasma INSL3 dynamics in dogs were investigated across a wide age range using a novel time-resolved fluorescence immunoassay (TRFIA), and compared with plasma testosterone concentrations. Furthermore, hormone concentrations were compared among cryptorchid, normal and castrated dogs to evaluate endocrine function of LC component in retained testes. Canine plasma INSL3 was measured by using a newly developed TRFIA. The minimum detection limit of the INSL3 assay was 0.02 ng/ml and the detection range was 0.02–20 ng/ml. Blood samples were taken from normal male dogs from pre-pubertal age to advanced age (4 months – 14 years, n=89) and from unilateral cryptorchid (n=31), bilateral cryptorchid (n=7) and castrated dogs (n=3); all were small-breed dogs. Plasma INSL3 concentrations increased (P<0.05) from pre-pubertal age (4–6 months) to pubertal age (6–12 months), and then declined (P<0.05) from pubertal age to post-pubertal age (1–5 years), reaching a constant level. Plasma testosterone concentrations increased (P<0.0001) drastically from pre-pubertal age to
pubertal age and seemed to plateau. INSL3 and testosterone concentrations were lower ($P<0.0001$ for each) in bilateral cryptorchid dogs than in normal and unilateral cryptorchid dogs. The INSL3 (range: 0.05–0.43 ng/ml) and testosterone (range: 0.10–0.94 ng/ml) concentrations were readily detected in bilateral cryptorchids, but not in castrated dogs (INSL3<0.02 ng/ml; testosterone<0.04 ng/ml). The present findings showed a transient surge in plasma INSL3 concentrations at pubertal age in male dogs, but not in testosterone concentrations. Lower plasma INSL3 and testosterone concentrations in bilateral cryptorchid dogs suggest impaired endocrine functions of LC component in paired retained testes. Peripheral plasma INSL3 and testosterone concentrations may be used for the diagnosis of bilaterally retained testes in male dogs.

Chapter 3: INSL3 and testosterone secretory responses to various effectors in cultured testicular interstitial cells of dogs

The influence of Leydig cell effectors on INSL3 secretion has not been previously investigated using cultured TICs in any domestic species, except in rodents. No successful regulator of INSL3 secretion has been found even in rodents. The experiments described herein were conducted to assess the INSL3 and testosterone secretory capacity in response to various regulatory substances in cultured TICs of dogs.

3.1. INSL3 and testosterone secretory responses to human chorionic gonadotropin in cultured interstitial cells from scrotal and retained testes

Differences in secretory capacity of INSL3 and testosterone between interstitial cells of scrotal and retained testes are plausible. Thus, levels of INSL3 and testosterone secretions in response to various doses of human chorionic gonadotropin (hCG) in cultured interstitial cells were compared between retained and scrotal testes in small-breed dogs. The testicular tissues were dispersed in Dulbecco’s Modified Eagle Medium with Ham’s nutrient mixture containing 2000 PU/ml dispase II and 10% fetal bovine serum. Fifty-thousand cells were plated with differing concentrations (0–10 IU/ml) of hCG for 18 h in multiwell-plates. INSL3 and testosterone in the same spent medium were measured by enzyme-immunoassays (EIA). A new EIA with a reliable detection range of 0.025–5 ng/ml was developed in order to measure canine INSL3 in culture medium.

In the cells of scrotal testis, INSL3 secretion was stimulated ($P<0.01$) at the highest hCG concentration (10 IU/ml) relative to unstimulated controls, but remained unchanged at lower hCG concentration (0.1 IU/ml). In contrast, testosterone was clearly stimulated by 0.01 IU/ml hCG or higher concentrations (0.1, 1 and 10 IU/ml) in a dose-dependent manner ($P<0.01$). In the cells of scrotal testes, percent stimulation of INSL3 was 6.8-times and 12.3-times less than that of testosterone at 0.1 and 10 IU/ml hCG, respectively. These results suggest that physiological concentrations of LH may acutely stimulate testosterone release, but not the INSL3 release, in scrotal testes of dogs.

In the cells of retained testes, the incremental rate of INSL3 at 10 IU/ml hCG was lower ($P<0.05$) than that of scrotal testes. The incremental rate of testosterone secretion was lower ($P<0.05$) at 0.1, 1 and 10 IU/ml hCG in the cells of retained testes than that of scrotal testes. In dogs, LH-induced secretory INSL3 and testosterone responses may be lower in the interstitial cells of retained testes than of scrotal testes.

3.2. Effects of estradiol-17β, monobutyl phthalate and mono-(2-ethylhexyl) phthalate on the secretion of INSL3 and testosterone by cultured testicular interstitial cells

Effects of estradiol-17β, monobutyl phthalate (MBP) and mono-(2-ethylhexyl) phthalate (MEHP) on INSL3 and testosterone secretions in canine TICs were also examined. TICs were isolated from scrotal and retained testes of small-breed dogs through enzymatic dispersion of the testicular tissues. Suspension cultures were treated with estradiol-17β (0, 10, and 100 ng/ml), MBP (0, 0.8, and 8 mM) or MEHP (0, 0.2, and 0.8 mM) for 18 h, in the presence or absence of 0.1 IU/ml hCG. INSL3 (basal) and testosterone (both basal and hCG-induced) concentrations were measured in spent medium. Effects of estradiol-17β, MBP, and MEHP on INSL3 and
testosterone secretions were not affected ($P > 0.15$) by cell source (scrotal versus retained testis); therefore, data were combined, and analyzed as percentage relative to the control. In TICs, basal INSL3 secretion was inhibited ($P < 0.01$) by 8 mM MBP and 0.8 mM MEHP. Basal testosterone secretion was increased ($P < 0.01$) by 100 ng/ml estradiol-17β. Among phthalates, 0.2 and 0.8 mM MEHP stimulated ($P < 0.01$) basal testosterone secretion. However, hCG-induced testosterone secretion was inhibited ($P < 0.01$) by 8 mM MBP, and tended to be inhibited ($P = 0.056$) by 0.8 mM MEHP. Therefore, it was inferred that certain phthalate monoesters and estradiol-17β had direct effects on secretions of INSL3 and testosterone in canine TICs, with no significant difference between scrotal and retained testes.

Chapter 4: Effects of INSL3 on canine and mouse testicular interstitial cells, and on purified mouse Leydig cells

*In vitro* effects of INSL3 on canine and mouse TICs were examined taking testosterone as an end-point. To test whether these effects are exerted directly on LCs, a series of experiments was conducted using purified mouse LCs, aiming both testosterone and cAMP measurements. Purified LCs were obtained from mouse TICs after centrifugation on a 3-step discontinuous gradients (specific gravities: 1.05, 1.06 and 1.08) of Percoll. The TICs or LCs were plated in the presence or absence of INSL3 from different species (mouse, human, canine or bovine; 0–100 ng/mL) for 18 h in multiwell-plates (96 wells). The effects of bovine INSL3 (100 ng/ml) on testosterone secretion by LCs were tested in the presence or absence of SQ 22536 (1 μM), an adenylate cyclase inhibitor, or INSL3 antagonist (bovine and human; 100 ng/ml). Canine and mouse TICs were plated in densities of 200,000 and 25,000 cells per well, respectively. For LCs, different cell densities (2,500, 5,000, 10,000 or 20,000 cells per well) were tested. Testosterone and cAMP in spent medium was measured by EIA. In canine TICs, INSL3 stimulated ($P < 0.0001$) the testosterone secretion compared with unstimulated control; canine and bovine INSL3 (100 ng/ml) showed a more than twofold stimulation. In mouse TICs, maximum stimulation (more than twofold) of testosterone secretion was observed with 100 ng/ml bovine INSL3 ($P < 0.0001$). In purified mouse LCs, INSL3 stimulated ($P < 0.05$) testosterone secretion, and the maximum stimulation (nearly twofold) was observed with 100 ng/ml bovine INSL3 at the lowest LC density (2,500 cells per well). Bovine INSL3 (100 ng/ml) stimulated ($P < 0.0001$) cAMP production from primary Leydig cells maximally at 1 h, and remained significantly elevated even at 18 h. SQ 22536, the specific adenylate cyclase inhibitor reduced ($P < 0.0001$) the bovine INSL3-stimulated testosterone secretion from LCs. The addition of INSL3 antagonists (both bovine and human) into culture medium markedly reduced ($P < 0.0001$) the stimulatory effect of bovine INSL3 on testosterone secretion by LCs. Taken together, the observed stimulatory effects of INSL3 on testosterone secretion from TICs and LCs is exerted via the activation of cAMP, and this would suggest a new autocrine function of INSL3 in males.

Conclusions

1. Immunoassay systems were developed to quantify plasma INSL3 in cattle and dogs. According to the data obtained, it is inferred that testis-derived INSL3 is the major source of circulating INSL3 in bulls and male dogs.
2. In normal bulls, plasma INSL3 concentrations were continuously increased from birth to pubertal age and this pattern differed from that of testosterone around pubertal ages.
3. In normal male dogs, plasma INSL3 concentrations showed a transient surge at pubertal age, but testosterone did not.
4. Lower plasma INSL3 concentrations in bilateral cryptorchid than normal dogs suggest impaired endocrine functions of Leydig cell component in paired retained testes. Furthermore, the higher INSL3 concentrations in bilateral cryptorchid than castrated dogs indicate the diagnostic value for INSL3 to predict the presence of retained testes.
5. Physiological concentrations of LH do not acutely stimulate INSL3 release in canine testicular interstitial cells whereas testosterone response was dramatically increased.
6. LH-induced INSL3 and testosterone secretory responses are lower in the interstitial cells of retained testes than that of scrotal testes in dogs.
審査結果の要旨

インスリン様ペプチド 3（INSL3）は、1993年に発見された精巣のライディッヒ細胞に特異的に発現するペプチドで、ラクシンやインスリンに類似した構造を示す。ヒトではINSL3の免疫測定法が開発され、男性の性成熟や加齢とともに血中動態が報告されており、血中INSL3濃度はライディッヒ細胞の機能を示す新しい臨床的指標になることが示唆されている。しかし、ウシやイヌなどの飼育動物ではINSL3の免疫測定法は確立されておらず、性成熟過程における動態は未だ不明である。一方、INSL3は、同じライディッヒ細胞から分泌されるテストステロンとともに、精巣下降において重要な役割を果たすことが示唆されている。精巣下降が障害される潜在精巣はイヌに多発するため、潜在精巣に罹患したイヌの血中INSL3濃度を測定することにより、潜在精巣とINSL3との関連についての多くの情報が得られるものと推測される。

男性ではINSL3の分泌は黄体形成ホルモン（LH）の長期的な作用によって促進されるが、短時間での血中濃度の変動はなく、LHによる即時的な調節を受けないとされており、テストステロンの分泌がLHの即時的な調節を受けることとは対照的である。INSL3を調節するLH以外の因子についてはヒトやげっ歯類においても不明な点が多い。また、ライディッヒ細胞自身にはINSL3の受容体であるラクシンラファミリーペプチド受容体2（RXFP2）の発現が報告されているが、ライディッヒ細胞の内分泌機能に及ぼすINSL3の効果についても不明である。

本研究では、正常な雄ウシと雄イヌの性成熟に伴う血中INSL3動態を解析して、テストステロン動態との比較を行った。また、潜在精巣罹患犬の血中INSL3濃度を正常例と比較した。さらに、イヌ精巣の培養細胞を用いてINSL3の分泌に影響を及ぼす因子を調べるとともに、マウスライディッヒ細胞の内分泌機能に及ぼすINSL3の効果を検討した。以下に研究成果の概要を示す。

1. ウシおよびイヌの血漿INSL3を定量するための免疫測定法を確立した。これらの雄動物の末梢血中INSL3の大部分は精巣由来であることが示唆された。
2. 正常雄ウシの血漿INSL3濃度は出生時から性成熟期まではほぼ連続的に増加し、性成熟前後
の INSL3 の動態はテストステロンのそれとは異なることが示された。

3. 正常雄イヌの血漿 INSL3 濃度は性成熟期に一過性の上昇（サージ）が観察されるが、テストステロンではそのようなサージはみられなかった。

4. 両側性潜在精巣犬では血漿 INSL3 濃度は正常犬に比べて低いことから、ライディッヒ細胞の内分泌機能は低下していることが示唆された。さらに、両側性潜在精巣犬の血漿 INSL3 濃度が去勢犬よりも高値を示すことから、血中 INSL3 濃度は精巣の存在を示す診断的価値があると考えられる。

5. イヌ正常精巣の培養細胞において、生理的濃度の LH は短時間でテストステロン分泌を顕著に促進するが、INSL3 分泌を促進しないことが判明した。

6. イヌ正常精巣の培養細胞において、LH 刺激による INSL3 分泌は、正常精巣のそれと比較して低下していることがわかった。

7. 環境性エストロジェン様物質であるフタル酸類はイヌ精巣細胞からの INSL3 分泌を抑制することが示唆された。

8. INSL3 はマウスライディッヒ細胞に直接作用し、環状アデノシン 1 リン酸 (cAMP) 経路を介して、テストステロン分泌を促進することが示唆された。

以上の研究は、雄ウシおよび雄イヌの性成熟過程における血中 INSL3 動態、ならびに潜在精巣犬の INSL3 濃度を初めて明らかにしたものであり、イヌの正常精巣と停滞精巣における INSL3 の分泌調節の一端を解明するとともに、ライディッヒ細胞における INSL3 の新たな役割を示唆した。これらの研究成果は、産業動物・伴侶動物における精巣機能の調節機構の解明や内分泌機能の維持法の開発、ならびに潜在精巣の病態解明とその診断法の開発に寄与すると考えられ、獣医繁殖学領域における生殖内分泌学の発展に資するものであり、本研究の審査ならびに最終試験の結果と併せて、博士（獣医学）の学位を授与することを適当と認める。