Studies on the regulatory effects of ghrelin on growth hormone secretion mechanisms in ruminants

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Contents

Abbreviations	Full name
ANOVA	analysis of variance
AUC	area under the curve
BBB	blood-brain-barrier
BSA	bovine serum albumin
BW	body weight
cDNA	complementary deoxyribonucleic acid
c.p.m	count per minute
CV	coefficient of variations
DCE	dichloroethane
DCM	dichloromethane
DIPCDI	diisopropylcarbodiimide
DIPEA	diisopropylethylamine
DMAP	dimethylaminopyridine
DMF	dimethylformamide
EC cells	enterocromaffin cells
ECL cells	enterocromaffin-like cells
EDT	ethanedithiol
EDTA	ethylenediamine-*N,N,N',N'*-tetraacetatic acid
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
Fmoc	9-fluorenylmethoxycarbonyl
G-protein	GTP-binding protein
GCP-R	G-protein-coupled receptor
GH	growth hormone
GH-R	growth hormone receptor
GHRH	growth hormone releasing hormone
GHRH-R	growth hormone releasing hormone receptor
GHRP	growth hormone releasing peptide
GHS	growth hormone secretagogue
GHS-R	growth hormone secretagogue receptor
GLP-1	glucagon-like peptide-1

Abbreviations	Full name
HBTU	2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyuronium hexafluorophosphate
hCS	human chorionic somatomammotropin
HOBt	N-hydroxybenzotriazole.H_2O
HPLC	high-performance liquid chromatography
IGF-1	insulin-like growth factor 1
KLH	keyhole limpet hemocyanin
MBHA	methylbenzyldrylamine
mRNA	messenger ribonucleic acid
NEFA	non-esterified fatty acid
NPY	neuropeptide Y
PC1/3	prohormone convertase 1/3
PCR	polymerase chain reaction
PEG	polyethylene glycol
PRL	prolactin
PYY	peptide tyrosine tyrosine
RIA	radioimmunoassay
RP-HPLC	reverse-phase high-performance liquid chromatography
RT-PCR	reverse transcriptase-polymerase chain reaction
SPPS	solid phase peptide synthesis
SPSS	Statistical Package for the Social Sciences
SRIF	somatostatin
TFA	trifluoroacetic acid
TIS	triisopropylsilane
USP	United States Pharmacopeia

Chapter 1 (General introduction)

1. Regulation of growth hormone secretion

Growth hormone (GH), a protein released in pulses from the somatotrophs of anterior pituitary gland, is a pleiotropic hormone that required for postnatal linear growth, carbohydrate and fat metabolism, and milk production. In dairy cows, administration of GH not only promoted growth but also enhanced milk synthesis (Peel *et al.* 1981, Etherton TD & Bauman DE, 1998). Moreover, administration of porcine GH improved the rapid growth and protein accretion in highly selected pigs for protein accretion (Campbell *et al.* 1988). GH was first characterized in 1920s when Evans and Simpson demonstrated the growth-promoting effects in rats treated with a crude extract from bovine pituitaries (Evans & Simpson, 1931; for review, see Ref. Etherton TD & Bauman DE, 1998, Lindholm J. 2006). It is a 191 amino acid peptide and the molecular weight of about 22,000 daltons in cattle, sheep and human (Seeburg *et al.* 1977, Martial *et al.* 1979, Miller *et al.* 1980, Warwick *et al.* 1989), and 190 amino acid peptide in pig (Kato *et al.* 1990). Bovine, procine and rat GH share a high degree (~90%) of amino acid sequence similarity, while ~35% of amino acid sequence of human GH is appreciably different from those (Miller *et al.* 1980, Etherton TD & Bauman DE, 1998, **Fig. 1-1**). GH, human chorionic somatomammotropin (hCS), and prolactin (PRL) are a group of homologous hormones with growth-promoting and lactogenic activity. GH molecule has two separate sites (site 1 and site 2) necessary for functional interaction with the extracellular domain site of GH receptor, GH-R. Dimerization of GH-R favors site 1 to interact with GH-R followed by site 2 binding. GH-R, which is a member of the class 1 cytokine receptor family and widely expressed in most tissues postnatally with highest abundance in liver and adipose tissue, orchestrates the diverse effects of GH on metabolism and nutrient partitioning.

Classically, it has been believed that circadian and pulsatile release of GH from anterior pituitary cells is under the control of hypothalamus. The role of hypothalamus in the release of GH from pituitary has been postulated by several studies in which hypophysectomy not only decreased the secretion of GH, and also slowed the growth of swine and ruminants (Anderson LL, 1977, Klindt *et al.* 1983). In 1973, Paul Brazeau and colleagues had isolated and characterized from ovine hypothalamus a peptide which inhibits the secretion of GH *in vivo* and *in vitro* (Brazeau *et al.* 1973). They named this peptide "somatotropin-release inhibiting factor" which is later known as SRIF or somatostatin. The term "somatostatin" refers to a family of peptides: somatostatin-14, somatostatin-28, and somatostatin-28 (1-12) that are all cleaved from a common, 116-amino acid primary translation product, preprosomatostatin (Patel YC & O'Neil W. 1988). Somatostatin immunoreactive neurons are widely expressed in the central and peripheral tissues and the amino acid sequence of SRIF is highly conserved among species **(Fig. 1-2)**.

The existence of the GH-releasing factor in the hypothalamus was firstly reported by Nair *et al* (1978) from the study of 6000 batches of bovine hypothalamic fragments for bioassay in rats. In 1982, GH-releasing hormone (GHRH) was successfully characterized from human pancreatic tumors by two different research groups (Guillemin *et al.* 1982, Rivier *et al.* 1982), and later followed by the immunohistochemical studies of Bloch *et al* (1983) who enabled to detect the existence of GHRH in primate hypothalamus. Subsequent studies have isolated and characterized the GHRH from rat, bovine, ovine, caprine, porcine and mouse hypothalamus (Bohlen *et al.* 1983, Esch *et al*, 1983, Spiess *et al.* 1983, Brazeau *et al.* 1984, Suhr *et al.* 1989). Generally, GHRH is a 44-residue carboxy-terminally amidated peptide with highly conserved sequences in amino terminal region among species **(Fig. 1-3)**, although in case of rat and mouse GHRH those are predicted to be 43 and 42 amino acid peptides, respectively, with free carboxy-termini. The GHRH appears to

stimulate the transcription of GH gene as well as stimulation of GH release from somatotrophs (Nair *et al.* 1978, Barinaga *et al.* 1983, Frohman LA & Jansson JO, 1986) via acting on its pituitary receptors, GHRH-R (Mayo KE, 1992). The cDNA sequence of bovine GHRH-R encodes a 423-amino acid protein containing seven hydrophobic domains characteristic of a G protein-coupled receptor. Bovine GHRH-R mainly expresses in anterior pituitary gland and hypothalamus, and predicted amino acid sequence shares 93, 90, 89, 87, and 85% identity with the ovine, porcine, human, rat and mouse sequences (Connor *et al.* 2002).

The episodic secretion of GH from somatotrophs is supposed to be initiated firstly by a reduction in the secretion of SRIF and an increase in the release of GHRH from the hypothalamus into the hypophysial portal system. Indeed, several studies have demonstrated that episodic secretion of GHRH and SRIF in hypophysial-portal blood do not always occur reciprocally with respect to each other and often are not synchronized with the secretion of GH (McMahon *et al.* 2001*b*). Frohman *et al* (1990) reported that the pulsatile patterns of GHRH and SRIF in hypothalamic-pituitary portal plasma of unanesthetized sheep appears to reflect the independent neural rhythmicity with a primary role of GHRH in determining pulsatile GH secretion. The best correlation of secretion occurs between GH and GHRH and ranges from 48% to 78%, but only where the percent of GH pulses began with or immediately after a pulse of GHRH. When secretion of SRIF has been included, the percent of the GH pulses those occur with increased secretion of GHRH and decreased secretion of SRIF is low, ranging from 26% to 40% (Drisko *et al.* 1998, Cataldi *et al.* 1994). Therefore, it is worth considering other controlling factors in addition to GHRH and SRIF in the regulatory mechanisms of the synthesis and/or secretion of GH from anterior pituitary gland.

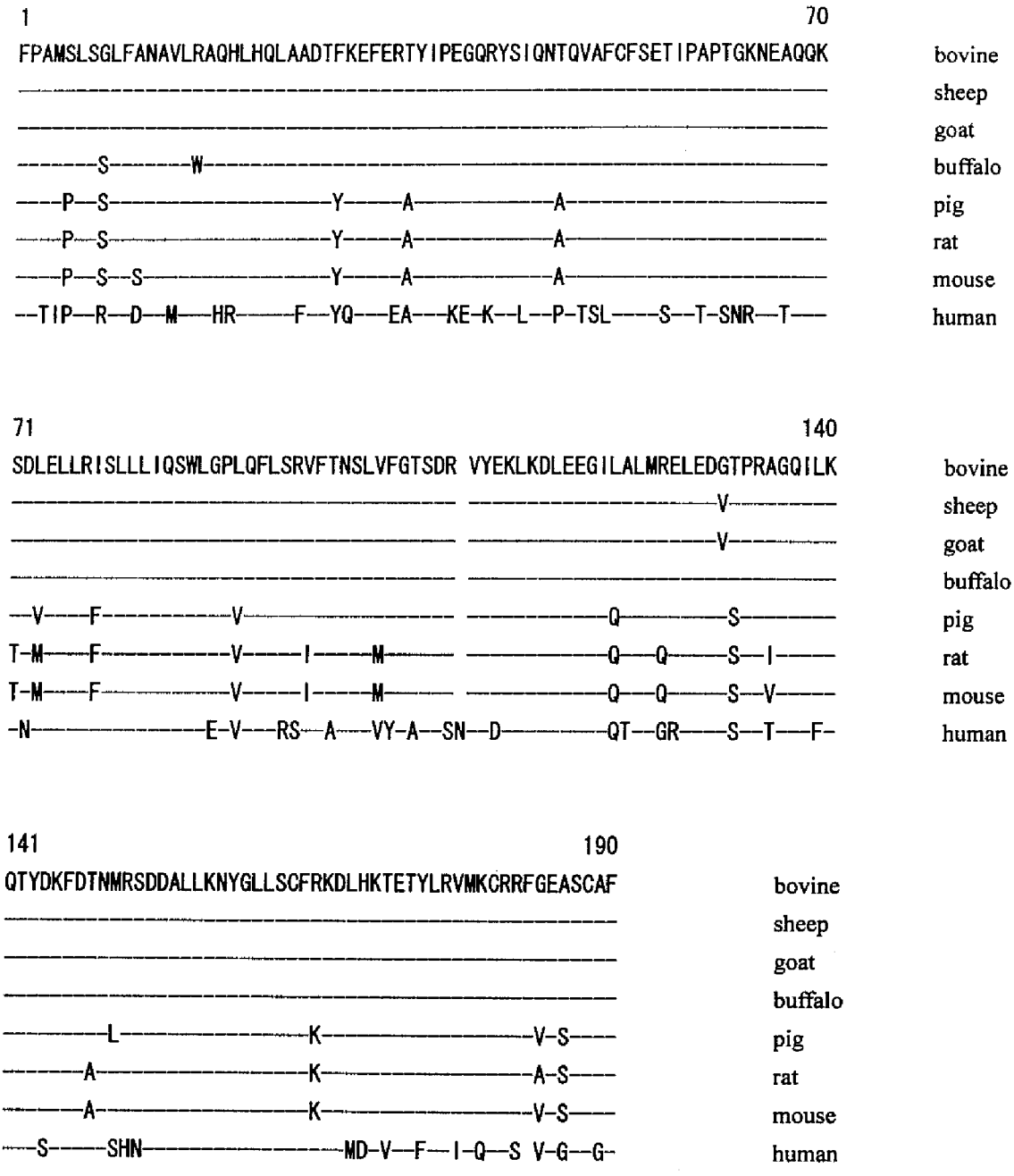

Fig. 1-1. Amino acid sequence alignment of bovine (UniProt accession number P01246), ovine (P67930), caprine (P67931), buffalo (O18938), pig (P01248), rat (P01244), mouse (P06880), human (P01241) growth hormone. The dashes (-) indicate the sequence identity with bovine sequence. See Table 1-1 for the translation of single-letter amino acid code.

1	28	
SANSNPAMAPRERKAGCKNFFWKTFTSC		bovine
SANSNPAMAPRERKAGCKNFFWKTFTSC		sheep
SANSNPAMAPRERKAGCKNFFWKTFTSC		human
SANSNPAMAPRERKAGCKNFFWKTFTSC		pig
SANSNPAMAPRERKAGCKNFFWKTFTSC		rat
SANSNPAMAPRERKAGCKNFFWKTFTSC		mouse

Fig. 1-2. The alignment of the amino acid sequence of somatostatin. UniProt accession numbers for references are; bovine, P26917; sheep, O46688; human, P61278; pig, P01168; rat, P60042; mouse, P60041. See Table 1-1 for the translation of single-letter amino acid code.

1	44	
YADAIFTNSYRKVLGQLSARKLLQDIMNRQQGERNQEQGAKVRL		bovine
----------		caprine
----------I----------		sheep
----------S------S---R--RA--		human
----------S----------R---		pig
H------S---RI----Y-----HE----------RSRFN		rat
-------TN---L-S--Y---VI-----K- ---I---R-RLS		mouse

Fig. 1-3. The alignment of the amino acid sequence of growth hormone-releasing hormone (GHRH). UniProt accession numbers for references are; bovine, P63292; caprine, P63293; sheep, P07217; human, P01286; pig, P01287; rat, P09916; mouse, P16043. Dashes (-) indicate identity with bovine sequence. See Table 1.1. for translation of single-letter amino acid code.

Table 1.1. Translation of the single-letter amino acid code

Single-letter code	Three-letter code	Amino acid
A	Ala	Alanine
R	Arg	Arginine
N	Asn	Asparagine
D	Asp	Aspartic acid
C	Cys	Cysteine
Q	Gln	Glutamine
E	Glu	Glutamic acid
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
L	Leu	Leucine
K	Lys	Lysine
M	Met	Methionine
F	Phe	Phenylalanine
P	Pro	Proline
S	Ser	Serine
T	Thr	Threonine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
V	Val	Valine

2. Discovery of ghrelin

In 1976, Bowers and colleagues reported the first unnatural GH-releasing peptide (GHRP), DTrp^2. It is a derivative of the natural opiate Met enkephalin pentapeptides (TyrGlyGlyPheMet and TryGlyGlyPheLeu) those were initially isolated from porcine brain by Hughes and coworkers (1975). The development of the GHRP is the first step for the discovery of natural ligand ghrelin. DTrp^2 is of low potency to release GH and is active only *in vitro*, but specific in action with lack of the opiate activity (Bowers *et al.* 1977). Premier tetra and penta peptides GHRPs have been classified into 4 groups according to the number, position and chairality of the Trp residue. They are TyrDTrp^{2}GlyPheMetNH$_2$ (DTrp^2), TyrAlaDTrp^{3}PheMetNH$_2$ (DTrp^3), TyrDTrp^{2}DTrp^{3}PheMetNH$_2$ (DTrp^{2,3}) and TyrDTrp^{2}AlaTrpDPheNH (DTrp^2, LTrp^4). Each of these four chemical general classes of GHRPs has been used as templates to synthesize many new more potent GHRPs (for review, see Ref. Bowers CY, 1998). Finally, the first *in vitro* and *in vivo* active hexapeptide, HisDTrpAlaTrpDPheLysNH2 (GHRP-6) has been evolved (Bowers *et al.* 1984). Other GHRPs, GHRP-1 and GHRP-2 also show the potent GH-releasing activity in domestic animals (Bowers CY. 1998). The stimulatory effect of GHRPs on GH release appears not to be species-specific. However, the differences in the GH releasing activity of GHRH versus GHRP (Badger *et al.* 1984) as well as intracellular signaling pathways of these two peptides strongly imply the presence of specific receptor different from GHRH-R. In 1993, Roy G. Smith and colleagues identified a nonpeptidyl mimetic of GHRP-6; L-692-429, with improved pharmacokinetics and oral bioavailability and the authors proved that L-692-429 synergizes with GHRH and acts through an alternative signal transduction pathway (Smith *et al.* 1993). Subsequently, an orally active mimetic, MK-0677, was developed (Patchett *et al.* 1995). The selective receptor for such GH-secretagogues was firstly cloned from the pituitary and the arcuate, ventro-medial and infundibular hypothalamus of the swine and

human (Howard *et al.* 1996).

GH-secretagogues receptor (GHS-R) is a heterodimeric GTP-binding protein (G-protein)-coupled receptor (GCP-R) with seven predicted transmembrane domains, three intracellular and three extracellular loops, and the GPC-R triplet signature sequence (**Fig. 1-4**). The GHS-R gene is composed by two exons encoding a seven transmembrane domain protein and one intron (Howard *et al.* 1996, McKee *et al.* 1997). Exon one encodes the amino-terminal extracellular domain, five transmembrane domains, three intracellular loops and two of the three extracellular loops; exon two encodes transmembrane domains 6 and 7, the third extracellular loop and the intracellular carboxyl terminal segment. Two GHS-R mRNA isoforms, defined as types 1a and 1b are encoded by the GHS-R gene and are produced by alternative mRNA processing. Translation of the GHS-R mRNA isoform, where the intron is removed intact, produces the functional receptor GHS-R1a, a polypeptide of 366 amino acids with a molecular mass of approximately 41 kDaltons (KDa). On the other hand, GHS-R1b mRNA encodes a C-terminally truncated 298 amino acid protein containing only the first five transmembrane domains plus a unique 24 amino acid "tail" encoded by alternative spliced intronic sequence that contains a short intron coding sequence. The amino acid sequence of the GHS-R1a is highly conserved among species (**Fig. 1-4**). GHS-R, which is also known as orphan GPC-R since natural ligand for this receptor has been unknown is widely expressed in the pituitary, hypothalamus and other several tissues of human and rat (Papotti *et al.* 2000, Dass *et al.* 2003). Therefore, it is suggested that GHS-R and its unnatural and/or putative ligands play a key role in physiology and energy homeostasis. As an example of "reverse pharmacology": synthesis of unnatural compounds lead to the discovery of a natural receptor and then to the identification of natural ligand, Dr. Masayasu Kojima and his colleagues from National Cardiovascular Center Research Institute of Japan successfully purified and identified the endogenous ligand for the GHS-R from rat and human stomach tissues in 1999, and they

designated it "ghrelin" (Kojima *et al.* 1999).

The word "ghrelin" is derived from "ghre", a word root in Proto-Indo-European languages meaning "grow", reflecting its role in stimulating GH release. Ghrelin is a 28-amino acid peptide in which Ser^3 residue is n-octanoylated (Kojima *et al*, 1999). This octanoyl modification seems to be essential for the bioactivity of ghrelin. Another form of ghrelin which lack of acylation (des-acyl ghrelin) also circulates in bloodstream with high concentrations (Hosoda *et al.* 2000a). Human ghrelin gene comprises five exons; the 20 bp first exon appears to be a nonconding region, exon 2 and 3 encode mature ghrelin (i.e., the 28-amino acid functional peptide) and exon 4 and 5 encode the remaining proghrelin sequence. Ghrelin precursors in the rat and human stomach are composed of 117 amino acids (Kojima *et al.* 1999). In the rat stomach, two isoforms of mRNA encoding pro-ghrelin has been produced from the gene by an alternative splicing mechanism. One mRNA encodes the ghrelin precursor, and another encodes a precursor for des-Gln^{14}-ghrelin, a peptide identical to ghrelin, but with a deletion of Gln^{14} (Hosoda *et al.* 2000*b*). Des-Gln^{14}-ghrelin also has an n-octanoyl modification at Ser^3 like ghrelin, which is also essential for its activity to release GH. In addition, purification of the human ghrelin from the stomach tissues showed the modification of Ser^3 residue by decanoic acid and possibly by decenoic acid (Hosoda *et al.* 2003). Bovine ghrelin gene is located on chromosome 22 and putative bovine preproghrelin consists of 116 amino acids, which contains the 27 amino acid mature ghrelin without alternative splicing (Kita *et al.* 2005). In addition, Dickin *et al* (2004) reported that the alternative splice site in ghrelin is missing in ruminants. Therefore, it is unlikely that the 28-amino acid peptide exists in ruminants in addition to the 27-amino acid peptide. The enzyme prohormone convertase 1/3 (PC1/3) is responsible for the cleavage of the proghrelin to ghrelin through limited proteolytic cleavage at a single arginine *in vivo*, and post-translational acylation is independent of proteolytic processing since octanoylated proghrelin was detected in the stomach of PC1/3-null mice (Zhu *et al*,

2006). Ghrelin has been identified in all species studied to date, and the first 11 amino acids of ghrelin sequence are highly conserved among species (**Fig. 1-5**) suggesting the essential role of ghrelin in the physiological processes.

Another novel neuropeptide derived from the ghrelin precursor, obestatin, has also been identified on the basis of bioinformatic approach (Zhang *et al.* 2005). Obestatin is a 23-amino acid peptide with an amidation at C-terminus (**Fig. 1-5**). Intraperitoneal administration of obestatin suppresses feed intake and the gastrointestinal functions in rat but it does not increase GH secretion like ghrelin. Zhang *et al.* (2005) reported that obestatin is the ligand of cognate orphan GPCR, GPR 39. However, recent studies have provided the independent evidence that obestatin does not affect feed intake (Gourcerol *et al.* 2007) and does not interact with GPR39 (Chartrel *et al.* 2007).

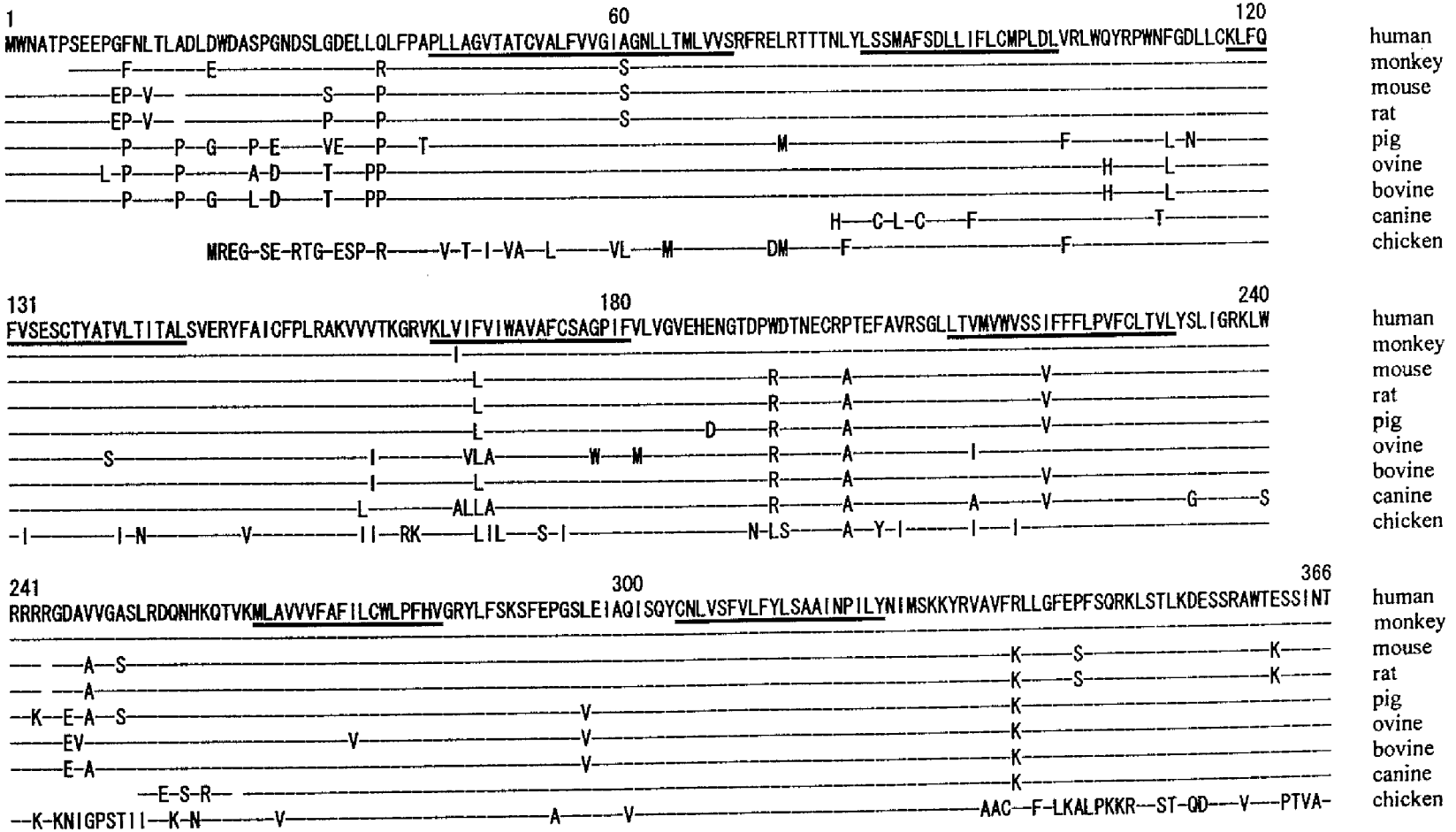

Fig. 1-4. The alignment of the amino-acid sequences of growth hormone secretagogue receptor type 1a (GHS-R 1a). The underlined sequences are the predited transmembrane domain according to the Howard *et al* (1996). Dashes (-) indicate the identity with human sequence.
Gene bank accession numbers for the references are; human: Q92847, monkey: Q6B7N9, mouse: Q99P50, rat: Q08725, pig: Q95254, ovine: Q8MH25, bovine: UPI00005BB8A8, canine: UPI0000EB0C35, chicken: Q7ZT14.
See Table 1-1 for the translation of single-letter amino acid code.

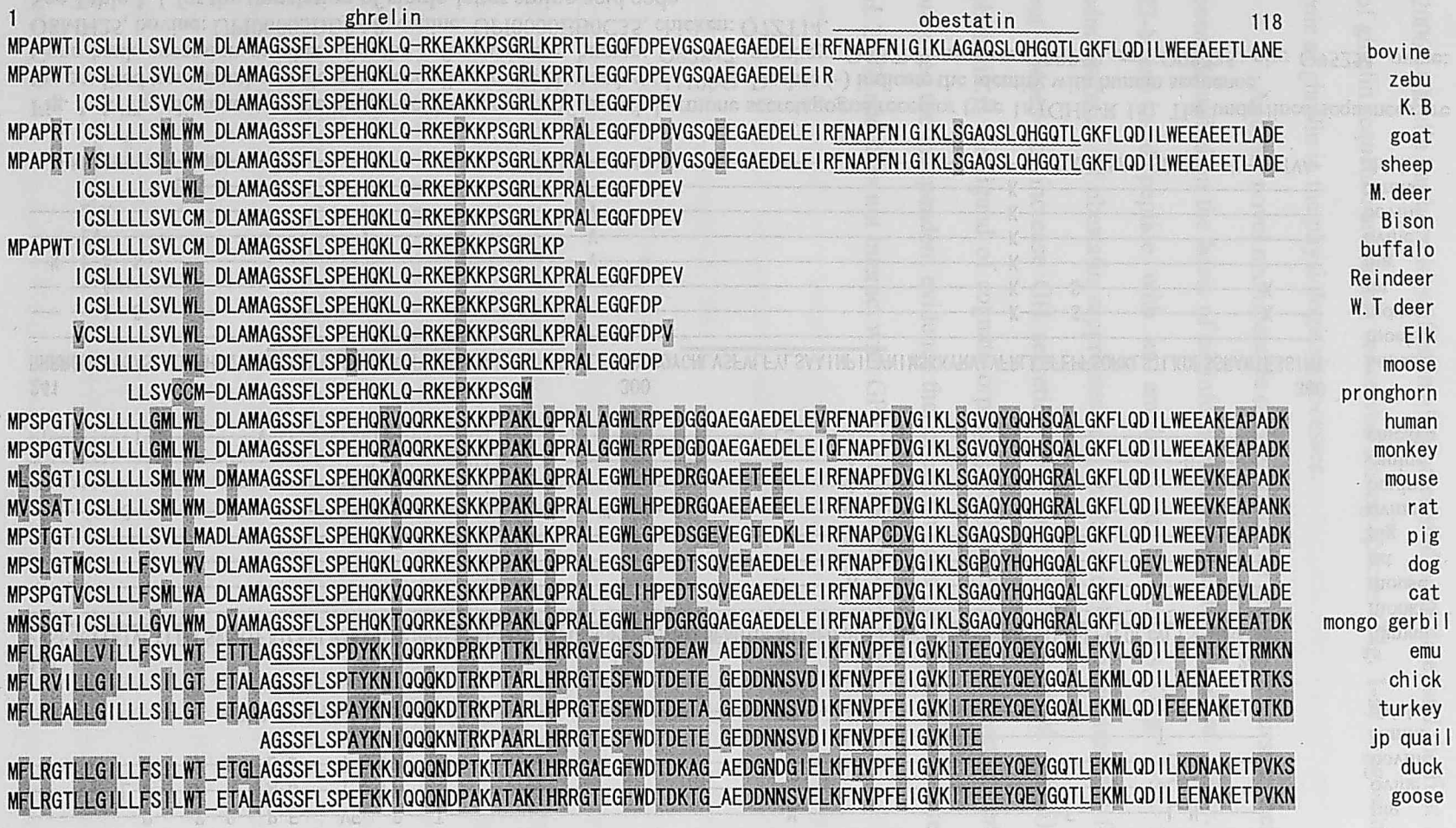

Fig. 1-5. The alignment of the amino-acid sequences of prepro-ghrelin. Sets of the residues different from bovine are shaded.

Fig. 1-5. continued: Reference numbers for the preproghrelin sequences. See Table 1-1 for the translation of single-letter amino acid code.

Name	Species	GenBank Accession No.
Holstein	*Bos taurus* (Bovine)	AF350329, Q9BDJ6
Zebu	*Bos indicus* (Bovine)	Q45RQ5_BOSIN
Whale	*Kogia breviceps* (Pygmy sperm whale)	Q6SLG4_KOGBR
Goat	*Capra hircus* (Caprine)	Q6BEG7
Sheep	*Ovris aries* (Ovine)	NM_001009721
Mule deer	*Odocoileus hemionus* (Black-tailed deer)	Q6SLF2_ODOHE
Bison	*Bison bison*	Q6SPC1_BISBI
Water buffalo	*Bubalus bubalis*	Q863L0_SHEEP
Reindeer	*Rangifer tarandus* (Caribu)	Q6SLF7_RANTA
W.tail deer	*Odocoileus varginianus* (White-tailed deer)	Q6SLF9_9CETA
Elk	*Cerus elaphus canadensis* (Wapiti)	Q6SLF5_CEREL
Moose	*Alces alces*	Q6SLF3_9CETA
Pronghorn	*Antilocapra americana*	Q6SPC3_ANTAM
Human	*Homo sapiens*	NM_016362, Q9UBU3
Monkey	*Macaca mulatta* (Rhesus Macaque)	Q6UDE7_MACMU
Mouse	*Mus musculus*	BC132230, Q9EQX0
Rat	*Rattus norvegicus* (Sparague-Dawley rat)	NM_021669, Q9QYH7
Pig	*Sus scrofa*	DQ355969, Q9GKY5
Dog	*Canis familiaris*	AB060700, Q9BEF8
Cat	*Felis silvestris*	AB089201, Q6BEG6
Mongo gerbil	*Meriones unguiculatus* (Mongolian jird)	AF442491
Emu	*Dromaius novaehollandiae*	AY338467
Chicken	*Gallus gallus*	AY299454
Turkey	*Meleagris gallopavo* (Common turkey)	AY333783
Jp quail	*Coturnix coturnix japonica* (Japanese quail)	Q2V0H4_COTJA
Duck	*Anas platyrhynchos* (Domestic duck)	AY338466
Goose	*Anser specie*	AY338465

3. Regulation of ghrelin concentrations

Most of the reports on the physiology of ghrelin have been obtained with the studies on human and laboratory animals rather than domestic animals. Ghrelin immunoreactive cells are primarily located in a scattered distribution from the neck to the base of oxyntic gland of the gastric fundus region in rat and human, and about 20% of the chromatogranin A-immunoreactive endocrine cells contain ghrelin mRNA (Date *et al.* 2000). Ghrelin cells are ultrastructurally and cytochemically different from other known endocrine cells of the oxyntic mucosa, such as histamine producing enterocromaffin-like cells (ECL cell), somatostatin D cell or serotonin EC cell. The ghrelin cells are morphologically correspondent to X cells in the dog, A-like or X cells in the rat, and P/D1 cells in man (Rindi *et al.* 2002). Two types of the ghrelin cells, closed- and opened-type cells have been found in rat gastrointestinal tract (Sakata *et al.* 2002) suggesting that the ghrelin cells are distinctly regulated by the different factors. Ghrelin cells are already detected in the developing human fetal gut at gestational week 10 (Rindi *et al.* 2002) and at day 18 in the fetal rat (Hayashida *et al.* 2002) with the increasing in numbers in an age-dependent manner from the neonate stage to the adult (Hayashida *et al.* 2002). Apart from gastrointestinal tissues, ghrelin expression has been identified in a number of tissues including the hypothalamus, pituitary, pancreas, heart, reproductive tissues and some neuroendocrine tumors at mRNA or protein level, or both (Papotti *et al.* 2000, Korbonits *et al.* 2001 & 2004). Circulating ghrelin levels have also been recently documented in human fetus (Cortelazzi *et al.* 2003) while mRNA for GHS-R1a is detected at 18- and 31-week gestation (Shimon *et al.* 1998). Moreover, GHS-R 1a is widely expressed in the hypothalamus, pituitary gland and many other tissues such as thyroid gland, stomach, intestine, adipose tissue and ovary (Papotti *et al.* 2000, Dass *et al.* 2003). Therefore, it is suggested that ghrelin/GH axis might be active in the early development and ghrelin inserts

its function through the GHS-R to regulate the energy homeostasis.

At the present time, ghrelin is the only known circulating orexigenic hormone with potential on the hunger and/or body weight regulation. The stomach is the major source of circulating ghrelin and gastrectomy reduces the plasma ghrelin concentrations to approximately half of pre-gastrectomy values (Ariyasu *et al.* 2001, Hosoda *et al.* 2003). Ghrelin is secreted 1-2 hours preprandially and its plasma concentration decreases drastically during the postprandium in the rat (Murakami *et al.* 2002). Ghrelin also shows diurnal rhythm, with the bimodal peaks occurring before dark and light periods in rats. These two peaks were consistent with maximum and minimum volumes of gastric content respectively (Murakami *et al.* 2002). We have reported the elevation of plasma ghrelin in fasted pigs while prominent increase was seen nocturnally (Inoue *et al.* 2005). Moreover, the gastrectomy-induced reduction in the plasma ghrelin levels gradually returned to pre-gastrectomy values (Hosoda *et al.* 2003) indicating that some other tissues, apart from the stomach, might involve in ghrelin secretion. Several studies have been reported that the circulating levels of ghrelin are regulated by short-term factors pertaining to food ingestion and longer term factors pertaining to body weight (Cummings & Overduin. 2004). Intraperitoneal administrations of gastric hormones such as gastrin and CCK in rats result in increase of both acylated and des-acyl ghrelin levels (Murakami *et al.* 2002). Recent study in ghrelin cells which were directly challenged by a wide variety of neurotransmitters and regulatory peptides *in situ* demonstrated that ghrelin release is stimulated by adrenaline, noradrenaline, endothelin and secretin, while the release is inhibited by somatostatin and gastrin-releasing peptide (de la Cour *et al.* 2007). Overall, it can be postulated that the mechanism of the release of ghrelin is critically complex, and the paracrine factors, apart from endocrine ones, also regulate ghrelin secretion.

The information related to the synthesis and release of ghrelin in the domestic animals is still scarce. Ghrelin immunoreactive cells are also present in the oxyntic gland of

cow, sheep, pig and horse stomachs (Hayashida *et al.* 2001, Govoni *et al.* 2005). Both ghrelin and GHSR-1a are present in the ovine tissues including abomasums, anterior pituitary, hypothalamic and hindbrain regions, testis and ovary, and ghrelin displays a developmentally-related pattern of expression in reproductive tissues (Miller *et al.* 2005, Harrison *et al.* 2007). It has been reported that the preprandial increase and postprandial reduction of the plasma acylated ghrelin levels have been detected in a scheduled meal-fed sheep (Sugino *et al.* 2002a), and the preprandial surge of ghrelin is modified by feeding regimens (Sugino *et al.* 2002b). The evidence of preprandial transient surge of plasma ghrelin has also been reported in humans (Cummings *et al.* 2001). However, other studies in ruminants have only shown the reduction in post-prandial plasma acylated ghrelin levels in cows (Hayashida *et al.* 2001, Miura *et al.* 2004). Govoni *et al.* (2005) also reported that the starvation and refeeding influence the plasma ghrelin levels in prepubertal gilts. Therefore, it is suggested that ghrelin participates in the regulatory process on feeding, and the vagal control is the important candidate for the ghrelin release in ruminant (Sugino *et al.* 2004) as well-documented in human (Broglio *et al.* 2004). On the other hand, periprandial changes of the plasma ghrelin concentrations were not seen in 3-month old calves (Miura *et al.* 2004).

4. Roles of ghrelin in energy homeostasis

A: Stimulation of GH release

Several studies have been reported the potent stimulatory effects of ghrelin on the GH secretion *in vitro* and *in vivo*, in a broad range of species including human (Takaya *et al.* 2000, Arvat *et al.* 2001), rodent (Seoane *et al.* 2000, Wren *et al.* 2002, Yamazaki *et al.* 2002), swine (Hashizume *et al.* 2003), ruminants (Hashizume *et al.* 2005, Itoh *et al.* 2005) and non-mammalian species such as birds, amphibians and fish (Kaiya *et al.* 2001, 2002 & 2003). Ghrelin directly stimulates the cAMP production and increased free intracellular Ca^{2+} levels in somatotrophs (Malagon *et al.* 2003). Moreover, ghrelin has a synergistic effect on the GH secretion *in vivo* when it is administered together with GHRH (Arvat *et al.* 2001). However, none of the additive or synergistic effect of ghrelin and GHRH on the GH secretion is not seen *in vitro* (Yamazaki *et al.* 2002, Hashizume *et al.* 2003), suggesting that the sites of action of GH release by the ghrelin might be at both hypothalamic and pituitary levels. This hypothesis is supported by the Mogi *et al.* (2004) in which intracerebroventricular administration of ghrelin induced a robust increase in GHRH release in the cerebrospinal fluid without affecting SRIF release. In fact, the route of ghrelin to promote the GH release is via GHS-R1a, which is coupled with the phospholipase C/inositol phosphate/ protein kinase C signaling pathway, while GHRH binds to GHRH-R which activates the adenylate cyclase / cAMP/ protein kinase A signaling pathway (Anderson *et al.* 2005). The vagus afferent neurons are likely to be responsible to the ghrelin-induced GH secretion in rats (Date *et al.* 2002a), however, the potency of ghrelin to stimulate the GH secretion in vagotomized patients are similar with that in normal subjects (Takeno *et al.* 2004). Somatostatin might suppress the stimulatory effect of ghrelin on GH by directly inhibiting the gastric ghrelin secretion (de la Cour *et al.* 2007, Seoane *et al.* 2007*a*) and decreased plasma ghrelin levels (Barkan *et al.* 2003). On the other hand, Di

Vito *et al* (2002) has reported that the endocrine response to ghrelin administration was only blunted by an exogenous somatostatin in humans indicating the existence of different pathways independent of somatostatinergic influence on ghrelin's endocrine effects.

Since ghrelin is the natural ligand of GHS-R, the interaction between ghrelin and other nonnatural GHSs is of interest. In humans, ghrelin appears to be more potent stimulant than nonnatural peptidyl GHSs such as hexarelin and GHRP-6, but also than natural stimulant GHRH to release GH (Arvat *et al.* 2001, Mimic *et al.* 2002). Ghrelin partially resistant to the homologous desensitization exerted by GHRP-6 (Mimic *et al.* 2002) but no interaction between ghrelin and hexarelin was also observed (Arvat *et al.* 2001) suggesting the existence of receptor subtypes for GHSs. The interactions of those natural and nonnatural GHSs to stimulate GH release in domestic animals have not available yet.

B: Effects on energy homeostasis

Apart from its strong GH releasing activity (Kojima *et al.* 1999), ghrelin has also been implicated in the regulation of other endocrine and exocrine properties including stimulation of the lactotroph and corticotroph secretion, appetite stimulation, glucose metabolism and control of gastric motility and acid secretion (Korbonits *et al.* 2004). Agouti-related protein/neuropeptide Y neurons that are clearly protected by the blood brain barrier (BBB) are the crucial target of the anabolic action of ghrelin (Nakazato *et al.* 2001). In fact, Banks *et al* (2002) reported that mouse acylated ghrelin crosses the mouse BBB in the brain-to-blood direction while mouse des-acyl ghrelin crosses only in the blood-to-brain direction. Human ghrelin which differs from mouse ghrelin by two amino residues only is transported in both directions in mice. Therefore, the extent and the direction for ghrelin to cross the BBB is influenced by at least two features of its primary amino acid sequence, its post-translationally added fatty acid side chain and its amino acid sequence.

Ghrelin is known as an anabolic hormone since it stimulates appetite (Cummings *et al.* 2001), induces feed intake (Nakazato *et al.* 2001, Murakami *et al.* 2002), and reduces fat oxidation (Tschop *et al.* 2000), thus leading to adiposity (Korbonits *et al.* 2004). Administration of the anti-ghrelin antiserum against the N-terminal region, and antagonism of the ghrelin receptor reduces feed intake and body weight gain in mice (Murakami *et al.* 2002, Asakawa *et al.* 2003) indicating that GHS-R1a is the cognate receptor for ghrelin's metabolic effects, and acyl-modification is critical for the activation of ghrelin receptor. The stimulatory effect of ghrelin on feed intake is partly explained by inhibition on glucagon like peptide 1 (GLP-1) and PYY-induced reduction in feed intake (Chelikani *et al.* 2006). However, we reported in fasted pigs that intravenous administration of PYY3-36 does not influence the plasma acylated ghrelin levels (Ito *et al.* 2006). In contrast, ghrelin-null mice showed similar in body size, growth rate, food intake, body composition, reproduction, gross behavior, and tissue pathology to that of wild-type littermates (Sun *et al.* 2003, Wortley *et al.* 2004). Otherwise, Wortley *et al* (2004) also suggested from analyses of ghrl(-/-) mice that endogenous ghrelin might play a prominent role in determining the type of metabolic substrate (i.e., fat vs. carbohydrate) that is used for maintenance of energy balance, particularly under the conditions of high fat intake. However, it must be noted that knockout animals are not able to represent the whole complex physiological process that control the energy expenditure.

In domestic animals, the controversial results have been reported on the effect of ghrelin on feed intake. Salfen *et al.* (2004) reported that body weight gain is higher in the ghrelin-administered pigs although feed intake is not different when compared with saline-injected control animals. Similarly, the voluntary feed intake was not affected by the central infusion of ovine ghrelin in sheep (Iqbal *et al.* 2006). On the other hand, intravenous administration of bovine ghrelin at a dose of 0.08 μg/kg BW results the greater length of time spent eating, and dry matter intake is also tended to be increased (Wertz-Lutz *et al.*

2006). Melendez *et al* (2006) also reported in their preliminary study in which intramuscular injection of ovine ghrelin at a dose of 3 μg/kg BW in pre-partum ewes increased the dry matter intake on the first day of treatment. More precise studies are necessary to clarify the role of ghrelin on feed intake or energy expenditure in the domestic animals.

The involvement of ghrelin in glucose metabolism and pancreatic peptide secretion is still unclear. There are some reports suggesting the stimulatory (Date *et al.* 2002b, Lee *et al.* 2002) or inhibitory (Broglio *et al.* 2001, Reimer *et al.* 2003) effects of ghrelin on insulin secretion in human and rats. Moreover, Salehi *et al* (2004) reported that the physiological dose of ghrelin has no effect on glucose-stimulated insulin release from isolated mouse islets while low doses inhibit and high doses stimulate. Furthermore, 1 nmol/kg and 10 nmol/kg BW ghrelin injections suppress the basal plasma insulin levels in the intact mice. Very recent study on ruminants reported that ghrelin injection with the concentration of 1μg/kg BW has a stimulatory effect on insulin in the lactating cows but not in calves (Itoh *et al.* 2006).

Another molecular form of ghrelin; des-acyl ghrelin, which is the major portion of circulating ghrelin is supposed to be the non-functional peptide. However, recent studies reported some non-endocrine effects of des-acyl ghrelin in monogastric animals. Peripheral administration of des-acyl ghrelin in the transgenic mice (Asakawa *et al.* 2005), and intracisternal administration of des-acyl ghrelin in free-feeding and food-deprived rats (Chen *et al.* 2005) induce negative energy balance by inhibiting feed intake and delaying gastric emptying. In contrast, Toshinai *et al.* (2006) reported that the exogenous des-acyl ghrelin does not inhibit, rather induce feed intake in rat by a different mechanism independent of ghrelin's functional receptor, GHS-R1a.

5. Objectives

Growth hormone participates in postnatal growth, energy partition and fatty acid metabolism in dairy production. The pulsatile release of GH from anterior pituitary is under the control of two hypothalamic hormones, GHRH and SRIF. Discovery of ghrelin from the stomach and identification of ghrelin as a potent GH stimulant opens the new era for the understanding of the mechanism of GH secretion and the endocrinology of growth. Ghrelin also induces appetite, improves feed intake and suppresses lipid degeneration in humans and laboratory animals. The octanoyl-modification on its Ser^3 residue is essential to activate the cognate receptor of ghrelin, GHS-R1a. Moreover, the N-terminal Gly-Ser-Ser(n-octanoyl)-Phe segment appeared to constitute the "active core" required for agonist potency at human GHS-R 1a (Bednarek *et al.* 2000). However, Torsello *et al.* (2002) demonstrated that truncated ghrelin analogs encompassing the first 5 amino acids are ineffective in the stimulation on GH release in neonatal rats and do not displace radiolabelled ghrelin from binding sites in the membranes from human hypothalamus and pituitary. Both acylated and des-acyl form of ghrelin circulate in the blood stream.

Ruminant ghrelin is composed of 27 amino acids with lack of Des^{14} and the C-terminal amino sequence is different with that of monogastric animals (**Fig. 1-5**). The recent studies on the physiology and roles of ghrelin in the ruminant have been indicating that acylated ghrelin levels change periprandially in scheduled meal-fed sheep (Sugino *et al.* 2002a), and peripheral and/or central administration of ghrelin potently stimulates GH release (Itoh *et al.* 2005, Hashizume *et al.* 2005). However, ghrelin is unlikely involved in the feed stimulation in the ruminants (Iqbal *et al.* 2006). The role of ghrelin in the energy homeostasis in dairy cattle is of interest. The GH response to ghrelin is higher in lactating cows compared with the responses in calves and non-lactating cows (Itoh *et al.* 2005). Moreover, ghrelin administration increases the plasma insulin concentrations (Itoh *et al.*

2006). Therefore, it is suggested that ghrelin plays in different roles in energy homeostasis in ruminants depending on the physiological status of an individual. The important fact to be noted is that all of the above studies have quantified the plasma ghrelin concentrations of the domestic animals by RIA specific for monogastric animals. As a result, only the concentration of circulating acylated ghrelin has been detected, and the concentration of des-acyl ghrelin in ruminants is still unknown. Therefore, the objectives of the present studies are;

(1) To establish the specific RIA systems for the measurements of acylated and des-acyl ghrelin concentrations in ruminants

(2) To investigate the effects of ghrelin on GH axis in cattle

(3) To study the effects of the structure of ghrelin, and the physiological stage and metabolic conditions of animals on the ghrelin-induced hormonal changes

(4) To investigate the metabolic and nutritional changes of plasma ghrelin concentrations in small and large ruminants.

Chapter 2 (Experiment 1)

Solid phase peptide synthesis and radioimmunoassay

INTRODUCTION

Livestock farming is one of the most important sectors which supply meat, milk, egg, skin, feather etc. for human consumption. Rapid growth and feed efficiency are the major aspects to achieve the maximal production and highest profits from domestic animals. It is known that multiple hormonal and metabolic signals from central and peripheral tissues including gastrointestinal tract involve in the growth mechanism and energy homeostasis of domestic animals. Many outstanding studies on the physiological and pathophysiological control mechanisms on the growth in farm animals have been carried out over the decade, however, the conductivity of studies on the regulatory effects of the gastrointestinal hormones in growth physiology of ruminants is still limited. One of the reasons is that most of these peptides or hormones of ruminant origin which are different in the amino acid sequences from those of monogastric animals are commercially easily unavailable. As a consequence, it is also difficult to get the specific and sensitive antibodies for the evaluation of hormonal measurements in ruminants. Furthermore, most of the commercially available radioimmunoassay (RIA) kits are not only expensive but they also are specific for human and/or laboratory animals rather than for domestic animals. Therefore, the pure peptides of ruminant origin which must be utilized for the proposed *in vivo* studies have firstly to be synthesized. Moreover respective polyclonal antibodies have been raised, and RIA systems that are not validated yet but essential for the studies in ruminant physiology have been evaluated.

CLONING OF THE BOVINE GHRELIN

At first, we tried to synthesize and purify the recombinant bovine ghrelin by molecular cloning technique based on the procedures which has already been established in our laboratory on bovine and ovine leptin. Stomach tissue from Holstein steer was obtained from local abattoir: the pieces of tissue (approximately 2 g weight) were incised from abomasums within 5 min post-slaughter and immediately placed in liquid nitrogen for transport to the laboratory. Then, the tissue was stored at -80℃ until used. Total ribonucleic acid (tRNA) extraction was performed using guanidine thiocyanate. The resulting aqueous phase was further purifed via phenol-chloroform extraction method to get pure RNA. Thereafter, RNA was precipitated with isopropanol and the pallet was washed with 80% ethynol, air dried, and resuspended in TE buffer containing 10 mM Tris-HCl (pH 7.5) and 1mM EDTA. The integrity of RNA was comfirmed by electrophoresis through 0.8% agarose gels stained with ethidium bromide. Thereafter, messenger RNA (mRNA) purification was done via OligotexTM–dT30 <super> (TAKARA). Then, ghrelin complimentary DNA (cDNA) was synthesized from mRNA mixture by using First-Strand cDNA Synthesis Kit (Amersham) and was amplified by reverse transcriptase (RT)-polymerase chain reaction (RT-PCR). The open reading frame of prepro-ghrelin peptide was obtained using following primers; sense (forward) primer: 5'-gcccgccccgtggaccatc-3', antisence (reverse) primer: 5'-gcatccatctgagcatttattc-3'. The forward and reverse primers were the partial sequence of bovine ghrelin (Genbank accession no. AF350329). The conditions for PCR were 30 cycles of 96℃ for 30 sec, 50℃ for 20 sec, 72℃ for 20 sec. The amplified product was loaded to the 0.8% agarose gel electrophoresis and a band of 483 base pairs long was obtained. **(Fig. 2-1).** The gene of ghrelin was recovered and subjected to the ligation with pGEM®-T Easy Vector (Promega) by DNA ligation kit Ver.2.0 (Takara).

Then, competent cells (INV 1-α F' E.coli, Invitrogen) were transformed and appropriate colonies were selected using blue and white selection procedure. Colonies were incubated in Luria-Bertani (LB) medium containing NaCl, tryptone, and bacto-yeast extract. Quick-screening of white colonies was done for the expected insert size via enzymatic cleavage of EcoR I site. Alkaline lysis method was used to purify the plasmid from appropriate colonies. For DNA sequencing, the inserts were amplified using T7 and SP6 promotors and dye-labeled using Thermo Sequence™ II Dye Terminator Cycle Sequencing Premix Kit (Amersham Biosciences Corp.). The complete sequence of proghrelin was confirmed **(Fig. 2-2)** by ABI PRISM™ 377 DNA sequencing system (PE Applied Biosystems).

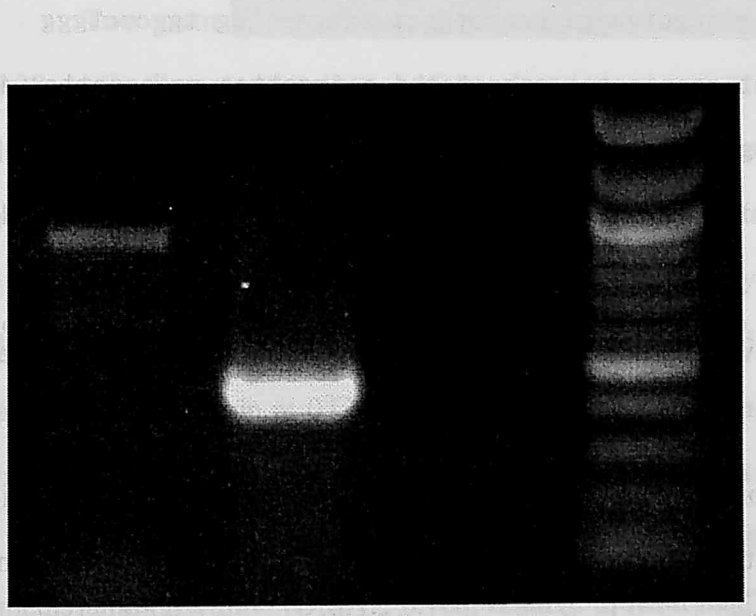

Fig.2-1. Gel electrophoresis of PCR product for bovine preproghrelin. mRNA extracted from bovine abomasums was amplified by RT-PCR using forward (F) and reverse (R) primers. This primer pair amplifies the entire 483-bp cDNA sequence reported (AF350329). FR; forward and reverse, S; molecular weight marker

*gcccgccccg tggaccat*ct gcagcctgct gctgctcagc gtgctctgca tggacttggc 60

catggcgggc tccagctttc tgagccccga acatcagaaa ctgcagagaa aggaagctaa

gaagccatca ggcagactga agccccggac cctggaaggc cagtttgacc cggaggtggg 180

aagtcaggcg gaaggtgcag aggacgagct ggaaatccgg ttcaacgccc cctttaacat

tgggatcaag ctagcagggg ctcagtccct ccagcatggc cagacgttgg ggaagtttct 300

tcaggacatc ctttgggaag aagctgaaga aaccctggct aacgagtgag tggccctggg

accaaccacc tgtccgttct cccaccctca gaagctctca cctggcttcc gggacacttc 420

cgagaccacg tggggctctg aggggtacta gagtaggcnt t*gaataaatg ctcagatgga*

tgc 483

Fig.2.2. The predicted sequence of bovine preproghrelin by cDNA/pGEM-T Easy Vector (483bp). The primers are shown in italic words. The sequence of proghrelin is shaded. Unidentified base is underlined.

However, preparation of the adequate amounts of recombinant peptide approximately 0.2 gram for the proposed *in vivo* experiments in Holstein cattle is not practical. Moreover, it is difficult to do the acyl-modification on the Ser^3 residue of recombinant bovine ghrelin with octanoic acid. Therefore, we synthesized chemically bovine ghrelin and other peptide hormones by solid phase peptide synthesis procedures.

SOLID PHASE PEPTIDE SYNTHESIS

Solid phase peptide synthesis (SPPS) is a method based on the sequential addition of α-amino and side-chain protected amino acid residues to an insoluble polymeric support. In this experiment, peptides shown in **Table 2.1** were synthesized by manual base-labile Fmoc (9-fluorenylmethoxycarbonyl) strategy in which Fmoc-group is used for N-α protection (Peptide synthesis protocol, NOVABIOCHEM, catalog 2000, Merck, Germany). Modifications and detail procedures are expressed in the correspondent sections.

(A) SPPS for ghrelin

Two gram Wang resin (p-Benzyloxybenzyl Alcohol resin, 200-400 mesh eq. to 75-38 μm in diameter, loading: 1.16 nmol per gram) was placed in a clean, dry flask and allowed to swell by addition with sufficient amount of dimethylformamide (DMF). In the another container, the first Fmoc amino acid (2 eq. relative to resin loading) was dissolved in dry dichloromethane (DCM) and a solution of diisopropylcarbodiimide (DIPCDI, 5 eq. relative to resin loading) in dry DCM was added to the amino acid solution. The mixture was stirred for 20 min at 0℃ in the moisture-free condition. After removing the DCM by evaporation under the reduced pressure using a rotary evaporator, the residue was dissolved in a minimum of DMF and added to the resin-containing flask. Finally, dimethylaminopyridine (DMAP, 0.1eq. relative to resin loading) in DMF was added to the resin/amino acid mixture. The mixture was allowed to stand at room temperature for 1 h with occasional swirling. To check the yield of first residue attachment, a small sample of resin (20mg) was removed, washed and dried.

Estimation of first residue attachment: Dried Fmoc amino acid resin (approximately 0.1 micromol with respect to Fmoc loading) was weighted and placed to two 3×10 mm matched silica UV cells. Freshly prepared 20% piperidine in DMF (3ml) was

placed into each of the UV cells. The cell contained only 20% piperidine was used as a reference. The resin mixture was agitated with the aid of a Pasteur pipette for 2-3 min and absorbance was read in a spectrophotometer at 290 nm. The estimate of first residue attachment (57.7%) was obtained from equation below:

Fmoc Loading : mmole/g = (Absorbance$_{sample}$-Absorbance$_{ref}$)/1.65 × mg of resin)

After measuring the attachment of first amino acid, capping procedure was run to reduce the expected enantiomerization or dipeptide formation. Resin/amino acid mixture was washed with DMF, dichloroethane (DCE) and DCM for several time and allowed to dry. DCE (0.08 eq. relative to resin loading), benzoyl chloride (0.06 eq.) and pyridine (0.06 eq.) were added to the dried resin and the mixture was allowed to stand at room temperature for 2 h with continuous stirring. Then, resin was washed with DMF and deprotection of N-terminal protecting Fmoc group was obtained by the treatment of resin beads with 10% piperidine in DMF. The presence of free amino groups was tested by the method of Kaiser (Kaiser *et al.* 1970) with slight modifications. A few resin beads were sampled and washed with 2 ml methyl alcohol/acetic acid. Supernatant was discarded after centrifugation and resin was washed twice with dichloromethane: methanol (6:4) solution. Then, 33 µl phenol:ethanol (phenol 4g in ethanol 1ml), 67 µl pyridine and 25 µl ninhydrin (ninhydrin 0.5g/10ml ethanol) were added to the resin beads and the mixture was incubated for 5 min in water bath. A positive test is indicated by blue resin beads. Second amino acid (2.5 eq. relative to resin loading) and N-hydroxybenzotriazole.H_2O (HOBt: 2.5 eq. relative to resin loading) were dissolved in DMF and the activation was enhanced by the addition of DIPCDI (2.5 eq. relative to resin loading). After 2 h incubation, the preactivated second amino acid was incubated with the resin/amino acid mixture in a beaker for 20 min with continuous stirring. The attachment of amino acid was confirmed by Kaiser Test. When

there is lack of free N-terminal amino groups, i.e. the coupling is successful, negative test was indicated. Then resin beads were placed to glass filter and washed 3 times with DMF prior to the deprotection of Fmoc group with 40% piperidine in DMF. The deprotection/coupling procedures were repeated until the desired peptide sequence was obtained.

In case of amino acids like Arg, Gln, Asn, His or Ser, activation was done by 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) method. Fmoc amino acid (2.5 eq. relative to resin loading), HBTU (2.45 eq.) and HOBt (2.5 eq.) were dissolved in DMF. Diisopropylethylamine (DIPEA, 5 eq. relative to resin loading) was added to the amino acid solution, stirred the mixture and the solution was added immediately to the resin. HBTU can proceed coupling smoothly, and the rates can be enhanced by addition of HOBt. A major problem in SPPS was peptide chain aggregation due to either hydrophobic interactions or interchain hydrogen bonding. This typically occurs between 5-15 residues from the C-terminus and can lead to incomplete coupling and deprotection. Protection of the peptide bond has been shown to disrupt the hydrogen bonding, which is thought to be the root cause of peptide chain aggregation. Therefore, side-chain protected Fmoc-amino acids; Fmoc-Arg(Pbf)-OH, Fmoc-Glu(otBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-His(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(tBu)-OH and Boc-Gly were used. Fmoc-Ser-OH was chosed for Ser^3 because it needed acylation with n-octanoic acid (Kojima *et al.* 1999).

For acylation on Ser^3 residue, the n-octanoic acid (20 eq. relative to resin loading) was firstly dissolved in dry DCM, and a solution of DIPCDI (10 eq. relative to resin loading) in dry DCM was added. The mixture was stirred for 20 min at 0℃ in moisture-free condition. After removing the DCM by evaporation under reduced pressure using an evaporator, dissolved the residue in a minimum amount of DMF and added to the resin/amino acid mixture. Finally, DMAP (0.1eq. relative to resin loading) in DMF was

added and the reaction mixture was allowed to stand at room temperature for 25 min with occasional swirling. Then, the resin was washed with DMF and the acylation procedure was repeated three times. Finally, resin compound was washed 5 times with DMF, 3 times with 2-isopropyl alcohol, 2 times with methyl alcohol, and dry ether. The weight of the resin was measured after ether was removed by evaporation. The resin was equally divided into four 10 ml screw-capped tubes and was treated with 95% Trifluoroacetic acid (TFA), 2.5% water, 2.5% Ethanedithiol (EDT) and 1% Triisopropylsilane (TIS) to cleave the N-terminal Boc-group and side-chain protected groups. After 3 h cleavage time, TFA was removed by a evaporator overnight prior to the ether precipitating step. Then, CH_3CN in TFA water was added and the supernatant containing crude peptide was separated after centrifugation. The resin support and ether was kept until the peptide analysis was completed. The crude peptide was purified by the reverse-phase high-performance liquid chromatography (RP-HPLC, semi-preparative column: TSK-GEL ODS-120A, pore size 10μm, 7.8mm I.D × 30cm, TOSOH). The flow rate was 2.0 ml/min under linear gradient for 60 min from 0 % to 60% CH_3CN containing 0.1% TFA and reference wavelengths were 214 nm for peptide and 260 nm for Phenylalanine. The acyl modification appeared to increase the hydrophobicity of the peptide, explaining the increase in retention time when it was subjected to HPLC: acylated ghrelin was eluted at fraction 47 while des-acyl ghrelin was eluted at fraction 37. The calculated yield of total pure peptide (≈ 1 g) showed that approximately 82.5% of crude peptide was acylated. In addition to the synthesis of bovine ghrelin [1-27], [Cys-12]-ghrelin [1-11], [Cys-12]-bovine ghrelin [13-27] and acylated porcine ghrelin [1-28] were synthesized for immunization procedures. Purified peptides were lyophilized and stored at -30℃ for further usage.

Table 2-1: List of the synthesized peptides and their amino acid† sequences.

	Amino acid sequences
	1 * 11 20 27
Bovine ghrelin [1-27]	GSSFLSPEHQKL_QRKEAKKPSGRLKPR
	1 * 11
[Cys12]-ghrelin [1-11]	GSSFLSPEHQKC
	1 11 20 27
[Cys0]-bovine ghrelin [11-27]	CKL_QRKEAKKPSGRLKPR
	1 * 11 20 28
Porcine ghrelin [1-28]	GSSFLSPEHQKVQQRKESKKPAAKLKPR
	1 11 21 29
Human GHRH amide [1-29]	YADAIFTNSYRKVLGQLSARKLLQDIMSR-NH_2
	1 11 21 31 36
Bovine PYY_{1-36}	YPAKPQAPGEHASPDELNRYYTSLRHYLNLVTRQRF-NH_2
	3 11 21 31 36
Bovine PYY_{3-36}	AKPQAPGEHASPDELNRYYTSLRHYLNLVTRQRF-NH_2
	4 11 21 31 36
Cys[0]-bovine PYY_{4-36}	CKPQAPGEHASPDELNRYYTSLRHYLNLVTRQRF-NH_2

* acylation with octanoic acid

†See Table 1-1 for the translation of the single-letter amino acid code.

(B) SPPS for GHRH and PYY

For the synthesis of human GHRH amide [1-29] and bovine PYY peptides (Table 2-1), Rink Amide MBHA resin (4-(2,4-Dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-MBHA resin, 100-200 mesh, 1.37 mmoles per gram for loading) was used. The detail procedures are similar with the synthesis of ghrelin. However, the special procedures for the attachment of first amino acid including subsequence capping step were omitted according to the C-terminal amidation of Rink Amide MBHA resin. Resin 1 mmoles (0.7138 mmoles/g) was primarily used and side-chain protected Fmoc-amino acids were Fmoc-Thr(tBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Glu(otBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-His(Trt)-OH, Fmoc-Lys(Boc)-OH, and Fmoc-Ser(tBu)-OH. Synthesized peptides were purified by RP-HPLC and peptide containing elusion were lyophilized and stored at -30℃ until use.

RADIOIMMUNOASSAY (RIA)

(A) Plasma ghrelin RIA

Rabbit anti-[Cys-12]-ghrelin [1-11] serum

Synthesized [Cys-12]-ghrelin [1-11] (6 mg) was conjugated to maleimide-activated mariculture keyhole limpet hemocyanin (mcKLH) according to the manufacturer's instruction (Pierce # 77600). Hapten-conjugated antigen was emulsified with same volume of Freund's complete adjuvant (FCA, Wako # 014-09541) for initial immunization. The skin of the rabbit was thoroughly disinfected and emulsified conjugated antigen (1mg per injection) was subcutaneously injected to several sites to minimize the painful inflammatory reactions. The procedure was repeated two weeks apart except Freund's incomplete adjuvant (FIA, Wako # 011-09551) was used for later immunizations. After each injection, the rabbit was placed in a rabbit restrainer to collect the 5~10 ml blood by transecting of marginal ear veins. After sixth injection, immunization procedure was terminated but intermittent blood samplings were continued up to twelve times. To identify the sensitivity of polycolonal antibody from collected blood samples, serially diluted sera in assay buffer (0.05 M sodium phosphosphate containing 0.25% BSA; pH 6.85) were treated with ^{125}I-bovine acylated full-length ghrelin (10000 c.p.m.). After 24 h incubation at 4℃, 10 μl of goat anti-rabbit serum in 990 μl precipitating reagent (0.05 M Sodium phosphate, 0.9% NaCl, 0.025 M EDTA.2Na, 0.08% Sodium Azide, 0.05% Triton X-100, 3% PEG-6000) was added to the reaction solution. After 30 min incubation period at 4℃, bound and free portions were separated by centrifugation (×3000 rpm ×30 min ×4℃). Sensitivity for the harvested serum is shown as values in bound per total c.p.m. in **Table 2-2**. Antibody titer started to increase after second immunization, peaked at 40 days after and maintained high levels until the end of sampling period (81 days after initial injection). Antibody-containing sera were stored at -80℃.

Guinea pig anti-[Cys-0]-bovine ghrelin [11-27] serum

Total of 4 mg synthesized [Cys-0]-bovine ghrelin [11-27] was conjugated to mcKLH and polyclonal antibody was generated in two guinea pigs (*Cavia porcellus*). Conjugated antigen was emulsified as described earlier and injected subcutaneously (0.3 mg per head per injection). Immunization procedures were repeated weekly. After seventh injection, blood was collected by cardiac puncture under ether anaesthesia. Sensitivity of the harvested sera was shown in **Fig. 2-3**. Serum from guinea pig-1 with high antibody titer was stored at -80°C.

Ghrelin RIA procedure

To the best of our knowledge, there is no report to date on the circulating total ghrelin level in ruminants. To remedy this, we validated two RIA systems for bovine ghrelin. Acylated bovine ghrelin [1-27] was radioiodinated by the chloramine-T method (Tai *et al.* 1975) at room temperature and purified by HPLC. In brief, 0.2 mCi ^{125}I-Na is pre-incubated for 5 min with 2.0 µl of sodium borate (1 M, pH 8.5) and 3.5 µg of chloramine-T dissolved to get the concentration of 5 mg/ml in 50 mM sodium borate (pH 8.5). Then, 1.0 µg of synthesized acylated bovine ghrelin [1-27] dissolved in 1mM HCl is added. After 60 seconds incubation, the reaction is stopped by adding with 50 µg sodium metabisulphite (5 mg/ml in 50 mM sodium borate). Then, 1 nmol of tyrosine (0.1mM in 1 mM HCl) is added after 90 seconds, and finally, 2.0 µl of 10% TFA and 2 nmol of sodium iodide (1mM) is added. Total reaction volume is 29 µl. The reaction solution was loaded to HPLC using 50 µl microsyringe and purified by Synchropack RP-8 HPLC column (0.346 ml bed; MICRA-silver; narrow-bore: 100 × 2.1 mm). The flow rate was 0.4 ml/min under linear gradient for 60 min from 0 % to 60% CH_3CN containing 0.1% TFA. The fraction was collected as 0.4 ml/tube. The elution containing iodo-ghrelin was stored at -80°C in 60% CH_3CN/0.1% TFA. This tracer was usually stable for two months, and used for both

active and total ghrelin assays with acylated bovine ghrelin [1-27] as standard.

Standards and plasma samples were incubated with correspondent antibodies diluted in assay buffer (0.05 M sodium phosphosphate, 0.01 M EDTA, 0.08% Sodium Azide, 0.1% gelatin, 0.25% BSA; pH 6.85). The rabbit anti-bovine ghrelin [1-11] antiserum for active ghrelin RIA and guinea pig anti-bovine ghrelin [11-27] antiserum for total ghrelin RIA were used at the final dilution of 1/240,000 and 1/15000, respectively. After 24 h incubation, ^{125}I-bovine ghrelin (8000 c.p.m./100 μl assay buffer containing 1% carrier serum) was added to all tubes. Bound and free ligands were separated by second antibody method after further 24 h incubation and radioactivity in the pellet was counted with a gamma counter (ARC-1000, Aloka, Japan).

In first RIA system, we used rabbit anti-[Cys-12]-ghrelin [1-11] serum for the measurement of active ghrelin and this antibody does not react with bovine and porcine des-acyl ghrelin **(Fig. 2-4A)**. Therefore, we consider that the values measured with the first RIA system specifically represent the acyl-ghrelin levels. On the other hand, antibody used for second RIA system (guinea pig anti-[Cys-0]-bovine ghrelin [11-27] serum) reacts with bovine acyl-ghrelin [1-27] and bovine des-acyl ghrelin, but does not react with porcine acyl-ghrelin, porcine des-acyl ghrelin and human acyl-ghrelin **(Fig. 2-4B)** indicating that this antibody specifically recognizes the C-terminal bovine ghrelin fragment [11-27]. Therefore, since the values measured by using this RIA system represents acyl-ghrelin, des-acyl bovine ghrelin, and all ghrelin fragments with intact bovine C-terminal region [11-27], we use the term "total ghrelin" for these values. These RIA systems favor us to report for the first instance the plasma ghrelin levels following synthesized bovine ghrelin administration and physiological changes in plasma ghrelin concentrations in ruminants.

Table 2-2. Sensitivity (titer) for polyclonal rabbit anti-[Cys-12]-ghrelin [1-11] serum.

Days after first immunization	Bound/ Total			
	1/40000	1/80000	1/160000	1/320000
27	0.57	0.48	0.33	0.21
40	0.62	0.56	0.51	0.39
42	0.64	0.58	0.46	0.33
54	0.62	0.59	0.53	0.37
68	0.62	0.54	0.44	0.29
70	0.61	0.53	0.42	0.26
71	0.63	0.53	0.42	0.26
73	0.61	0.53	0.39	0.25
75	0.63	0.53	0.38	0.22
77	0.61	0.52	0.39	0.24
79	0.60	0.49	0.33	0.22
81	0.65	0.50	0.39	0.23

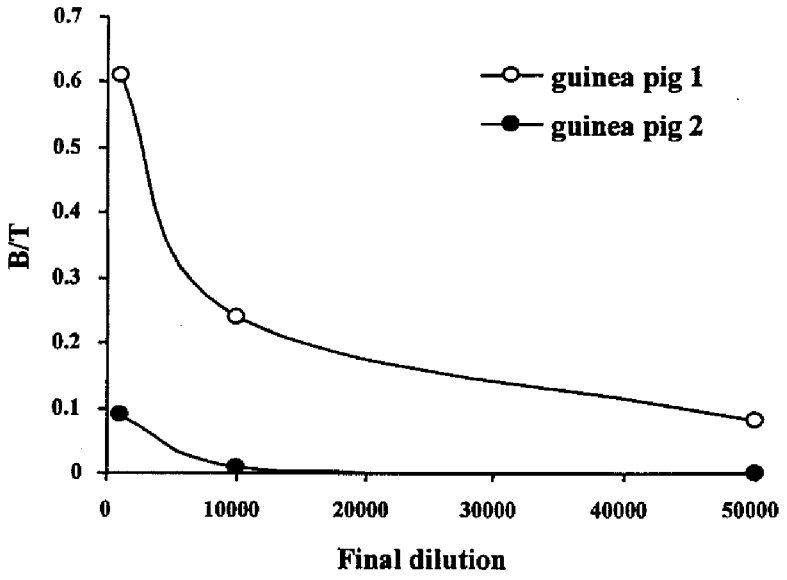

Fig. 2-3. Sensitivity of guinea pig anti-[Cys-0]-bovine ghrelin [11-27] serum. See text for detailed immunization procedures. Serum was treated with ^{125}I-bovine acylated full-length ghrelin for 24 h and bound and free portions were separated by goat anti-guinea pig serum. B/T = Bound/ Total.

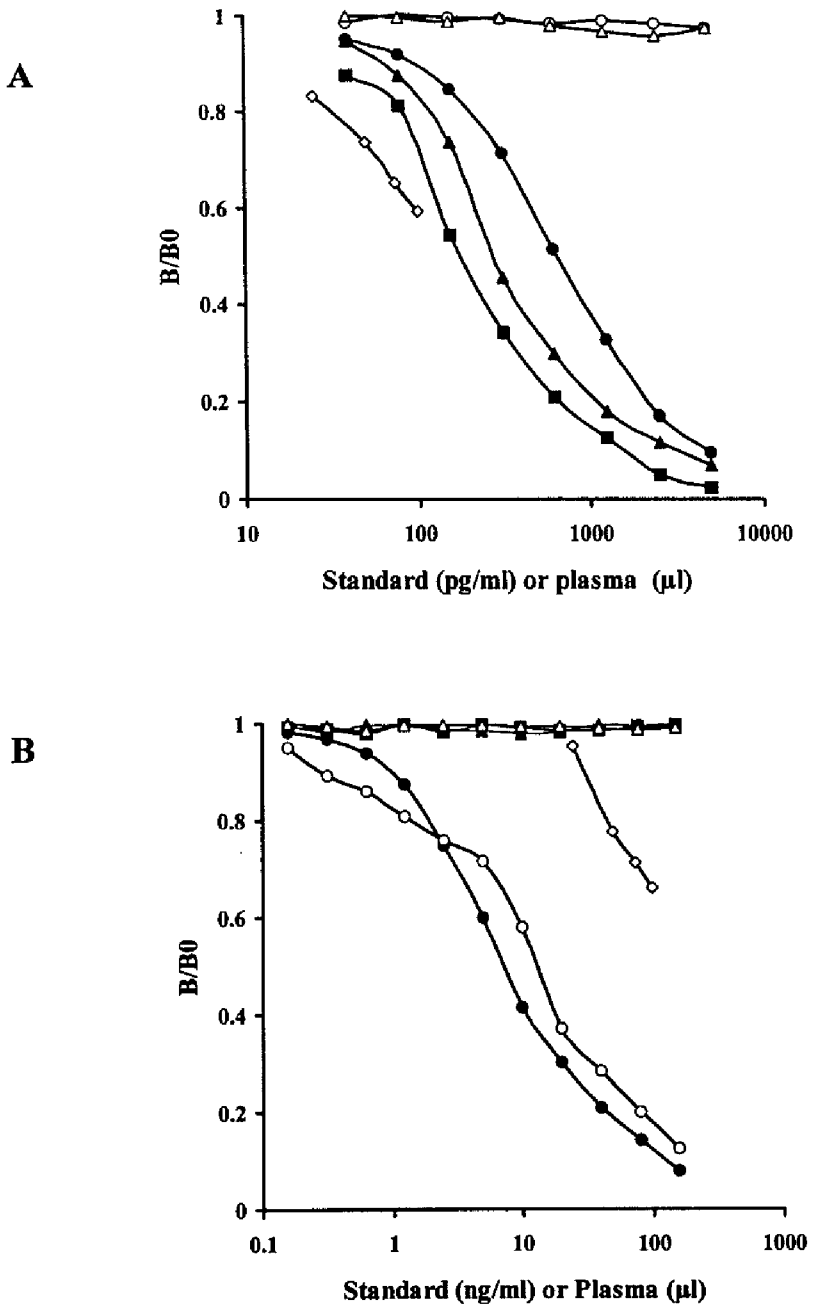

Fig. 2-4. Standard RIA curves for bovine ghrelin. Inhibition of ^{125}I-labeled bovine ghrelin binding to rabbit anti-bovine ghrelin [1-11] antiserum (**A**), and guinea pig anti-bovine ghrelin [11-27] antiserum (**B**) by serial dilution of bovine acylated ghrelin (●), bovine des-acyl ghrelin (○), porcine acylated ghrelin (▲), porcine des-acyl ghrelin (Δ) and human acylated ghrelin (■). Inhibition of ^{125}I-labeled bovine ghrelin binding to both antisera by serial dilution of pooled bovine plasma (◊) parallel to the curve obtained using control full-length bovine ghrelin as standard. Each point is the mean for triplicate determinations. B/B_0, bound/bound in zero standard.

2. Plasma PYY RIA

Guinea pig anti-[Cys-0]-bovine PYY_{4-36} serum

Two guinea pigs were immunized by the subcutaneous injection of emulsified conjugated [Cys-0]-bovine PYY_{4-36}. Detailed procedures are similar to those for anti-bovine ghrelin [11-27] serum. Both serum harvested from two guinea pigs showed the similar potency to bind the radioiodinated bovine PYY_{3-36} (**Fig. 2-5**). Therefore, serum from both animals was stored at -80°C for further use.

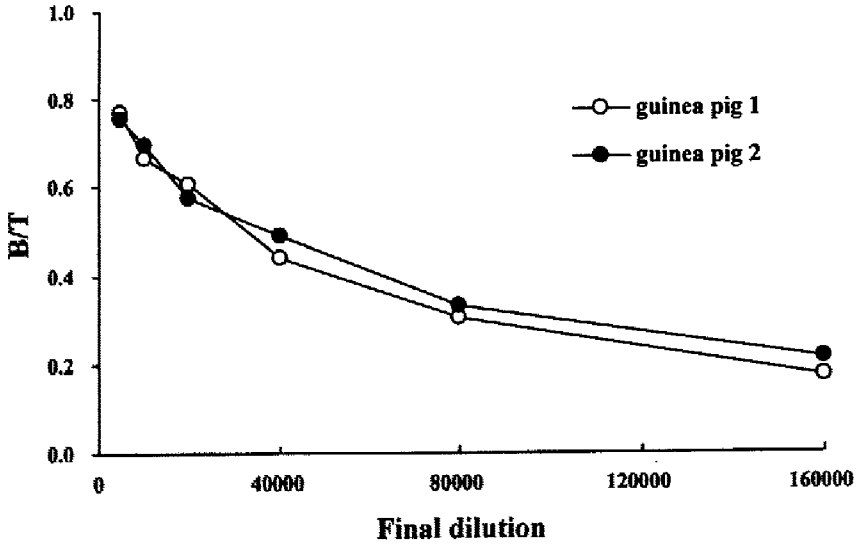

Fig. 2-5. Sensitivity of guinea pig anti-[Cys-0]-bovine PYY_{4-36} serum. See the text for detailed immunization procedures. Serum was treated with ^{125}I-bovine PYY_{3-36} for 24 h and bound and free portions were separated by goat anti-guinea pig serum. B/T = bound/ total.

PYY RIA procedure

Synthesized bovine PYY_{3-36} was radioiodinated by the chloramine-T method. Detail procedures were similar to those of bovine ghrelin iodination. Radio-labeled peptide was purified by HPLC. Iodo-PYY was eluted at fraction # 27 and stored at -80°C in 60% CH_3CN/0.1% TFA. This tracer was usually stable for two months, and used for RIA with bovine PYY_{3-36} as standard.

On the day one, standards and plasma samples were incubated with antibodies diluted in 100 µl of the assay buffer (0.05 M sodium phosphosphate, 0.9% NaCl, 0.025 M EDTA, 0.08% sodium azide, 1.0% BSA; pH 7.4). The rabbit anti-bovine PYY_{4-36} antiserum was used at the final dilution of 1/50000. Subsequently, ^{125}I-bovine PYY_{3-36} (8000 c.p.m. in 100 µl assay buffer containing 1% carrier serum) was added to all tubes. After 24 h incubation, 1% goat anti-guinea pig serum in 1 ml precipitating buffer (0.05 M Sodium phosphate, 0.9% NaCl, 0.025 M EDTA.2Na, 0.08% Sodium Azide, 0.05% Trition X-100, 3% PEG-6000, pH 7.4) was added. After 30 min incubation, bound and free ligand were separated by centrifugation (×3000 rpm ×30 min ×4℃) and radioactivity in the pellet was counted with a gamma counter (ARC-1000, Aloka, Japan).

The guinea pig anti-bovine PYY_{1-36} antiserum does not react with porcine PYY_{1-36}, Human PYY_{1-36}, human pancreatic polypeptide and neuropeptide Y (**Fig.2-6**) indicating that this antibody specifically recognizes the bovine PYY_{1-36} and bovine PYY_{3-36}. Therefore, the term "PYY" will be used for these values since the values measured by this RIA system represent bovine PYY_{1-36} and bovine PYY_{3-36}.

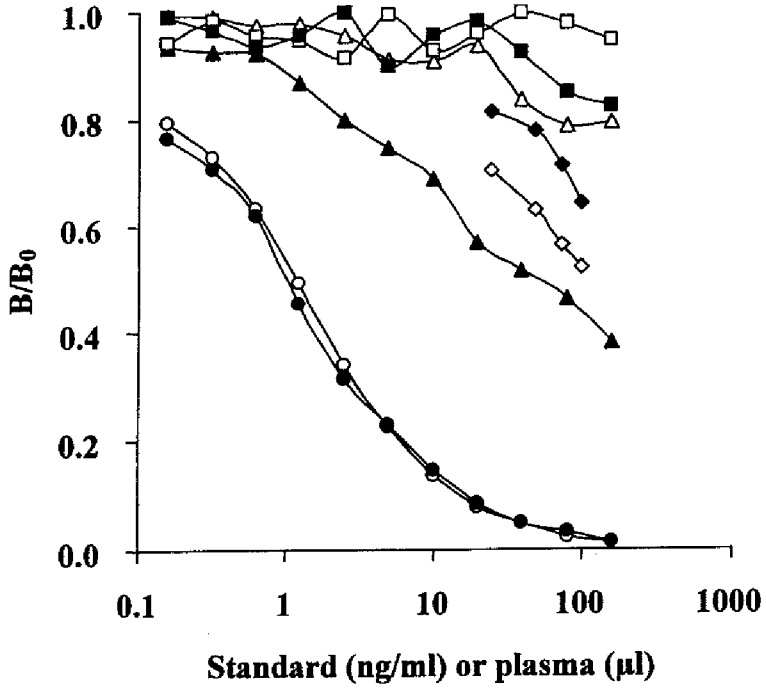

Figure 2-6. Standard RIA curve for bovine PYY. Inhibition of ^{125}I-labeled bovine PYY_{3-36} to guinea pig anti-bovine [Cys-0]-PYY_{4-36} antiserum by serial dilution of bovine PYY_{1-36} (●), bovine PYY_{3-36} (○), porcine PYY_{1-36} (▲), human PYY_{1-36} (Δ), human pancreatic polypeptide (□) and human NPY (■). Inhibition of ^{125}I-labeled rbleptin to antiserum by serial dilution of pooled bovine (◊) or caprine plasma (♦) parallel to the curve obtained using bovine PYY_{3-36} as standard. Each point is the mean for triplicate determinations. B/B_0, bound/bound in zero standard.

3. Plasma leptin RIA

Guinea pig anti-bovine recombinant leptin serum

Three rabbits and two guinea pigs were immunized by the subcutaneous injection of emulsified recombinant bovine leptin (1.0 and 0.5 mg per head per injection for rabbit and guinea pig, respectively). The procedures for the injection and blood sampling are similar as described above. Among the collected sera, the serum collected from guinea pig 2 binds the ^{125}I-bovine rleptin to 31% at the final dilution rate of 1/200000 (**Fig. 2-7**) while serum from other animals failed to bind the tracer. Therefore, serum from guinea pig 2 was stored at -80°C for later usage.

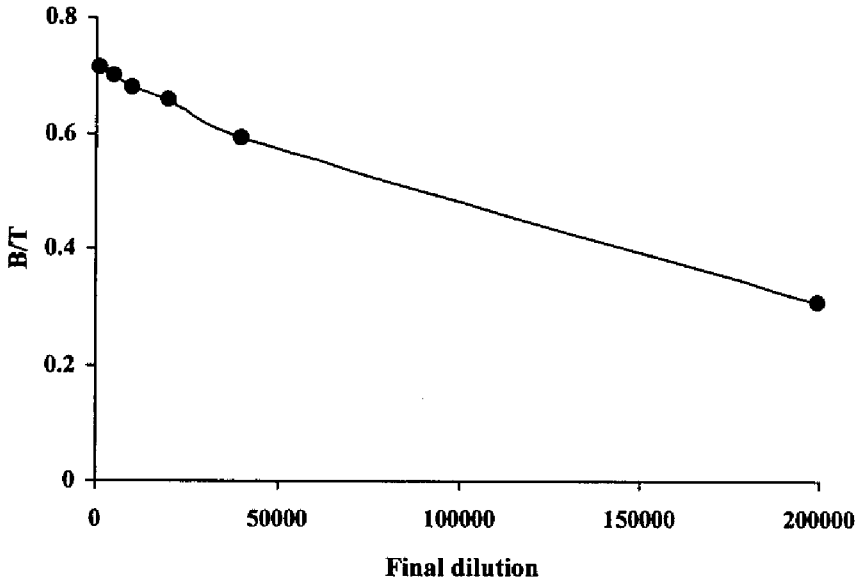

Fig. 2-7. Sensitivity of guinea pig anti-bovine recombinant leptin (rleptin) serum. See the text for detailed immunization procedures. Serum was treated with ^{125}I-bovine rleptin for 24 h and bound and free portions were separated by goat anti-guinea pig serum. B/T = bound/ total.

Leptin RIA procedure

The bovine recombinant leptin (rleptin) was freshly purified by gel-filtration (Sephadex G 25) and the concentration of leptin was calculated as follows:

Concentration = the absorbance at 280nm/ 0.149 (absorption coefficient for bovine leptin)

Then the leptin was subjected to radioiodination. In brief, 0.2mCi ^{125}I was pre-incubated for 5 min together with 15 μg chloramine-T (5 mg/ml in 50 mM sodium phosphate, pH 7.5). Then, 2.0 μg of rleptin (0.1mg/ml in 50mM sodium phosphate) was added to the reaction tube. After 40 seconds incubation, the reaction was stopped by adding of 125 μg of sodium metabisulphite dissolved in 50 mM sodium phosphate solution at 5 mg/ml. 1 nmol of tyrosin (0.1mM in 1 mM HCl) was added after 90 seconds followed by the addition of 2 nmol sodium iodide (1mM). Total reaction volume was 67 μl. The iodinated ligand was purified by gel filtration using Sephadex G-50, equilibrated with 50 mM sodium phosphate/ 1% BSA (pH 7.4) and stored at -80℃. Each RIA incubation mixture was composed of 100 μl standard or plasma samples, 100 μl assay buffer (0.05 M sodium phosphate, 0.9% NaCl, 0.025 M EDTA.2Na, 0.05% Triton X-100, 0.08% NaN_3, 1.0% BSA, pH 7.4) and 100 μl antiserum diluted in assay buffer. The guinea-pig anti-bovine rleptin serum obtained from guinea pig 2 (**Fig. 2-7**) was used at final dilution of 1/200000. After 24 h incubation at 4℃, ^{125}I-leptin (≈ 10000 c.p.m.) in assay buffer containing 1% carrier serum was added to all tubes. After an additional 24 h incubation, second antibody (goat anti-guinea pig antiserum) diluted in precipitation buffer (0.05 M sodium phosphate, 0.9% NaCl, 0.025 M EDTA.2Na, 0.05% Triton X-100, 0.08% NaN_3, 3% PEG-6000, pH 7.4) was added. Finally, bound and free ligands were separated after 24 h by centrifugation (×3000 rpm ×30 min ×4℃) and radioactivity in the pellet was counted with a gamma counter (ARC-1000, Aloka, Japan). Typical standard and plasma dilution curve for bovine leptin RIA is shown in **Fig. 2-8**. Displacement curves for serially diluted bovine and caprine plasma are parallel to the standard curve.

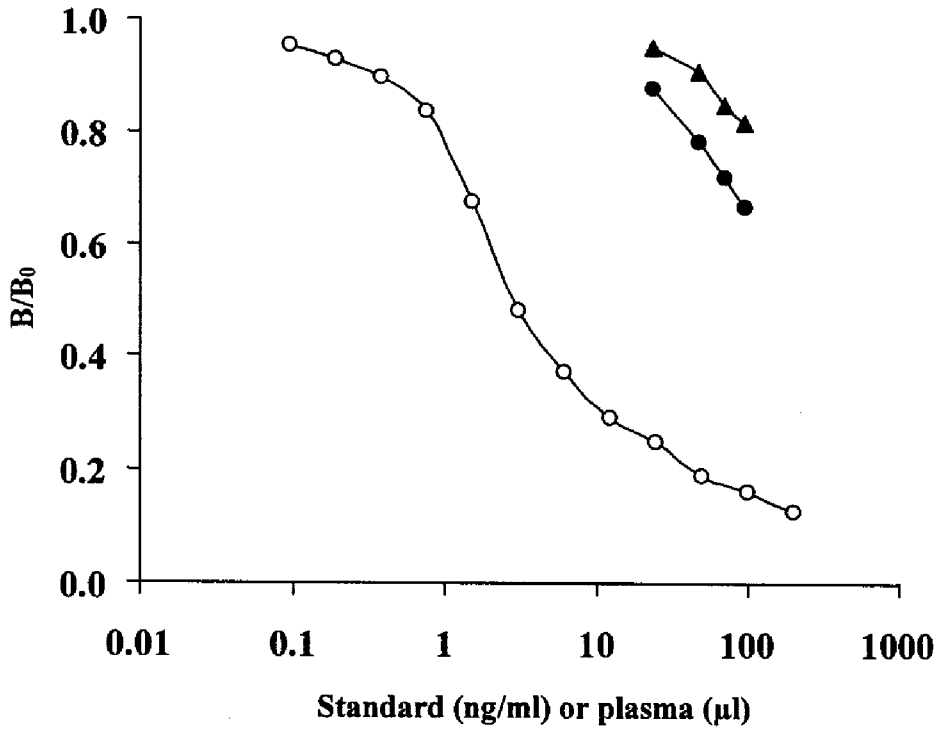

Fig. 2-8. Standard RIA curves for bovine leptin. Inhibition of ^{125}I-labeled bovine leptin binding to guinea pig anti-bovine leptin antiserum by serial dilution of bovine recombinant leptin (○). Inhibition of ^{125}I-labeled bovine leptin binding to antiserum by serial dilution of pooled bovine plasma (▲) and caprine plasma (●) parallel to the curve obtained using control bovine recombinant leptin as standard. Each point is the mean for triplicate determinations. B/B_0, bound/bound in zero standard.

Summary

We synthesized bovine acylated ghrelin [1-27], human GHRH-NH_2 [1-29], bovine PYY-NH_2 [1-36] and bovine PYY-NH_2 [3-36] for in vivo experiments, and [Cys-12]-acyl ghrelin [1-11], [Cys-0]-bovine ghrelin [13-27] and [Cys-0]-bovine PYY-NH_2 [4-36] for immunization procedures via Fmoc-strategy of solid phase peptide synthesis. In accordance, rabbit anti-ghrelin anti-serum which specifically recognizes the acylated ghrelin, guinea pig anti-bovine ghrelin anti-serum which recognizes the acylated ghrelin, des-acyl ghrelin and all ghrelin fragments with intact C-terminal region in ruminants, guinea pig anti-bovine PYY anti-serum which specifically recognizes both ruminant PYY [1-36] and ruminant PYY [3-36], and guinea pig anti-bovine leptin anti-serum are raised. By using these antisera, we have successfully validated the total of four radioimmunoassay (RIA) systems including ghrelin RIA which is applicable for the measurements of serum/plasma active ghrelin concentrations in both monogastric and ruminant species, and other RIA systems specific for the ruminant total ghrelin, PYY and leptin concentrations. The values measured by using these RIA systems will aid us to understand more deeply the physiology of growth and energy homeostasis in ruminants.

Chapter 3 (Experiment 2)

Dose-dependent response of plasma ghrelin and growth hormone concentrations to bovine ghrelin in Holstein heifers

INTRODUCTION

The gastric-derived endogenous ligand of GHS-R, ghrelin, has been reported as a novel peripheral GH stimulant in human as well as in rodents and domestic animals (Kojima *et al.* 1999, Hayashida *et al.* 2001), and acylation on its Ser^3 residue with octanoic acid is essential to activate its cognate receptor, GHS-R1a. Considerable amount of another isoform of ghrelin which is lack in acylation (des-acyl ghrelin) is also circulating in bloodstream (Hosoda *et al.* 2000*a*). Des-acyl ghrelin does not possess endocrine activities but promotes bone marrow adipogenesis *in vivo* (Thompson *et al.* 2004) and inhibits glucose output by primary hepatocytes (Gauna *et al.* 2005). Ghrelin is also known as orexigenic hormone and its effect on feeding is independent of the GH stimulatory pathway (Nakazato *et al.* 2001). Two molecular types of circulating ghrelin, i.e., ghrelin and des-Gln^{14}-ghrelin, have been purified and characterized from rat stomach and both peptides can bind to GHS-R1a (Hosada *et al.* 2000*b*). Monogastric animals have both forms of peptide, while ruminants only possess des- Gln^{14}-ghrelin because the first splicing acceptor site within intron 1 has been lost (Dickin *et al.* 2004). Moreover, although N-terminal region of ghrelin is homologous among species, 7 or 8 amino acids of C-terminal region of ruminant ghrelin are different from those of monogastric animals (Dickin *et al.* 2004).

Recent studies investigating the involvement of ghrelin in ruminant physiology have reported that ghrelin immunoreactive cells are present in abomasum of cow (Hayashida *et al.* 2001), and that the preprandial increase and postprandial decrease in

plasma ghrelin levels are regulated by cholinergic neurons in sheep (Sugino *et al.* 2004). Furthermore, *in vivo* and *in vitro* studies on the effect of ghrelin in adenohypophysial axis of ruminants have been performed by utilizing supraphysiological doses of rat or human ghrelin (Hayashida *et al.* 2001, Hashizume *et al.* 2003 & 2005, Itoh *et al.* 2005), and it has been reported that ghrelin stimulates GH secretion in dairy cattle of different physiological stages. A very recent study using 1 μg human ghrelin/kg BW showed that ghrelin also has different regulatory effect on plasma pancreatic hormone levels and on glucose concentration (Itoh *et al.* 2006). However, in our knowledge, there is no report for the dose-dependent effect of bovine ghrelin, especially of the physiological dose, on central and peripheral hormones regulating energy homeostatis in ruminants. In this experiment, we studied the effect of intravenous injection of synthesized acylated bovine ghrelin [1-27] on plasma ghrelin, GH, insulin, leptin and metabolite levels in Holstein heifers.

MATERIALS AND METHODS

All animal-related procedures used in this experiment were approved by the Institutional Animal Care and Use Committee of the Obihiro University of Agriculture and Veterinary Medicine, Japan.

Animals

Six Holstein heifers born at the Field Science Center (FSC), Obihiro University, were housed under a natural light-darkness cycle. Heifers were 6 month old with an average body weight (BW) of 178.4 ± 6.4 kg (± S.E.M) at the beginning of experiment. They were fed twice daily (0800 h & 1600 h) with concentrates for growing calves (crude protein 14%, crude fat 1.5%, crude fiber 8%, and crude ash 7%, Hokuren, Sapporo, Japan) under the routine management practice of FSC, Obihiro University. Animals finished eating the concentrate within 20min. Timothy hay, mineral salt block and water were available *at libitum*. Body weight was measured one day prior to each injection. At the end of experiment, heifers had an average BW of 196.2 ± 7.8 kg and the average daily gain during the experiment was 0.89 kg.

Peptides

N-acylated bovine ghrelin [1-27] (GSSFLSPEHQKLQRKEAKKPSGRLKPR), [Cys-12]-ghrelin [1-11] with acylation and [Cys-0]-bovine ghrelin [11-27] were synthesized by manual Fmoc solid phase peptide synthesis procedures. Detail of the procedures for ghrelin synthesis and octanoylation on the Ser^3 residue are presented at chapter 2. Peptidyl resin was cleaved with TFA followed by purification on a TSK ODS-120A column (7.8 mm ID×30 cm) by HPLC, linear gradient of 0-60% CH_3CN and fractions containing peptides were lyophilized and stored at -80°C. Lyophilized bovine

ghrelin (1-27) was dissolved in de-ionized water and diluted to 1mg/ml with sterilized 0.1% BSA-saline for the *in vivo* study.

Peptide injection and sampling procedures

Animals were subjected to a complete blocked Latin Square Design (six animal × six dose of injection× six day of sampling with one or two day recovery period) to obtain the minimal residual effects. For both injection of peptide and blood sampling procedures, a jugular vein catheter was inserted to each heifer one day before the start of experiment, and patency of the catheter was maintained with heparinized saline. Bovine ghrelin [1-27] was freshly diluted with sterilized solution of 0.1% BSA saline to reach the desired concentration for each animal before the injection. Animals were loosely chained to the stanchion during sampling and were injected with vehicle (5 ml of 0.1% BSA saline as control) or vehicle containing synthesized bovine ghrelin (0.1, 0.5, 1.0, 5.0, 10.0 μg/kg BW) at 1100 h (3 h after morning feeding) via jugular catheter followed immediately by flushing out of catheter with 5 ml heparinized saline. Blood samples were collected at -30, -15, 0, 5, 10, 15, 20, 30, 45, 60, 90, 120, 150, 180 min relative to the time of injection and moved into pre-ice-chilled heparinized tubes. Plasma was isolated after centrifugation (3000 r.p.m × 20 min at 4°C) and stored at -30°C until assay. For plasma ghrelin assay, 600 μl plasma were separately acidified with 30 μl 1 M HCl and stored at -30°C.

RIA for bovine ghrelin, GH, insulin and leptin

RIAs for bovine active ghrelin and for bovine total ghrelin have previously been described in chapter 2. Triplicate standards and duplicate samples were run within a single assay at 4°C, except samples where the concentrations exceeded the range of the total ghrelin assay. These samples were adjusted after re-assay with diluted plasma. Displacement curves of diluted bovine plasma with ^{125}I-bovine ghrelin were parallel to the

standard curve for both active and total ghrelin. Average recovery rate of 3 known amounts of bovine ghrelin added to the bovine plasma pool were 105% and 106% for active and total ghrelin, respectively. Sensitivities of the present assay were 14.2 pg/ml and 0.14 ng/ml, IC_{50} were 264.1 pg/ml and 8.5 ng/ml, and intra assay coefficient of variation (CV) were 6.3% and 7.3% for active and total ghrelin RIA, respectively.

Plasma GH concentration was measured by double antibody RIA procedures (Lee *et al.* 2000) using bovine GH (USDA-bGH, AFP-9884C) and anti-ovine GH (USDA-anti-oGH, AFP-0802201). Sensitivity and intra-assay coefficient of variations were 0.25 ng/ml and 7.3% respectively. Plasma insulin concentration was quantified as previously described (Inoue *et al.* 2005) by using bovine insulin (28.5 USP units/mg, Code I-5500, Sigma Chemical Company, St. louis, MO, USA) and guinea-pig anti-bovine insulin antiserum (Code I-6136, Sigma Chemical Company). Sensitivity and the intra-assay coefficient of variations were 0.05 ng/ml and 4.0%, respectively. Plasma leptin concentrations were measured by in-house double antibody RIA procedures (See chapter 2). Sensitivity and the intra-assay coefficient of variations were 0.46 ng/ml and 5 %, respectively. All samples were run in duplicate at 4°C for each assay.

Measurements of plasma metabolites

Plasma glucose and non-esterified fatty acid (NEFA) levels were measured by commercially available kits (Code No. 439-90901 and 279-75401 respectively, Wako, Japan).

Statistics

All data are expressed as means ± S.E.M. Statistical difference between averaged pre-injected values (from -30 to 0 min) and post-injected concentration at each time points were analyzed using one way ANOVA, followed by multiple comparisons of general linear

mixed model. Area under the curve (AUC) of each hormone for the 0 to 60 min response to injection of vehicle or ghrelin was calculated using the trapezoid method and differences were evaluated by Student's paired *t*-test. All analyses were performed using SPSS for Windows, version 10.0.0 (SPSS, Chicago, IL, USA). $P < 0.05$ was considered significant.

RESULTS

The changes in plasma active and total ghrelin responses to synthesized bovine ghrelin are presented in **Fig. 3-1**. The RIA for active ghrelin utilizing the rabbit anti-bovine ghrelin [1-11] antiserum showed that there were no differences in pre-injected plasma active ghrelin concentrations in any of the groups (159.0 ± 19.1 pg/ml). Exogenous ghrelin administration increased plasma active ghrelin levels in a dose-dependent manner (**Fig. 3-1A**). Significantly higher peak amplitude was seen 5 min after 0.5 μg/kg BW injection compared with pre-injected levels (363.8 ± 123.1 vs. 195.1 ± 33.7 pg/ml, $P < 0.01$) and increased dose-dependently. Thereafter, the elevated active ghrelin levels quickly returned to the pre-injected levels within 10 min. On the other hand, the RIA for bovine total ghrelin utilizing the antibody against bovine ghrelin [11-27] showed that the basal concentration of bovine total ghrelin was 2.8 ± 0.7 ng/ml and this value is approximately 16 times higher than the basal active ghrelin level. Total ghrelin was also increased transiently following synthetic bovine ghrelin injection and returned to the pre-injected values within 5-15 min (**Fig. 3-1B**). Similar to the changes in active ghrelin levels, significantly higher peak amplitude was also seen 5 min after 0.5 μg/kg BW injection compared with pre-injected levels (24.1 ± 5.1 vs. 3.9 ± 1.5 ng/ml, $P < 0.001$) and increased dose-dependently.

The response of plasma GH concentration to bovine ghrelin is presented in **Fig. 3-2**. Plasma GH concentration in the vehicle-injected group did not change throughout the experiment (2.6 ± 0.6 ng/ml). Acylated bovine ghrelin [1-27] injection stimulated GH secretion dose-dependently. Plasma GH level peaked significantly 5 min after injection of 0 .1 μg ghrelin/kg compared with pre-injected values (6.1 ± 1.9 vs 2.1 ± 0.5 ng/ml, $P < 0.05$). The peak amplitude and duration of the GH response were dependent on the dose of ghrelin, i.e., higher dose of bovine ghrelin resulted in increased GH level over a longer period (**Fig. 3-2**).

The response of plasma insulin to exogenous bovine ghrelin is shown in **Fig. 3-3**. Vehicle injection did not modify the plasma insulin level throughout the study (0.7 ± 0.1 ng/ml). The plasma insulin concentration increased, in general, about 10-15 min after ghrelin administration and decreased within 45 min post-injection. Transient but significant peaks were seen only in groups injected with higher doses of ghrelin (5.0 μg/kg BW and 10.0 μg/kg BW).

Average basal glucose concentration in bovine plasma was 86.5 ± 2.9 mg/dl in the vehicle-injected group. Plasma glucose levels increased after ghrelin injection compared with pre-injected values but did not reach significance and the response was independent of the dose-given. Moreover, there was a wide range in variation among animals (data not shown).

Figure 3-4 shows the effect of synthetic bovine ghrelin on plasma NEFA level in Holstein heifers. Basal NEFA concentration in the control group (35.7 ± 3.8 μEq/l) was lower than that of ghrelin injected groups and significant changes were not seen throughout the experiment. Injection of bovine ghrelin tended to increase plasma NEFA level but the values did not reach significance compared with pre-injected values when ghrelin was given in low doses (0.1 μg/kg BW & 0.5 μg/kg BW). On the other hand, plasma NEFA concentration increased as early as 5-10 min after injection with the higher doses of ghrelin (from 1.0 μg/kg BW to 10.0 μg/kg BW) and returned to pre-injected values 60 min later.

Plasma leptin levels did not change in vehicle-injected groups (1.37 ± 0.07 ng/ml), and administration of bovine ghrelin did not modify the basal plasma leptin concentrations (**Fig. 3-5**). However, plasma leptin levels increased 120 min later after ghrelin injection at a dose of 10.0 μg/kg BW (average peak values is 2.28 ± 0.30 ng/ml).

Table 3-1 summarizes the AUCs of plasma hormones and metabolites 0-60 min after ghrelin injection. Mean AUCs for six animals were pooled after subtraction of pre-injected AUCs and expressed as a percentage of the AUC for the vehicle-injected group.

Active ghrelin AUC pooled for 0-60 min started to increase significantly when the dose of ghrelin was increased to 0.5 μg/kg BW compared with that of the vehicle injected group (11934.2 ± 1470.6 vs 9472.6 ± 1091.2 pg/ml/min). Total ghrelin AUC after ghrelin injection at 0.1 μg/kg BW was 110% of the values of vehicle-injected group (172.2 ± 42.7 vs. 155.9 ± 35.1 ng/ml/min) and increased dose-dependently ($P < 0.001$). Reflective to the changes in GH concentration, GH AUC for 1h post-injection with the smallest dose of bovine ghrelin (0 .1 μg/kg) was significantly greater than that of the control group ($P < 0.01$). The GH AUC after injection with the highest dose of ghrelin (10.0 μg/kg BW) increased 797% over that of the vehicle-injected group (1186.66 ± 16.95 vs. 148.86 ± 1.43 ng/ml/min, $P < 0.001$). However, none of the glucose AUCs 1 h after injection with several doses of ghrelin significantly differed compared with the vehicle-injected group (5221.3 ± 112.8 mg/dl/min). Moreover, insulin AUC did not increase significantly in any of the ghrelin-injected groups compared with the vehicle-injected group (44.8 ± 5.6 ng/ml/min). In all ghrelin-injected groups, pooled NEFA AUCs for 0-60 min post-injection were significantly higher than that of vehicle-injected group (vs. 2208.9 ± 165.5 μEq/l/min). However, no clear effect of ghrelin on leptin AUCs was observed.

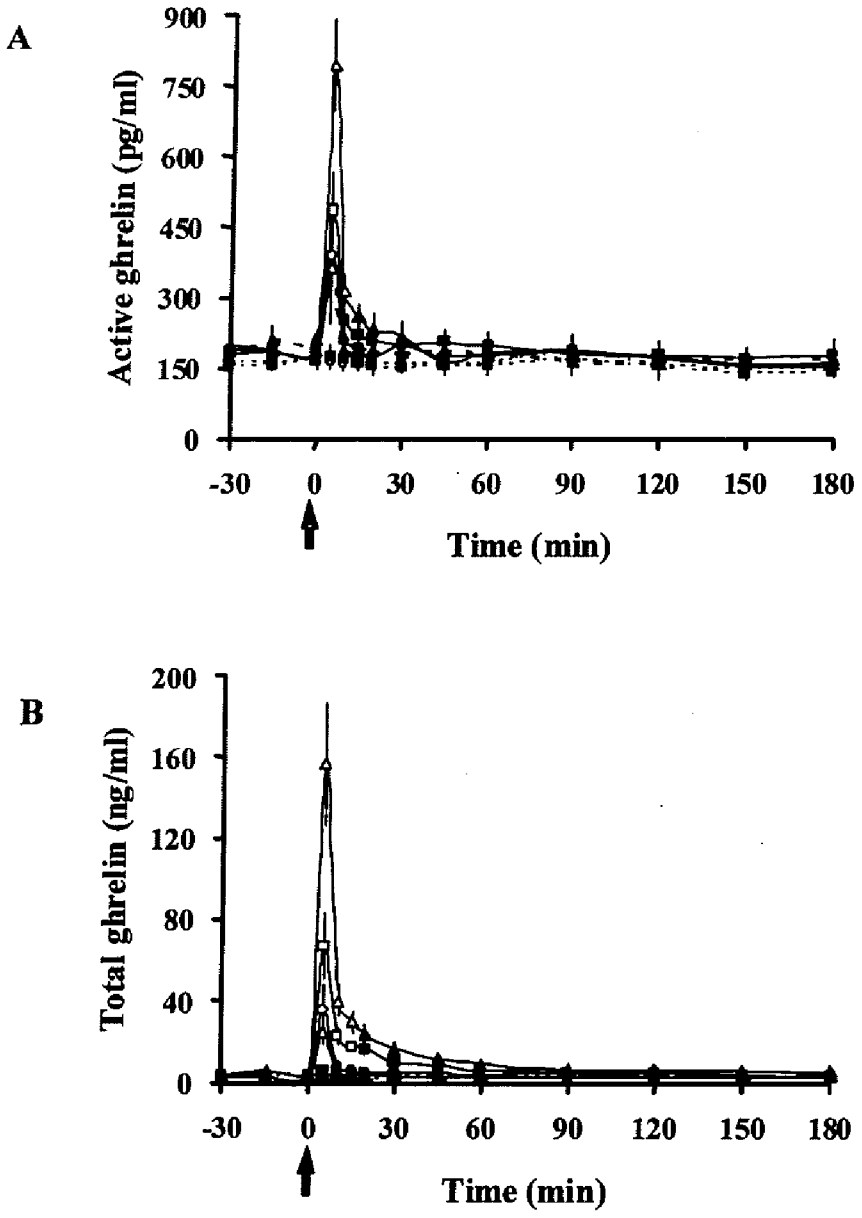

Fig. 3-1. Changes of plasma active (A) and total (B) ghrelin concentration in response to synthetic bovine ghrelin in Holstein heifers. Animals were intravenously injected with acylated bovine ghrelin (--●--; 0, --■--; 0.1, --▲--; 0.5, —●—; 1.0, —■—; 5.0, —▲—; 10.0 μg/kg BW) dissolved in 5 ml 0.1% BSA-saline. Plasma ghrelin concentrations were measured by RIA. The arrow indicates the time of injection. Values are the means ± SEM of 6 animals. Opened symbols indicate a significant difference ($P < 0.05$) compared with mean pre-injected concentrations (-30 to 0 min).

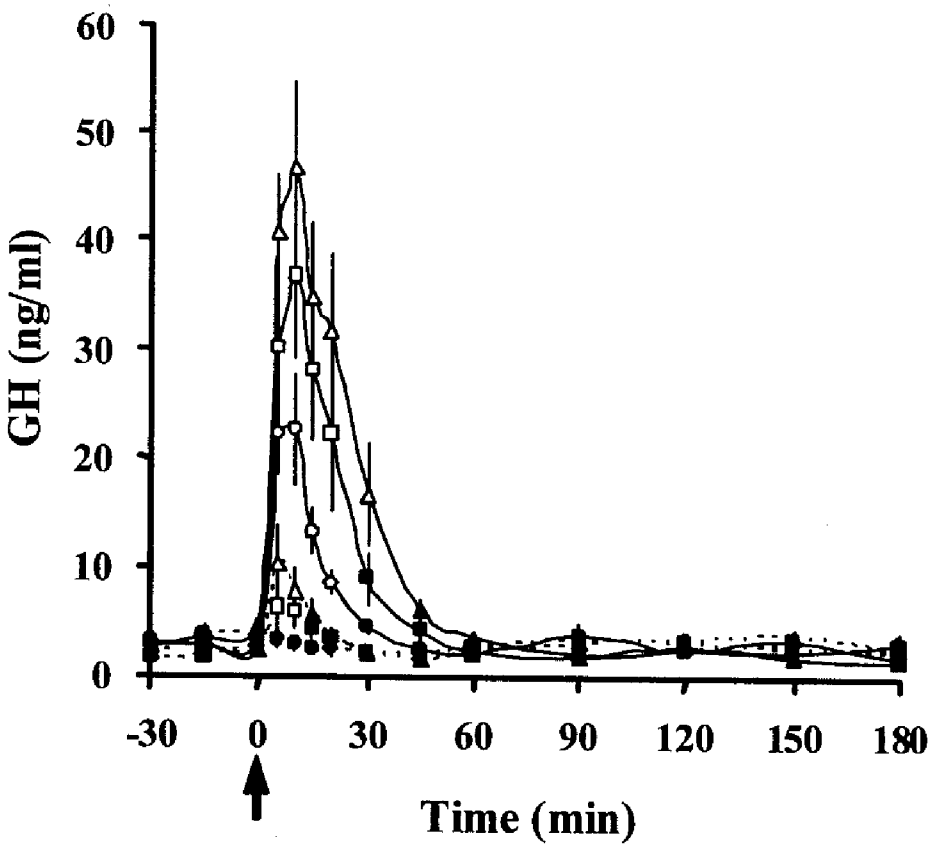

Fig 3-2. Dose-dependent stimulatory effect of ghrelin on plasma GH concentration in Holstein heifers. Animals were intravenously injected with acylated bovine ghrelin (--●--; 0, --■--; 0.1, --▲--; 0.5, —● —; 1.0, —■—; 5.0, —▲—; 10.0 μg/kg BW) dissolved in 5 ml 0.1% BSA-saline. The arrow indicates the time of injection. Values are the means ± SEM of 6 animals. Opened symbols indicate a significant difference ($P < 0.05$) compared with mean pre-injected concentrations (-30 to 0 min).

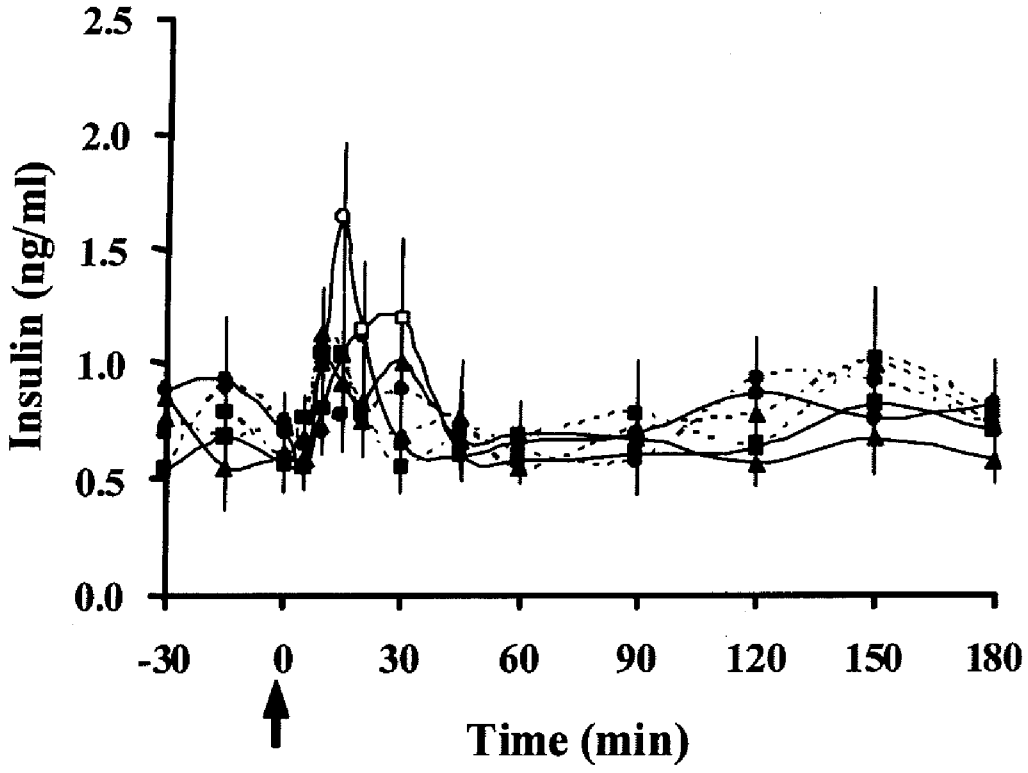

Fig. 3-3. Response of plasma insulin to ghrelin in Holstein heifers. Animals were intravenously injected with acylated bovine ghrelin (--●--; 0, --■--; 0.1, --▲--; 0.5, —●—; 1.0, —■—; 5.0, —▲—; 10.0 μg/kg BW) dissolved in 5 ml 0.1% BSA-saline. The arrow indicates the time of injection. Values are the means ± SEM of 6 animals. Opened symbols indicate a significant difference ($P < 0.05$) compared with mean pre-injected concentrations (-30 to 0 min).

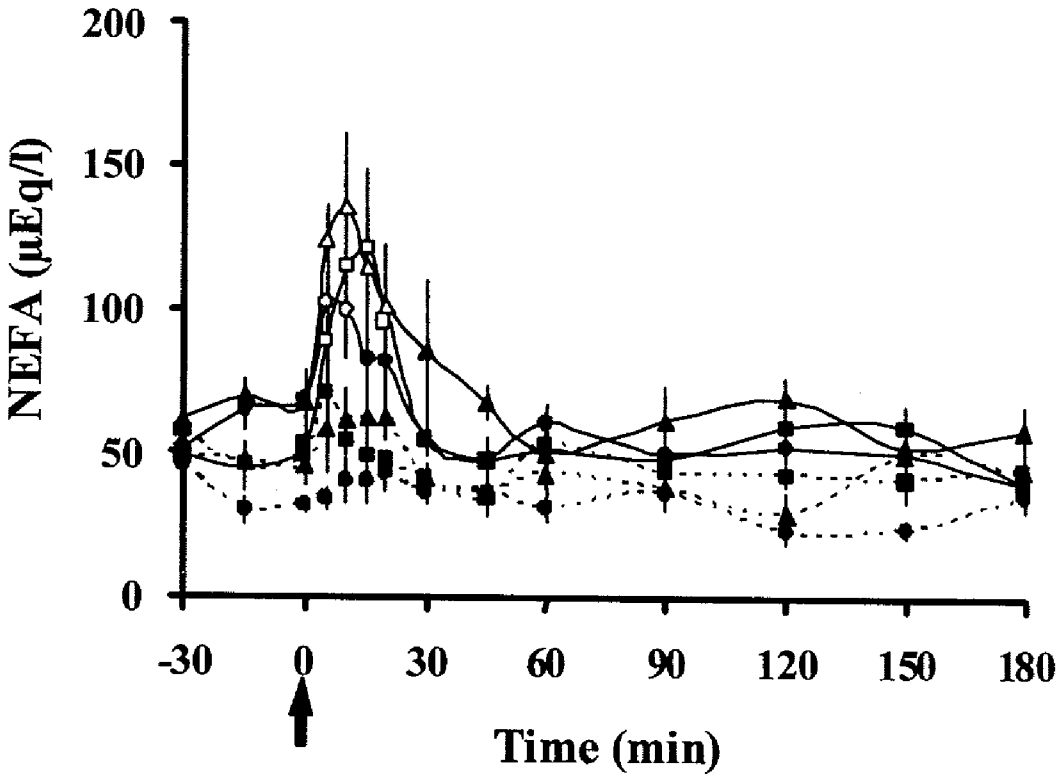

Fig. 3-4. Dose-dependent effect of ghrelin on non-esterified fatty acids (NEFA) in Holstein heifers. Animals were intravenously injected with acylated bovine ghrelin (--●--; 0, --■--; 0.1, --▲--; 0.5, —●—; 1.0, —■—; 5.0, —▲—; 10.0 µg/kg BW) dissolved in 5 ml 0.1% BSA-saline. The arrow indicates the time of injection. Values are the means ± SEM of 6 animals. Opened symbols indicate a significant difference ($P < 0.05$) compared with mean pre-injected concentrations (-30 to 0 min).

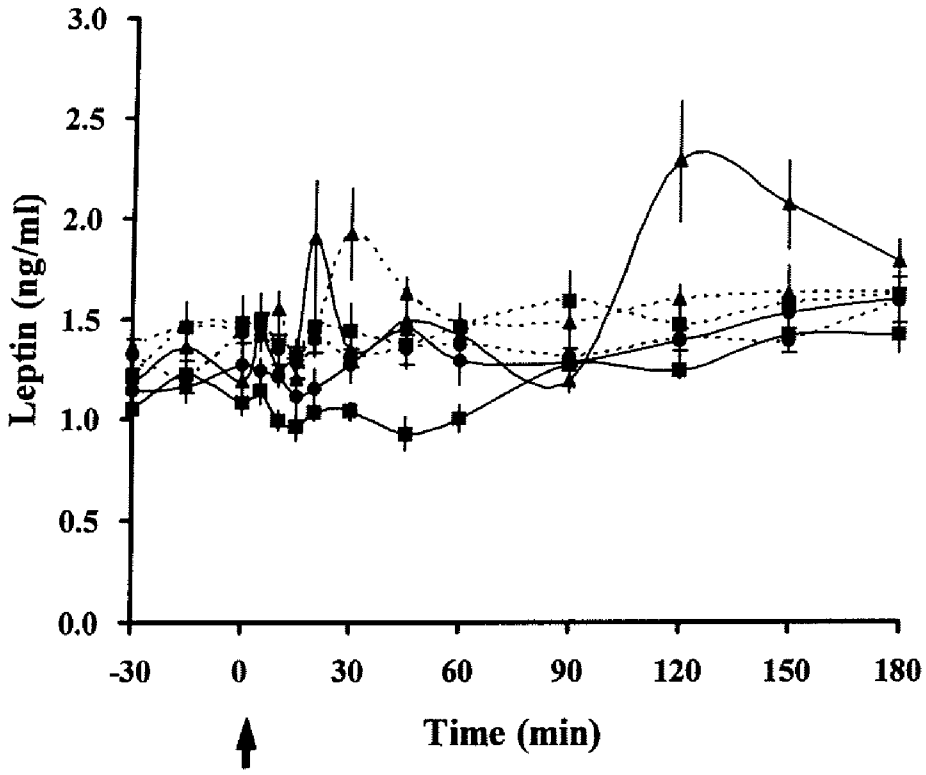

Fig. 3-5. Dose-dependent effect of ghrelin on plasma leptin levels in Holstein heifers. Animals were intravenously injected with acylated bovine ghrelin (--●--; 0, --■--; 0.1, --▲--; 0.5, —●—; 1.0, —■—; 5.0, —▲—; 10.0 μg/kg BW) dissolved in 5 ml 0.1% BSA-saline. The arrow indicates the time of injection. Values are the means ± SEM of 6 animals. Opened symbols indicate a significant difference ($P < 0.05$) compared with mean pre-injected concentrations (-30 to 0 min).

Table 3-1. Area under the curve of plasma hormones and metabolites from 0 to 60 min after injection of ghrelin in six Holstein heifers.

	Dose of synthetic bovine ghrelin (μg/kg BW)				
	0.1	0.5	1.0	5.0	10.0
GH	128 ± 23*	149 ± 32*	313 ± 45***	562 ± 124***	797 ± 143***
Active ghrelin	100 ± 8	126 ± 16***	127 ± 14***	146 ± 14***	163 ± 16***
Total ghrelin	110 ± 27	185 ± 31***	281 ± 61***	612 ± 96***	1127 ± 210***
Insulin	98 ± 16	102 ± 15	108 ± 18	112 ± 15	107 ± 25
Glucose	94 ± 2	97 ± 3	98 ± 3	96 ± 3	101 ± 3
NEFA	126 ± 7***	131 ± 10***	185 ± 22***	193 ± 30***	238 ± 38***
Leptin	105 ± 9	116 ± 6	93 ± 6	74 ± 4	107 ± 6

Bovine ghrelin was dissolved in 5 ml 0.1% BSA-saline and intravenously injected at 0 min. AUCs were presented as percentage (± SEM) of vehicle-injected group after subtraction of pre-injected AUC (-30 min to 0 min).
*$P < 0.05$, ***$P < 0.001$ vs. vehicle-injected control group.

DISCUSSION

Feeding time, energy balance, physiological and some pathological states such as obesity influence the plasma ghrelin levels in domestic animals as well as in humans (Korbonits *et al.* 2004). Our previous study demonstrated that plasma ghrelin level showed the nocturnal periodicity and tended to be increased by fasting in pigs (Inoue *et al.* 2005). In ruminants, circulating active ghrelin levels are lower in 3-month-old calves than in mature cows (Miura *et al.* 2004). It has also been reported that plasma active ghrelin levels increased preprandially and decreased after feeding in schedule meal-fed sheep (Sugino *et al.* 2004) but these periprandial changes were not evident in 3 month old calves (Miura *et al.* 2004). In the present study, the bovine ghrelin RIA showed that both isoforms of ghrelin (acylated and des-acyl ghrelin) are circulating in bloodstream of Holstein heifers with considerable amounts. This result is in agreement with other reports in human and animal models (Hosoda *et al.* 2000*a*, Asakawa *et al.* 2005). The basal level of total ghrelin is approximately 16 times higher than that of active ghrelin, but it should be noted that this ratio can only be used for the six-month-old heifers and not for animals of other physiological stages such as pre-ruminant animals, i.e., calves. In pre-ruminant stage, ingested liquid feeds by-pass the reticulorumen and enter directly to the abomasum where ghrelin immunoreactive cells exist. After weaning, ingested dry feeds are slowly digested by ruminal microbes, and the nature of nutrients absorbed by abomasum become different from the pre-ruminant stage. Therefore, weaning may alter the basal plasma levels of gastric-derived hormone, ghrelin, the secretion of which is influenced by ingested nutrients (Korbonits *et al.* 2004). This hypothesis is supported by the study in which basal gut hormones such as gastrin, cholecystokinin, pancreatic polypeptide and vasoactive intestinal peptide levels increased, while secretin and somatostatin levels decreased after weaning in Holstein calves (Toullec *et al.* 1992). Moreover, orally ingested glyceryl trioctanoate rich in

milk lipids significantly increases the active ghrelin levels in murine stomach leading to the results that the amount of active ghrelin in stomach tissues increasing gradually during the suckling period and decreasing sharply after the initiation of weaning, while plasma total ghrelin levels do not change across this transitional period (Nishi *et al.* 2005). Therefore, it can be suggested that plasma active and total ghrelin concentrations and their ratio in plasma may alter with different physiological stages like before and after weaning in ruminants.

On the other hand, the role of des-acyl ghrelin on ruminant physiology is still not known. In rat, administration of des-acyl ghrelin decreased food intake and gastric emptying rate through an action on the paraventricular nucleus and the arcuate nucleus in the hypothalamus (Asakawa *et al.* 2005). Des-acyl ghrelin is supposed to induce negative energy balance, in contrast to acylated ghrelin (Tschöp *et al.* 2000, Nakazato *et al.* 2001), and possesses some peripheral effects (Thompson *et al.* 2004, Gauna *et al.* 2005). Recently, Iqbal *et al* (2006) reported that injection of acylated ghrelin failed to induce voluntary feed intake but significantly stimulated GH secretion in sheep while Toshinai *et al* (2006) reported that intracerebroventricular injection of des-acyl ghrelin stimulated feeding during light phase via distinct pathway from GHS-R in rats fed *ad libitum*. Therefore, it is suggested that dominant amount of circulating des-acyl ghrelin may also play an important role in mechanisms such as regulation of feeding in ruminants. Further extended studies emphasizing the effects of feeding, physiology and energy states on plasma ghrelin levels, and studies on the role of des-acyl ghrelin in ruminant physiology are necessary.

In this study, both active and total ghrelin levels were only temporally elevated in plasma after injection of synthetic bovine ghrelin and were degraded/eliminated in the bloodstream within 15 min. This result was in consistent with other reports in which the half-life of plasma ghrelin was supposed to be very short (Tschöp *et al.* 2000, Gauna *et al.* 2004). The similar results has reported in weaned pigs; serum ghrelin level peaked within

15 min of intravenous infusion of human ghrelin (2 μg/kg BW) and decreased to nearly half-maximal values by 30 min after infusion (Salfen *et al.* 2004).

In dairy industry, it is of economical importance to increase plasma GH secretion because it is associated with faster growth, less fat stores and improved milk production (McMahon *et al.* 2001b). Two hypothalamic hormones, GHRH and SRIF are known to be the main regulators of episodic GH release from anterior pituitary gland where GHRH stimulates and SRIF inhibits the GH secretion. Other synthetic enkephalin derivatives, GHRPs, (also known as GHSs) also stimulate the secretion of GH (Bowers CY 1998). The endogenous ligand of GHS-R, ghrelin, has been of interest as a peripheral potent GH stimulator since it was first purified from rat stomach (Kojima *et al.* 1999), and several studies have been published on the physiological and pharmacological effects of ghrelin in human and domestic animals (Korbonits *et al.* 2004). It has also been reported that the octanoylation on Ser^3 residue of the N-terminal region is important for ghrelin to activate its receptor, GHS-R1a (Kojima *et al.* 1999), and short peptides encompassing the first 4-5 residues of ghrelin can activate the GHS-R1a almost as efficiently as the full-length ghrelin (Bednarek *et al.* 2000). In contrast, truncated peptides of octanoylated ghrelin such as EP-01037 (Gly-Ser-Ser(O-octanoyl)-Phe-NH_2) and EP-00775 (Gly-Ser-Ser(O-octanoyl)-Phe-Leu-Ser-Pro-Glu-NH_2) neither stimulate GH secretion *in vivo* nor replace radiolabelled ghrelin from binding sites (Torsello *et al.* 2002). Moreover, bovine ghrelin is a 27 amino acid peptide and 8 amino acids of C-terminal region [11-27] are heterologous to those of monogastric animals (Dickin *et al.* 2004). Based on these facts, it is preferable to use a specific peptide to investigate the physiological effect of ghrelin on the mechanisms of GH secretion in ruminants.

In this study, we firstly reported the dose-dependent stimulatory effect of synthetic acylated bovine ghrelin [1-27] on GH secretion in Holstein heifers. We used 6 doses of synthetic bovine ghrelin from 0.1 μg/kg BW to 10 μg/kg BW (approximately 0.033

nmol/kg BW to 3.3 nmol/kg BW) in single bolus injections. The minimal dose of bovine ghrelin used in this study induced significantly greater GH AUC 1 hour after injection compared with that of vehicle-injected control group. The GH peak amplitude also reached significant as early as 5 min after injection. Itoh *et al* (2005) also reported that intravenous bolus injection of human ghrelin (0.3 nmol/kg BW, approximately equivalent to 1 μg/kg BW) significantly stimulated GH secretion in dairy cattle, and the effect seemed to be more potent in early lactating cows than in calves and in late lactation cows. In contrast, Hashizume *et al* (2005) reported that intra-arterial injection of rat ghrelin at 1μg/kg BW was not able to stimulate GH secretion in ovariectomized goats but injection of ghrelin at 3 μg/kg BW significantly stimulated GH release. Moreover, Iqbal *et al* (2006) reported that a bolus injection of ovine ghrelin at the dose of 1, 5, 10 and 20 μg to third cerebral ventricle induced a weaker GH response compared with DAP-octanoyl^3 human ghrelin injection in ovariectomized ewes. These conflicting results of different responses of GH to ghrelin injection might be due to the differences in experimental condition, differences in physiological stages of the experimental animals such as age, different preparations of peptide for injection and the use of human/rat ghrelin which have quite different amino acid residues at C-terminal region compared with ruminant ghrelin (Dickin *et al.* 2004). Our results from dose-dependent study clearly indicated that a physiological dose of bovine ghrelin is sufficient to induce GH secretion in Holstein heifers and this is consistent with the results of previous works in human and rats (Takaya *et al.* 2000, Hataya *et al.* 2001, Tolle *et al.* 2001). Moreover, the highest dose of ghrelin used in this study (10.0 μg/kg BW) still stimulated the secretion of GH significantly than the 5.0 μg/kg BW indicating that 10.0 μg/kg BW is not the situation dose for the maximum GH release in Holstein heifers. Altogether, these data suggest that ghrelin is a potent GH releaser in ruminants.

The increase of plasma insulin concentration in response to synthetic bovine ghrelin injection reached significance at 10 min when the heifers were injected with 1.0

μg/kg BW while lower doses of ghrelin failed to stimulate insulin secretion. However, none of the insulin AUCs for 1 h post-injection in ghrelin-injected groups reached significant compared with the vehicle-injected groups. The effect of ghrelin on pancreatic hormones is still unclear. There are some reports suggesting that ghrelin has stimulatory (Date *et al.* 2002b, Lee *et al.* 2002) or inhibitory (Broglio *et al.* 2001, Reimer *et al.* 2003) effects on insulin secretion in human and rats. Moreover, Salehi *et al* (2004) reported that a physiological dose of ghrelin has no effect on glucose-stimulated insulin release from isolated mouse islets while low doses inhibit and high doses stimulate. Furthermore, injection of 1 and 10 nmol ghrelin/kg BW suppresses basal plasma insulin levels in intact mice. A very recent study on ruminants reported that injection of 1μg ghrelin/kg BW has a stimulatory effect on insulin in lactating cows but not in calves (Itoh *et al.* 2006). Combining these results with ours in which physiological doses of ghrelin failed to elevate insulin levels, it seems that physiological concentrations of ghrelin do not influence insulin secretion. Coincidentally, the injection of physiological or supra-physiological doses of ghrelin failed to induce apparent changes in plasma glucose concentration in this study. This result is in agreement with other reports in ruminants studies on the effects of KP 102, another potent GH secretagogue (Roh *et al.* 1996) and ghrelin (Itoh *et al.* 2006). On the other hand, it was reported that injection of acyl ghrelin induces a rapid rise in insulin and glucose levels but decreases insulin sensitivity in human models (Gauna *et al.* 2004). These authors also reported in another *in vitro* study that acylated ghrelin induced while des-acyl ghrelin inhibited glucose output from primary porcine hepatocytes (Gauna *et al.* 2005). From this dose-dependent study, it is not possible to extrapolate the precise regulatory mechanism of ghrelin, insulin and glucose in ruminants and more extended studies are needed in these area.

In humans, a 2-h intravenous infusion of acylated human ghrelin (7.5 pmol/kg/min) significantly increased not only serum GH concentrations, but also serum NEFA,

adenocorticotropin, epinephrine and prolactin levels when ghrelin was given at a dose of 15 pmol/kg/min (Lucidi *et al.* 2005). In our results in ruminants, a bolus injection of bovine ghrelin stimulated plasma NEFA level (**Fig. 3-4**), but the response was slightly delayed compared with the time-course of ghrelin-induced GH release. This result is similar to a previous report (Roh *et al.* 1996) in which serial injections of KP102 resulted in elevation of plasma NEFA level in Holstein calves. In rodents, chronic ghrelin administration has been shown to increase body fat content and to stimulate the activity of glycerol-3-phosphate dehydrogenease and the differentiation of rat parametrial preadipocytes *in vitro* (Tschöp *et al.* 2000, Korbonits *et al.* 2004). These controversial effects of ghrelin on lipid metabolism might be due, at least in part, to the differences in species, length (chronic and acute) and dose of peptide administration. One possible explanation for our result is that elevated plasma ghrelin may mimic the temporary negative energy balance in heifers because plasma ghrelin levels elevated in the conditions of energy insufficiency (Korbonits *et al.* 2004, Anderson *et al.* 2005). In addition, ghrelin-induced elevation of plasma GH concentration may promote fatty acid degradation (McMahon *et al.* 2001*b*). As the result, reserved body fat is mobilized to maintain the energy balance leading to the subsequent increase in plasma NEFA concentration (Chilliard *et al.* 2000). Other factors such as leptin (adipose tissue derived protein), insulin and glucagons may participate in the counter-regulation of fat metabolism with ghrelin.

In conclusion, our dose-dependent study clearly demonstrates that both acylated and des-acyl ghrelin are circulating in the bloodstream in considerable quantities in ruminant species. Moreover, our data show that intravenous injection of ghrelin at physiological doses is sufficient to induce endogenous GH secretion and confirm the potent stimulatory effect of acylated bovine ghrelin on plasma GH secretion in Holstein heifers. Ghrelin also has considerable effect on plasma insulin and NEFA levels indicating that ghrelin plays an important role in ruminant physiology.

SUMMARY

The stimulatory effect of novel gastric-derived hormone, ghrelin, on GH secretion has been reported in domestic animals as well as in human and rats. The effects of pharmacological doses of rat and/or human ghrelin on GH secretion have been reported recently in ruminants; however, physiological effect of exogenous bovine ghrelin on its own plasma level and on GH secretion is still unknown. In this study, the dose-dependent effects of synthesized acylated bovine ghrelin [1-27] on plasma active and total ghrelin, GH, insulin and metabolites were measured in Holstein heifers. Six animals were intravenously injected with synthesized acylated bovine ghrelin (0, 0.1, 0.5, 1.0, 5.0, 10.0 μg/kg BW and plasma hormone concentrations were measured from serially collected samples. Bovine ghrelin RIA showed that basal level of total ghrelin is approximately 16 times higher than that of active ghrelin in bovine plasma. Both forms of ghrelin were increased in dose-dependent manner responsive to the bovine ghrelin injections, peak values were reached at 5 min after administration and returned to pre-injected values within 15 min. Plasma GH was responsive to all doses of bovine ghrelin in dose-dependent manner, peaked as early as at 5-10 min after injection and returned to the basal value within 60 min. The GH area under curve 1 h after injection of smallest dose of ghrelin used in this experiment (0.1 μg/kg BW) was significantly higher than that of vehicle (0.1% BSA saline)-injected control group ($P < 0.05$). GH response to the highest dose of ghrelin (10.0 μg/kg BW) was greater than the response to 5.0 μg/kg BW ghrelin ($P < 0.001$). Plasma glucose concentrations were not significantly altered by the administration of bovine ghrelin while plasma insulin levels were transiently stimulated by higher doses of ghrelin (1.0, 5.0, 10.0 μg/kg BW). Plasma NEFA levels also increased following the ghrelin administration. This study confirms that ghrelin is not only a potent stimulator for GH secretion but also plays a considerable role in energy homeostasis of Holstein heifers.

Chapter 4 (Experiment 3)

Effect of pretreatment of des-acyl ghrelin on ghrelin-induced hormonal changes in Holstein steers

INTRODUCTION

In Chapter 3, we have clearly demonstrated the potent stimulatory effect of ghrelin on the GH secretion in Holstein heifers (ThidarMyint *et al.* 2006). Moreover, intravenous administration of the supraphysiological doses of ghrelin stimulated insulin and NEFA concentrations. Two types of ghrelin molecular forms, acyl- and des-acyl ghrelin (Hosoda *et al.* 2000*a*) circulates in the blood stream in all species studied to-date and their ratio is about 1:16 in Holstein heifers (ThidarMyint *et al.* 2006). The acyl-modification on its Ser^3 residue is supposed to be indispensable for the binding of ghrelin to the cognate receptor, GHS-R1a (Kojima *et al.* 1999). Moreover, N-terminal Gly-Ser-Ser(n-octanoyl)-Phe segment appeared to constitute the "active core" required for agonist potency at human GHS-R 1a (Bednarek *et al.* 2000). In contrast, Torsello *et al.* (2002) demonstrated that truncated ghrelin analogs encompassing the first 5 amino acids are ineffective in the stimulation on GH release in neonatal rat. Moreover, recent studies have demonstrated in monogastric animals that des-acyl ghrelin inhibits cell death in cardiomyocytes (Baldanzi *et al.* 2002) and central administration of des-acyl ghrelin reduces cumulative food intake in 16 h feed-deprived rats (Chen *et al.* 2005). Heijboer *et al* (2006) also reported that simultaneous administration of des-ghrelin abolishes the inhibitory effect of ghrelin on hepatic insulin action. Therefore, it can be hypothesized that des-acyl ghrelin acts as a natural antagonist of ghrelin. The objective of the present study was to determine the effect of des-acyl ghrelin on ghrelin-induced endocrine effects in ruminants.

MATERIALS AND METHODS

Animals

Five Holstein steers (10 months of age with 280 ± 9 kg BW) and six Holstein male calves (3 month of age with 86.8 ± 3.2 kg BW) were housed in group under natural dark-light cycle at beef cattle barn, Obihiro University of Agriculture and Veterinary Medicine. They were fed twice daily (0800 h & 1600 h) with concentrates for growing calves (crude protein 14%, crude fat 1.5%, crude fiber 8%, and crude ash 7%, Hokuren, Japan) according to the Japanese nutritional standard for beef-type dairy cattle. Animals finished eating concentrate within 20min. Timothy hay, mineral salt block and water were free-choice. Body weight was measured one day prior to each injection.

Experimental design

The experimental procedures were approved by the Institutional Animal Experimentation Ethics Committees of Obihiro University. A catheter was inserted to one of the external jugular vein of each steer one day before starting the experiment, and patency of the catheter was maintained with heparinized saline. Bovine acylated ghrelin [1-27] and bovine des-acyl ghrelin [1-27] were freshly diluted with sterilized solution of 0.1% BSA saline to reach the desired concentration for each animal before the injection. Animals were loosely chained to the stanchion during sampling. They were randomized to receive one of the following three intravenous injections: vehicle (5 ml of 0.1% BSA saline as control) injection 5 min prior to the injection of vehicle (v/v group), acylated ghrelin injections at a dose of 1.0 μg/kg BW with pre-injection of vehicle (v/ghr group), or 10.0 μg des-acyl ghrelin /kg BW (des-ghr/ghr group). Treatments were done at 0955 and 1000 h via jugular catheter followed immediately by flushing the catheter out with 5 ml heparinized saline. Blood samples were collected at -15, -10 (prior to the first injection), 0 (prior to the

second injection), 5, 10, 15, 20, 30, 45, 60, 75, 90, 105, 120 min, and moved into pre-ice-chilled heparinized tubes. Plasma was treated as previously described in chapter 3 and stored at -30°C until assay. Experiments were repeated with 2-days recovery period.

In addition, six male calves were randomly assigned to be intravenously injected with vehicle, acylated bovine ghrelin [1-27] or acylated porcine ghrelin [1-28]. Bovine and porcine ghrelin show six different amino acids sequences at their C-terminal region (**Table 2-1**). Peptides were injected at a dose of 1.0 μg/kg BW at 1000 h and serial blood samples were collected. Treatment and storage of plasma was done as described earlier. Experiments were repeated with 2-days recovery period.

Measurements

Plasma acyl-ghrelin, total ghrelin, GH, insulin and PYY levels were measured by RIA as previously described in Chapter 2. All samples were measured in a single assay at 4°C. Sensitivity was 20.0 pg/ml, 0.10 ng/ml, 0.04 ng/ml, 0.04 ng/ml and 0.01 ng/ml for acyl-ghrelin, total ghrelin, GH, insulin and PYY RIA, respectively. Intraassay CV was less than 10% for each assay. Plasma glucose and NEFA levels were evaluated by commercially available kits as previously described in Chapter 3.

Statistics

All data are expressed as means ± S.E.M. Statistical difference between pre- and post-injected values at each time points were analyzed using one way ANOVA, followed by multiple comparisons of general linear model. All analyses were performed using SPSS for Windows, version 10.0.0. $P < 0.05$ was considered significant.

RESULTS

The changes in plasma acyl- and total ghrelin concentrations in Holstein steers from -15 to 120 min after injections are shown in **Fig. 4-1.** Vehicle injections did not change the basal plasma acyl-ghrelin and total ghrelin concentrations (103.15 ± 16.11 pg/ml & 3.16 ± 0.46 ng/ml respectively). Plasma acyl-ghrelin levels promptly and transiently increased after acyl-ghrelin injections both in v/ghr group and des-ghr/ghr group (**Fig. 4-1A**). Plasma total ghrelin levels peaked at 5 min after acyl-ghrelin injections in v/ghr group (14.05 ± 2.02 ng/ml) and returned to the preinjected values within 10 min after injection. On the other hand, plasma total ghrelin levels elevated 5 min after des-acyl ghrelin injection (51.03 ± 13.81 ng/ml for des-ghr/ghr group) and the level steadily recovered to the baseline values 60 min after acyl-ghrelin administration (**Fig. 4-1B**).

The plasma GH concentrations immediately increased (peak value of 45.16 ± 10.42 ng/ml) from basal value of 3.36 ± 1.07 ng/ml after intravenous administration of acyl-ghrelin (**Fig. 4-2**). The peak values and recovery time of plasma GH between v/ghr group and des-ghr/ghr group were not differed. The plasma insulin levels transiently increased from basal values of 0.51 ± 0.08 ng/ml 10 min after acyl-ghrelin injections but the effects of preinjections of vehicle or des-acyl ghrelin on these changes were not observed (**Fig. 4-3A**). There was no significant change in plasma PYY levels in all groups (**Fig. 4-3B**), however, average plasma PYY levels were differed among groups (0.70 ± 0.05, 0.76 ± 0.03 & 0.87 ± 0.05 ng/ml in v/v, v/ghr & des-ghr/ghr groups respectively, $P < 0.05$). Plasma glucose concentration was not altered in v/v group showing average concentration of 85.45 ± 1.32 mg/dl (**Fig. 4-4A**). The transient peak was seen 5 min after ghrelin injection in v/ghr group (97.35 ± 3.04 mg/dl) or 10 min after injection in des-ghr/ghr group (97.21 ± 4.57 mg/dl). Plasma NEFA concentrations were elevated from the basal values of 76.36 ± 16.33 μEq/l after ghrelin injections and peaked at 10 min post-injection (175.73 ± 21.25 &

172.47 ± 47.72 μEq/l for v/ghr and des-ghr/ghr groups respectively, **Fig. 4-4B**).

Figure 4-5 shows the responses of plasma GH to the intravenous bolus injections of bovine acyl-ghrelin [1-27] and porcine acyl-ghrelin [1-28] in 3 month old calves. Both bovine and porcine ghrelin significantly stimulated GH release with similar potency (the peak values were 49.73 ± 15.37 and 42.42 ± 10.69 ng/ml 5 min after bovine and porcine ghrelin, respectively).

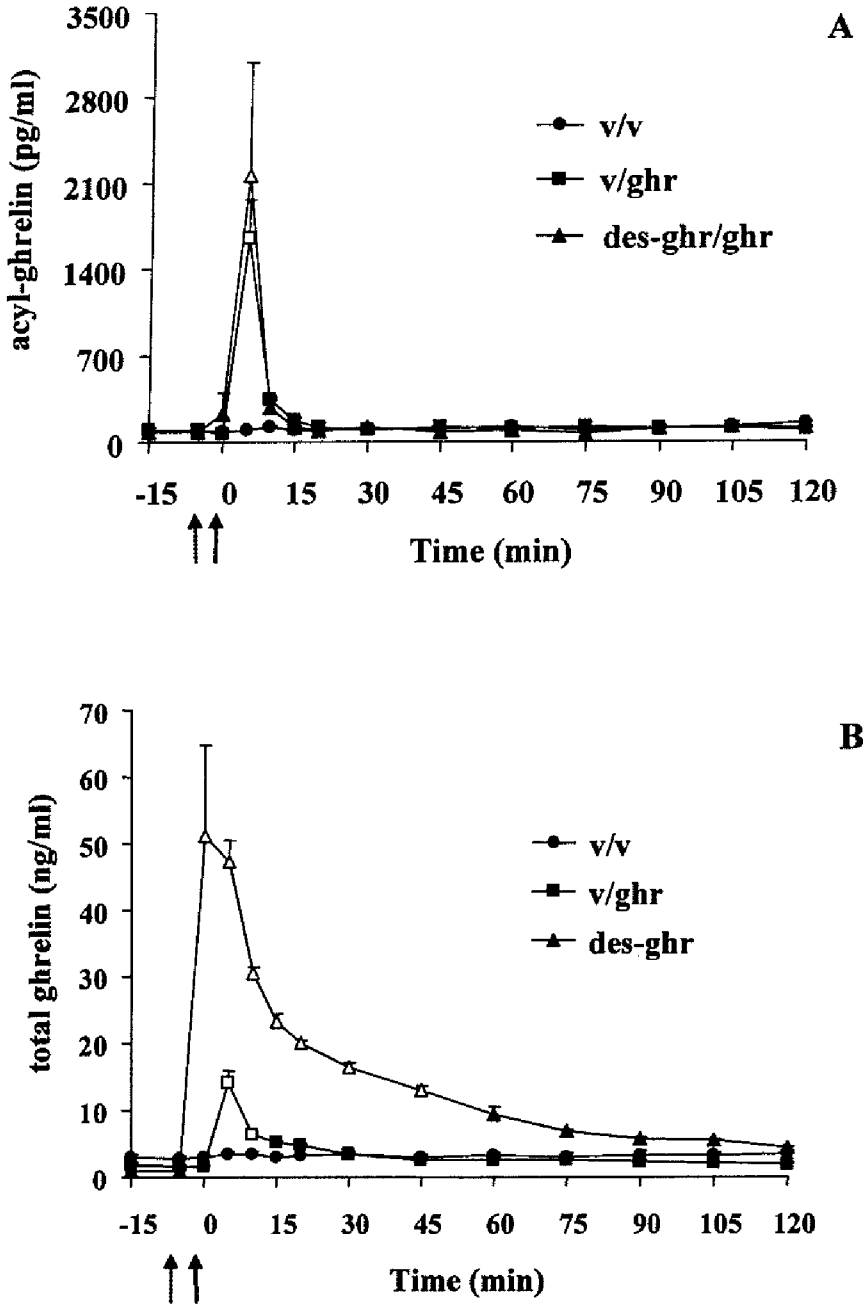

Fig. 4-1. Effect of preinjection of des-acyl ghrelin on ghrelin-induced plasma acyl-ghrelin (**A**) and total ghrelin (**B**) levels in Holstein steers. Animals were intravenously injected with vehicle followed by vehicle (v/v, ●); vehicle followed by acyl-ghrelin (v/ghr, ■); des-acyl ghrelin followed by acyl-ghrelin (des-ghr/ghr, ▲). The arrows indicate the time of injection. Values are the means ± S.E.M for 5 animals. Opened symbols indicate a significant difference ($P < 0.05$) compared with pre-injected concentrations (-15 min).

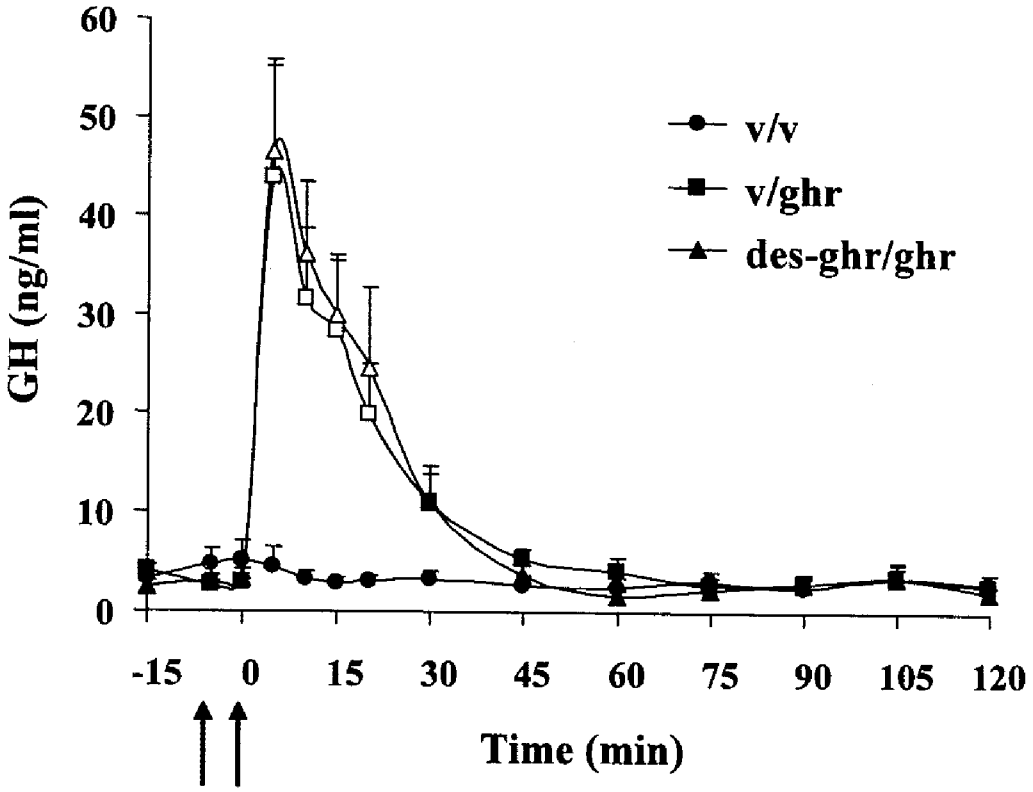

Fig. 4-2. Effect of preinjection of des-acyl ghrelin on ghrelin-induced plasma GH levels in Holstein steers. Animals were intravenously injected with vehicle followed by vehicle (v/v, ●); vehicle followed by acyl-ghrelin (v/ghr, ■); des-acyl ghrelin followed by acyl-ghrelin (des-ghr/ghr, ▲). The arrows indicate the time of injection. Values are the means ± S.E.M for 5 animals. Opened symbols indicate a significant difference ($P < 0.05$) compared with pre-injected concentrations (-15 min).

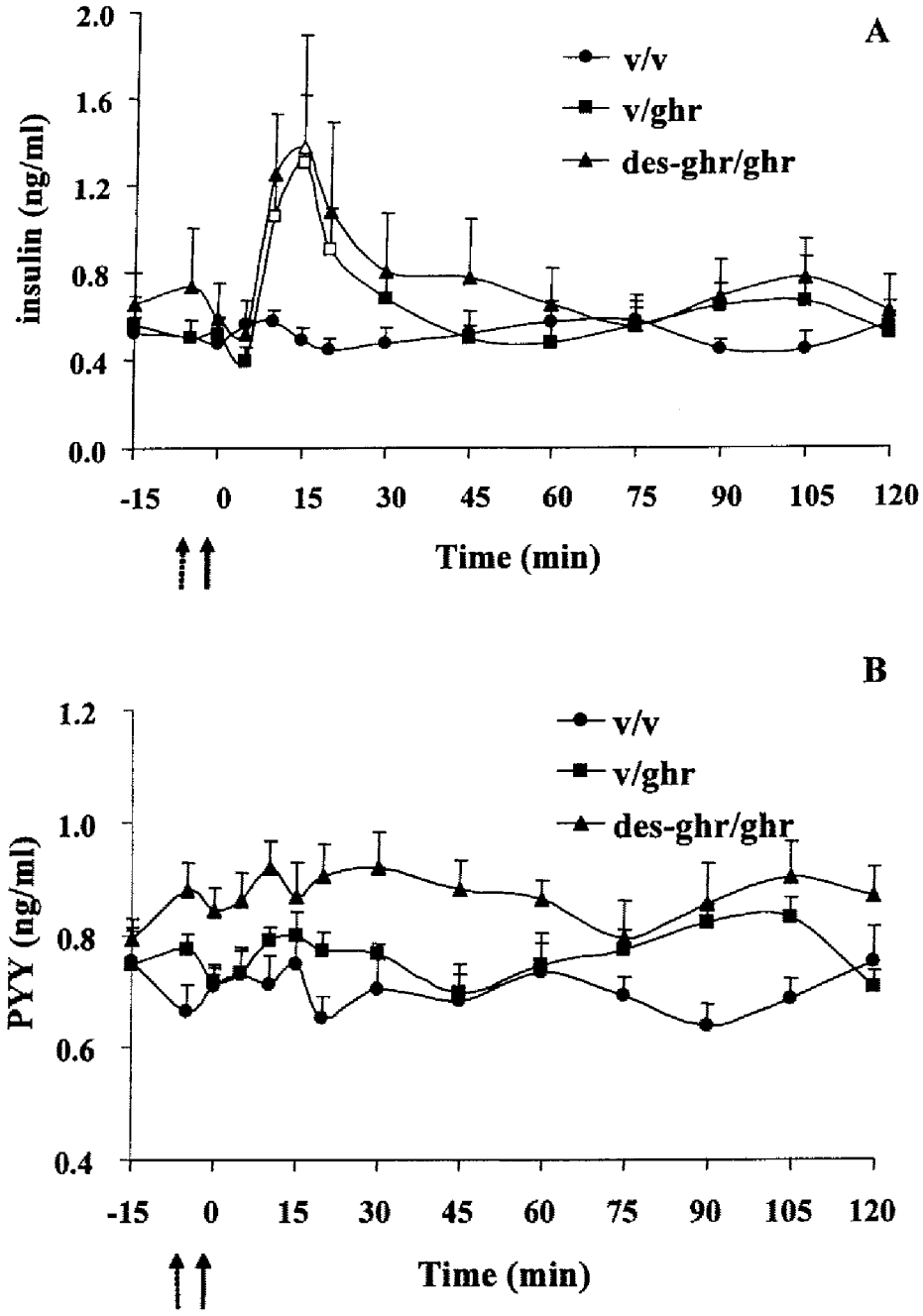

Fig. 4-3. Effect of preinjection of des-acyl ghrelin on ghrelin-induced plasma insulin (**A**) and PYY (**B**) levels in Holstein steers. Animals were intravenously injected with vehicle followed by vehicle (v/v, ●); vehicle followed by acyl-ghrelin (v/ghr, ■); des-acyl ghrelin followed by acyl-ghrelin (des-ghr/ghr, ▲). The arrows indicate the time of injection. Values are the means ± S.E.M for 5 animals. Opened symbols indicate a significant difference ($P < 0.05$) compared with pre-injected concentrations (-15 min).

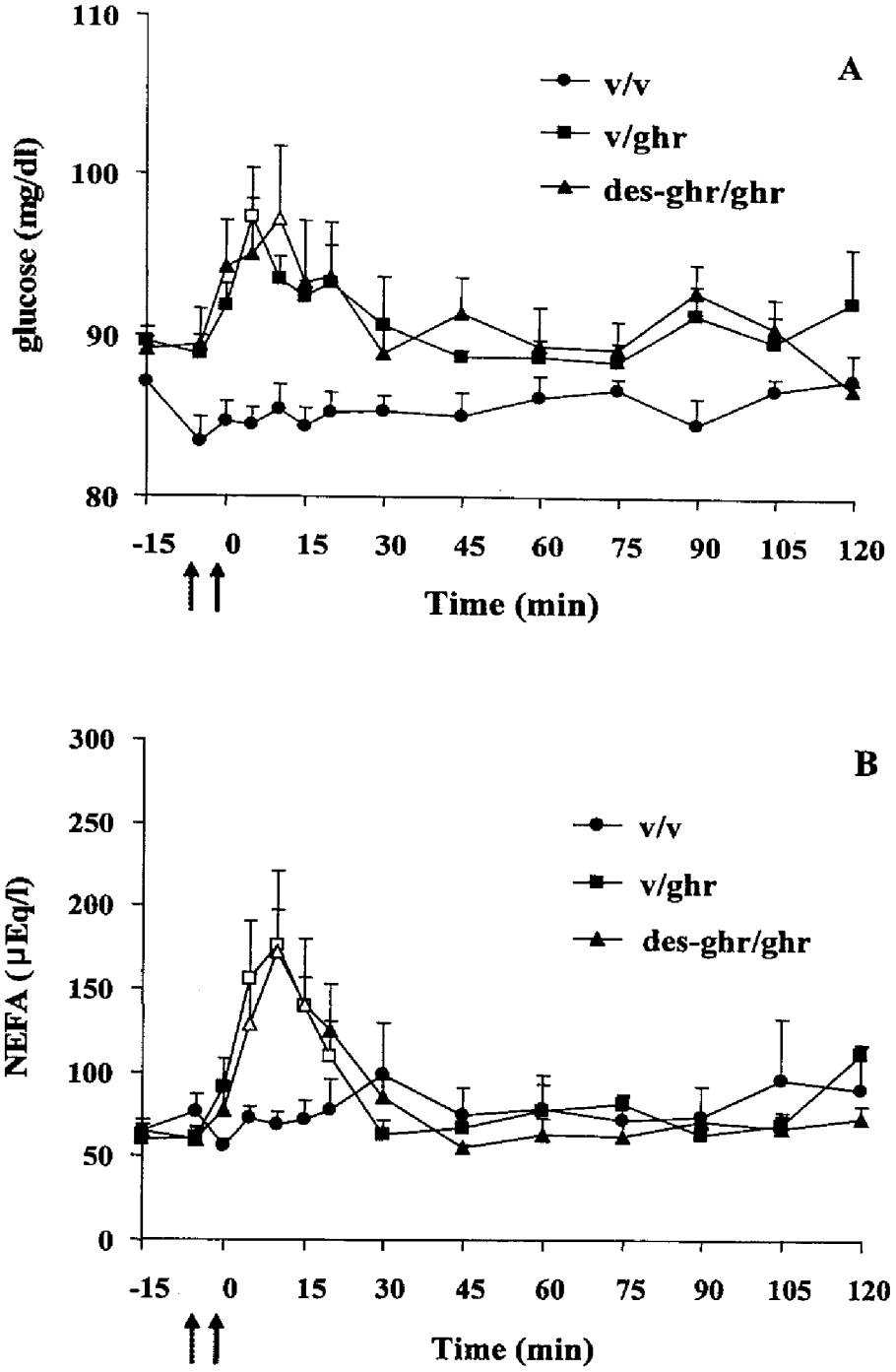

Fig. 4-4. Effect of preinjection of des-acyl ghrelin on ghrelin-induced plasma glucose (**A**) and NEFA (**B**) levels in Holstein steers. Animals were intravenously injected with vehicle followed by vehicle (v/v, ●); vehicle followed by acyl-ghrelin (v/ghr, ■); and des-acyl ghrelin followed by acyl-ghrelin (des-ghr/ghr, ▲). The arrows indicate the time of injection. Values are the means ± S.E.M for 5 animals. Opened symbols indicate a significant difference ($P < 0.05$) compared with pre-injected concentrations (-15 min).

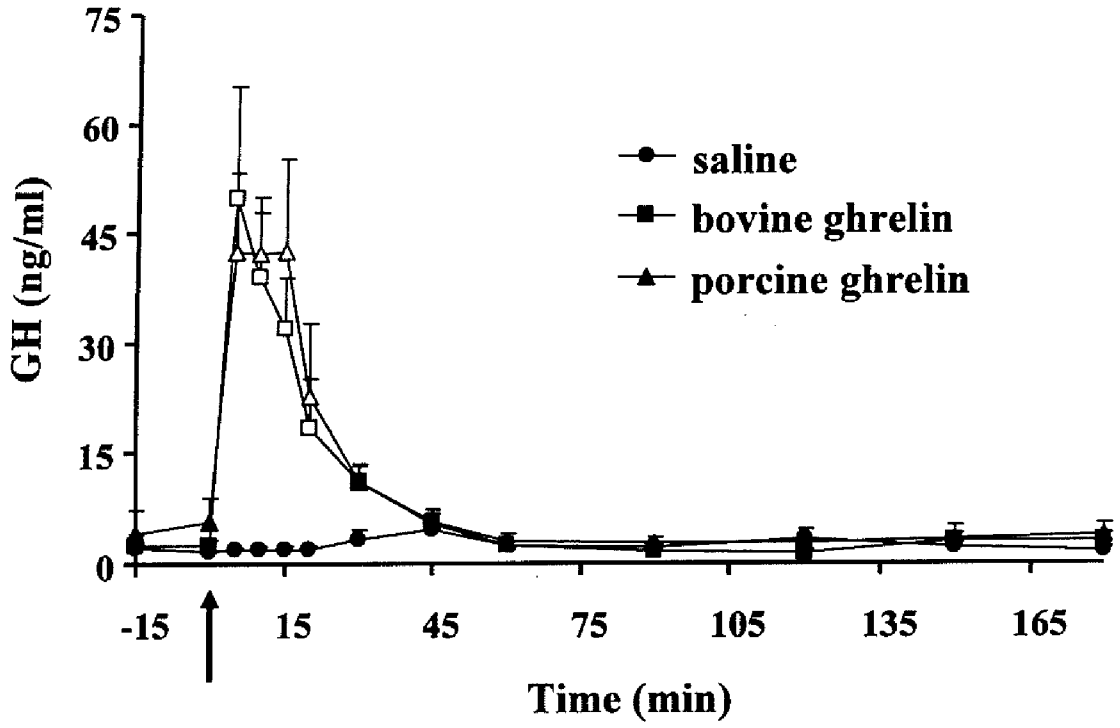

Fig. 4-5. Comparison on the effects of bovine and porcine acylated ghrelin on GH secretion in Holstein steers. Animals were intravenously injected with 5 ml 0.1% BSA-saline (●); bovine acyl-ghrelin (■); and porcine acyl-ghrelin (▲). The arrow indicates the time of injection. Values are the means ± S.E.M for 6 calves. Opened symbols indicate a significant difference ($P < 0.05$) compared with pre-injected concentrations (-15 min).

DISCUSSION

In the circulation and stomach tissues, ghrelin can be found as at least two major molecular forms: acyl-ghrelin with n-octanoylation on the Ser^3 residue, and des-acyl ghrelin which is lack in acylation (Hosoda *et al.* 2000*a*). Des-acyl ghrelin occupies the major portion of circulating ghrelin in Holstein heifers (ThidarMyint *et al.* 2006) and also in monogastric animals (Hosoda *et al.* 2000*a*). In the present study, basal plasma total ghrelin levels (including acyl-ghrelin, des-acyl ghrelin and intact C-terminal ghrelin fragments) were approximately 24 times higher than the acyl-ghrelin levels. This ratio of plasma acyl- and total ghrelin levels are differed from the results of the previous study (ThidarMyint *et al.* 2006) while total ghrelin concentrations are similar between these two experiments. Therefore, it can be postulated that plasma acyl-ghrelin may readily fluctuate with the changes in physiological conditions of animals compared with des-acyl ghrelin levels. More precise studies are in necessity on this aspect.

The finding of interest in the present study is that the rate of degradation/elimination of total ghrelin was relatively delayed; plasma total ghrelin levels increased promptly but declined gradually to the basal values 60 min post-injection when bovine des-acyl ghrelin was administered at a dose of 10 μg/kg BW (3.24 nmol/ kg BW; **Fig. 4-1B**). In our previous study, it was observed that the degradation/elimination rate of plasma total ghrelin concentrations induced by 10 μg (3.09 nmol) acylated bovine ghrelin/kg BW was obviously rapid, i.e., within 15 min post-injection (**Fig. 3.1B**). We can not explain the reason for these differences in the present time but the rapid degradation or short half-life of ghrelin has been reported in humans (Tschöp *et al.* 2000, Gauna *et al.* 2004) and in pigs (Salfen *et al.* 2004). Therefore, it can be suggested that the rate of degradation of ghrelin in circulation is also rapid in ruminants and perhaps the structure of ghrelin (acylated or des-acyl form) may affect the degradation rate of exogenous ghrelin.

The results from the present study again demonstrated the potent stimulatory effect of ghrelin on GH release in steers. On the other hand, intravenously pre-administered des-acyl ghrelin did not influence the ghrelin-induced GH elevation. In addition, intravenous bolus injections of bovine and porcine ghrelin of which different amino acid sequences at C-terminal region showed the similar potency on GH stimulation in calves. These findings are in consistence with the results of the study by Bednarek *et al* (2000) in which short peptides encompassing the first 4 or 5 residues of ghrelin were capable of increasing intracellular calcium levels in cells transfected with the human GHS-R1a about as efficiently as the full-length ghrelin. In deed, acyl-modification on its Ser^3 residue seems to be critical for the ghrelin to react with GHS-R1a (Kojima *et al.*1999). However, the controversial results have been reported by Torsello *et al* (2002) in which ghrelin analogs that were truncated in C-terminal region were ineffective to stimulate GH release in neonatal rats and failed to displace radiolabelled ghrelin from binding sites in membranes from human hypothalamus and pituitary. Nevertheless, the results from the present study support the critical role of acyl-modification for ghrelin to stimulate GH secretion in ruminants.

While the controversial effects of ghrelin on carbohydrate metabolism has been reported by numerous *in vivo* and *in vitro* studies (Date *et al.* 2002*b*, Lee *et al.* 2002, Broglio *et al.* 2001, Reimer *et al.*2003), the present study showed that intravenous bolus injection of ghrelin at a dose of 1 μg/kg BW increased plasma insulin levels in steers. These data supports the results of previous studies in Holstein heifers (ThidarMyint *et al.* 2006) and lactating cows (Itoh *et al.* 2006*b*). Plasma NEFA levels also significantly increased followed by ghrelin injection and only transient increase for plasma glucose levels was observed. The precise regulatory role of ghrelin on carbohydrate and fatty acid metabolism in ruminants can not be fully explained from the present results, but it is noteworthy that the responsive time of insulin and NEFA levels was less rapid compared with the GH

response to ghrelin in the present study. The similar result was seen in the previous study (ThidarMyint *et al.* 2006). It can be suggested that ghrelin-induced GH release may be responsible for the increase in plasma insulin and NEFA levels. Again, pre-injection of des-acyl ghrelin did not affect to those changes. Recent study by Heijboer *et al* (2006) showed that ghrelin hampers insulin's capacity in mice to suppress endogenous glucose production, whereas it reinforces the action of insulin on glucose disposal, independently of food intake and body weight. These metabolic effects are unlikely to be mediated by the GHS receptor. Therefore, more precise studies are necessary to understand the involvement of ghrelin and ghrelin receptor in the regulation of glucose and insulin levels in ruminants.

In contrast to the orexigenic effect of ghrelin, PYY secreted form L-cells of intestinal tract inhibits feed intake in monogastric animals (Batterham *et al.* 2002). Two molecular forms of PYY with opposite biologic actions are circulating in the bloodstream: PYY_{1-36} stimulates feed intake while PYY_{3-36} inhibits (Grandt *et al*, 1994). Both ghrelin and PYY immunoreactive cells are present throughout the gastrointestinal tract (Date *et al.* 2001, Onaga *et al.* 2000) suggesting the possible counter-regulation of secretion and function between these peptides. Although the information about this area and the effect of PYY on feed intake in ruminants is still limited, Chelikani *et al* (2006) has reported that intravenous infusion of ghrelin at dark onset attenuated the inhibitory effects of PYY_{3-36} on food intake and gastric emptying in freely feeding rats. The present study showed that the injection of ghrelin alone or pre-injection with des-acyl ghrelin had no clear effect on plasma PYY levels while the group differences in average plasma PYY levels were observed. We also have reported that plasma ghrelin levels did not change in pigs administered with porcine PYY_{3-36} (Ito *et al.* 2006). Moreover, plasma ghrelin levels fluctuated around feeding in scheduled meal-fed sheep (Sugino *et al.* 2002a) while plasma PYY levels were not altered over 48 h in both roughage and concentrate-fed sheep (Onaga *et al.* 2000). Therefore, it can be suggested that the roles of ghrelin and PYY are

independent in ruminant physiology, and at least in part, ghrelin is not a regulator of plasma PYY concentrations in ruminants.

Although none of the endocrine effects of des-acyl ghrelin has not available to date, numerous studies have shown the central effect of des-acyl ghrelin in the respect of feed regulation. Secretion of des-acyl ghrelin from the rat hypothalamus increased in glucoprivic states induced by fasting or treatment with 2-deoxy-D-glucose (2-DG), a selective inhibitor of carbohydrate metabolism (Sato *et al.* 2005). Toshinai *et al* (2006) also reported that central administration of des-acyl ghrelin stimulates feed intake in GHS-R deficient mice, but not in orexin deficient mice suggesting the regulatory effect of des-acyl ghrelin through interactions with a target protein distinct from the GHS-R. The controversial study by Asakawa *et al* (2005) has shown the anorexigenic and catabolic effect of centrally administered des-acyl ghrelin in rats. In that study, administration of des-acyl ghrelin decreased food intake and gastric emptying rate through an action on the paraventricular nucleus and the arcuate nucleus in the hypothalamus of rat. Moreover, des-acyl ghrelin overexpressing mice showed moderately decreased linear growth and exhibited a decrease in body weight, food intake, and fat pad mass weight. However, we did not observe the involvement of des-acyl ghrelin in the endocrine effects of ghrelin in cattle. At least in part, the site of the injection might not be the reason because des-acyl ghrelin can enter into the brain (Banks *et al.* 2002).

In conclusion, our present data in ruminants is in agreement with previously reported studies in monogastric animals showing the critical role of acyl-modification for the endocrine effects of ghrelin. Moreover, this is the first report, as in our knowledge, on the effect of exogenous ghrelin on plasma PYY levels in ruminants. The possible metabolic effects and the specific receptor for des-acyl ghrelin in ruminants have to be studied in advance.

SUMMARY

In this experiment, we study the effect of preinjection of des-acyl ghrelin on ghrelin-induced endocrine changes in Holstein steers. Five Holstein steers were intravenously injected with vehicle (5 ml 0.5% BSA-saline) or synthesized acylated bovine ghrelin at a dose of 1 μg/kg BW. They were preinjected with vehicle or des-acyl bovine ghrelin (10 μg/kg BW) and serial blood sampling were done. Plasma acyl-ghrelin level increased after ghrelin injection and rapidly returned to the basal values within 10 min post-injection. The elevated plasma total ghrelin levels induced by des-acyl ghrelin injection less rapidly returned to preinjected values (within 60 min). Bolus administration of bovine acylated ghrelin stimulates plasma GH and NEFA levels. There was a transient increase in plasma insulin and glucose levels followed by the ghrelin injection but plasma PYY levels did not change. Pre-injection of des-acyl ghrelin did not influence the endocrine effects of ghrelin in Holstein steers. Moreover, i.v. bovine and porcine acylated ghrelin injection to calves at a dose of 1 μg/kg BW induced equal and potent GH release. These data indicates that acyl-modification of ghrelin at Ser^3 residue is critical for the endocrine effects of ghrelin in ruminants.

Chapter 5 (Experiment 4)

Combined administration of ghrelin and GHRH synergistically stimulates GH release in Holstein preweaning calves

INTRODUCTION

The circadian and pulsatile secretion of GH from the anterior pituitary gland is mainly regulated by two hypothalamic hormones: the stimulatory GHRH and the inhibitory somatostatin SRIF (McMahon *et al.* 2001*b*). Other synthetic hexa- or penta-peptides, known as GHRP or nonpeptidyl GHSs, also stimulate GH release via the specific G-protein coupled receptor GHS-R (Bowers *et al.* 1984, Smith *et al.* 1993). In 1999, the endogenous ligand of GHS-R, ghrelin, was firstly purified from stomach of rat and reported as a potent GH release stimulant (Kojima *et al.* 1999). Ghrelin is also known as an anabolic hormone because it stimulates feeding and reduces fat oxidation, thus leading to adiposity (Tschöp *et al.* 2000). To date, several studies in monogastric animals showed that ghrelin is the third regulator of the adenohypophysial axis since it acts synergistically *in vivo* or additively *in vitro* with GHRH to release GH (Anderson *et al.* 2005).

Ruminant ghrelin is a peptide consisting of 27 amino acids with octanoylation on Ser^3 residue of N-terminal region (Kita *et al.* 2005) which modification is essential for the bioactivity of ghrelin on GH secretion (Kojima *et al.* 1999). Another form of ghrelin, des-acyl ghrelin, represents the major portion of circulating ghrelin (Hosoda *et al.* 2000*a*, ThidarMyint *et al.* 2006). Ghrelin was detected in bovine oxyntic glands of abomasal tissues (Hayashida *et al.* 2001) and preprandial surges of plasma active ghrelin concentrations were observed in scheduled-meal fed sheep (Sugino *et al.* 2002a), suggesting that ghrelin may be involved in appetite regulation in ruminant species. In our

previous study, we have shown that both active and total ghrelin circulate in the ruminant blood stream and that exogenous ghrelin dose-dependently stimulates GH secretion in Holstein heifers (ThidarMyint *et al.* 2006). Another study also reported that ghrelin-induced GH secretion is higher in early lactating cows than in calves and late-lactating cows (Itoh *et al.* 2005). Therefore, ghrelin is an important regulator of the somatotropic axis in ruminants and the effect of ghrelin might be altered by the physiological status of animals. Nevertheless, a recent *in vitro* study showed no synergistic or additive effect of ghrelin and GHRH on GH secretion from ruminant adenohypophysial cells (Hashizume *et al.* 2003). To the best of our knowledge, there is no report on associations between ghrelin and GHRH *in vivo* in ruminant species. In this study, we investigated the effects of ghrelin, GHRH and their combined administration on plasma GH, IGF-1, insulin, glucose and NEFA levels in Holstein bull calves. Moreover, we examined whether or not the interaction of ghrelin and GHRH on these parameters were altered after weaning. We also studied the possible changes of plasma active and total ghrelin concentrations in the pre- and postweaning periods because weaning induces changes in endocrine regulators in ruminants (Katoh *et al.* 2004, Yonekura *et al.* 2002).

MATERIALS AND METHODS

Animals

Six Holstein bull calves (18 ± 2 days of age, 40.5 ± 1.7 kgBW) were purchased from a commercial producer and housed in individual pens in natural light-darkness cycle. Calves were bottle-fed with 300 g milk substitute (crude protein 26%, crude fat 20%, crude fiber less than 1%, crude ash 10%, Calf milk AT, Snow Brand, Japan) in 2 liters of lukewarm water per meal two times per day (0900 h & 1700 h). Calf starter (crude protein 25%, crude fat 2%, crude fiber 7%, crude ash 10%, Calf Manna, Manna pro-corporation, St.Louis, USA), Timothy hay and water were supplied *ad libitum*. After weaning at 6 weeks of age, calves were raised in flock and offered concentrate for growing calves (crude protein 14%, crude fat 1.5%, crude fiber 8%, crude ash 7%, Hokuren, Sapporo, Japan) instead of milk substitute. Water and hay were provided *ad libitum* with mineral salt. Average daily gain during the experiment was 0.77 kg. Obihiro University Animal Use and Care Committee approved the experimental procedures of the study.

Peptides

n-Octanoylated bovine ghrelin (1-27) and human GHRH-NH_2 (1-29) were synthesized with Fmoc strategy of solid phase peptide synthesis procedures. Peptidyl resin was cleaved with TFA followed by purification on a TSK ODS-120A column (7.8 mm ID × 30 cm) by HPLC (linear gradient of 0-60% CH_3CN) and fractions containing peptides were lyophilized. Peptides were dissolved in sterile de-ionized water and diluted with sterile 0.1% BSA dissolved in physiological saline to reach 1 mg/ml and stored at -80°C. The amino acid sequence of first 29 residues of GHRH is reported as the shortest fragment required for full capacity of GH-releasing activity *in vivo* (Ling *et al.* 1984, Hodate *et al.* 1986) and represents the high degree of sequence conservation among species (Frohman

LA & Jansson JO, 1986).

Peptide administration and sampling procedures

To study the effect of ghrelin and/or GHRH on plasma GH levels during the pre-weaning period, a sterilized polyethylene catheter was inserted nonsurgically into an external jugular vein of each calf 1 day prior to the start of experiments when calves were 5 weeks old and weighed 51.2 ± 2.3 kg (± SEM). The catheter was fixed to the skin with a silk ligature and the patency was maintained with heparinized saline. This catheter was used for both peptide injection and blood sampling. Body weight was measured one day before each injection to allow the determination of peptide doses. Calves were assigned randomly to injection of vehicle (5 ml 0.1% BSA-saline), ghrelin (1 μg/kg BW equivalent to 0.3 nmol per kg), GHRH (0.25 μg/kg BW equivalent to 0.074 nmol per kg) or a combination of ghrelin (1 μg/kg BW) and GHRH (0.25 μg/kg BW) separated by 1 or 2 days between experiments. All calves received all treatments. Therefore, the number of animals in each treatment group was 6. The dose of ghrelin (1 μg/kg) was chosen from our previous study (ThidarMyint *et al.* 2006). Preliminary studies showed that the stimulatory effects of two doses of synthesized human GHRH (0.25 and 1.0 μg/kg BW) on GH secretion were similar in Holstein bull calves (ThidarMyint *et al.* unpublished data). Therefore the dose of 0.25 μg/kg BW was chosen for GHRH. Peptides were injected at 1200 h and serial blood samples (5 ml each) were withdrawn immediately before (-15 and 0) and at 5, 10, 15, 20, 30, 45, 60, 90, 120 min after each injection. Plasma was transferred to tubes and stored in triplicate at -30°C for later analysis. One portion of plasma was acidified by 1M HCl for ghrelin assays. Experimental procedures were repeated when the calves reached 10 weeks of age (post-weaning period, 73.1 ± 3.9 kg BW), but in contrast to the preweaning period, calves were loosely attached during blood sampling.

Measurement of plasma hormones and metabolites

Plasma active ghrelin, total ghrelin, GH and insulin concentrations were measured by double antibody RIA as previously described (ThidarMyint *et al.* 2006). The IGF-1 concentration was measured by RIA (Lee *et al.* 2000) after extraction of plasma with acid-ethanol (Daughaday *et al.* 1980). All samples were measured in duplicate at 4°C. Average inter-assay and intra-assay coefficients of variation were 12% and 11% for active ghrelin, 13% and 6% for total ghrelin, 5% and 5% for GH, 15 % and 11% for insulin, and 9% and 5% for IGF-1, respectively. Plasma glucose and NEFA levels were measured by commercially available kits (Code No. 439-90901 and 279-75401 respectively, Wako, Japan).

Statistics

All data are presented as means ± S.E.M. Values of the time points at -15 and 0 min were averaged and used as pre-injected values. The significant differences in the plasma concentrations at each time points, and changes in the effects of treatments before and after weaning were analyzed using repeated measures ANOVA. The values of AUC of each hormone for 120 min after the injection of vehicle, ghrelin, GHRH or their combination were calculated by using the trapezoid method and compared with Student's paired *t*-test after subtraction of correspondent pre-injected AUC (from -15 to 0 min). All analyses were performed using SPSS for Windows, version 10.0.0. $P < 0.05$ was considered significant.

RESULTS

Basal plasma concentrations around weaning

Basal plasma concentrations of active ghrelin, total ghrelin, GH, insulin, IGF-1, glucose and NEFA are summarized in **Table 5-1**. Plasma insulin, glucose and NEFA concentrations were higher during the preweaning period ($P < 0.001$), while the concentrations of active ghrelin, total ghrelin, GH and IGF-1 levels were similar during the pre- and postweaning periods.

Effect of peptide injections on plasma GH concentrations

Figure 5-1. shows the response of plasma GH concentrations to vehicle, ghrelin, GHRH and their combined administration around weaning. Vehicle injection did not affect basal plasma GH levels. In the preweaning period (**Fig. 5-1A**), plasma GH levels reached peak values at 5 min after injection and returned to pre-injected values within 30 min: the peak values in calves injected ghrelin or GHRH alone were similar (29.6 ± 5.3 ng/ml after ghrelin; 25.9 ± 5.4 ng/ml after GHRH), but the GH response to ghrelin plus GHRH was greater (102.3 ± 35.7 ng/ml; $P < 0.001$) than the sum of the GH responses to ghrelin and GHRH alone. After weaning (**Fig. 5-1B**), plasma GH levels increased immediately after peptide injections and peaked at 10 min (53.2 ± 14.5 ng/ml after ghrelin; 77.9 ± 31.2 ng/ml after GHRH; 92.9 ± 19.7 ng/ml after ghrelin plus GHRH). Thereafter, plasma GH levels gradually returned to pre-injected values at 30, 45 and 90 min after injection of ghrelin, GHRH and ghrelin plus GHRH, respectively.

Likewise, the values of GH AUCs for 120 min after injection in ghrelin-alone or GHRH-alone groups were similar (811.5 ± 37.5 vs 934.8 ± 136.8 ng·min/ml) in the preweaning period (**Fig. 5-2**). GH AUC in ghrelin plus GHRH group was significantly greater (2941.1 ± 529.3 ng·min/ml, $P < 0.05$) than the sum of GH AUCs responsive to the

injection of ghrelin alone and GHRH alone. Therefore, we considered that ghrelin and GHRH synergistically stimulate GH secretion. When calves were weaned, combined administration of ghrelin and GHRH induced a higher GH AUC (4086.1 ± 455.5 ng·min/ml) than that of ghrelin alone (1665.9 ± 215.3 ng·min/ml, $P < 0.001$), but similar to that of GHRH alone (3047.4 ± 823.6 ng·min/ml, $P = 0.295$). The GH AUCs in the postweaning period were greater than those of the preweaning period when peptides were injected alone ($P < 0.05$). However, the values of GH AUCs of ghrelin plus GHRH groups were similar ($P = 0.18$).

Effects on other hormones and metabolites

Plasma active ghrelin and total ghrelin were transiently elevated after administration with ghrelin alone or in combination with GHRH (average peak values; 913.2 ± 88.9 pg/ml and 18.9 ± 2.3 ng/ml respectively), however; injection of GHRH alone or in combination with ghrelin did not modify the plasma active or total ghrelin concentrations. Moreover, plasma IGF-1, insulin, glucose, or NEFA concentrations were not significantly changed by vehicle or peptide administration (data not shown).

Table 5-1. Basal[a] plasma concentrations of hormones and metabolites in Holstein bull calves before and after weaning[b].

	Preweaning period (5 wk of age)	Postweaning period (10 wk of age)
Active ghrelin (pg/ml)	408.1 ± 86.8	598.2 ± 113.4
Total ghrelin (ng/ml)	2.8 ± 0.3	3.1 ± 0.8
GH (ng/ml)	2.4 ± 0.8	3.4 ± 1.0
IGF-1 (ng/ml)	41.7 ± 9.7	39.2 ± 7.2
Insulin (ng/ml)	1.7 ± 0.7	0.4 ± 0.1***
Glucose (mmoles/L)	5.2 ± 0.2	4.0 ± 0.2***
NEFA (mEq/L)	0.16 ± 0.02	0.06 ± 0.01***

Values are presented as means ± S.E.M. for six calves.
[a] Averaged value of vehicle (0.1% BSA-saline)-injected group from -15 to 120 min.
[b] Calves were weaned at 6 weeks of age.
*** $P < 0.001$ vs correspondent value of pre-weaning period

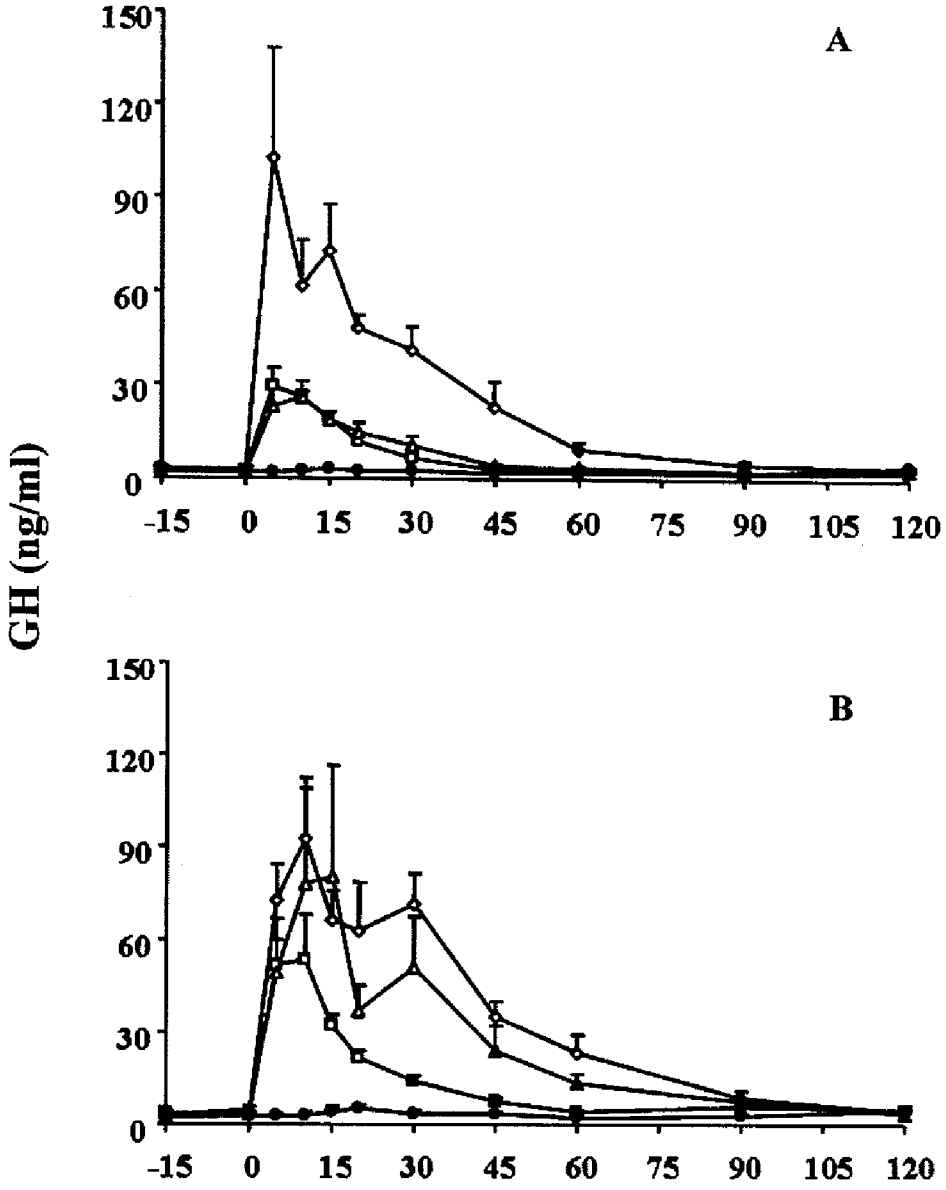

Fig. 5-1. Plasma GH responses to the iv injection of vehicle (●), n-octanoylated bovine ghrelin at 1 μg/kg BW (■), human GHRH amide (1-29) at 0.25 μg/kg BW (▲) and their combination (♦) in Holstein bull calves at 5 weeks of age (A; preweaning period) and at 10 weeks of age (B; postweaning period). Calves were weaned at 6 weeks. The bolus injections were done at 0 min. Data are expressed as means ± S.E.M. for 6 animals. Open symbols indicate a significant difference ($P < 0.05$) compared with the mean of pre-injected values (-15 to 0 min).

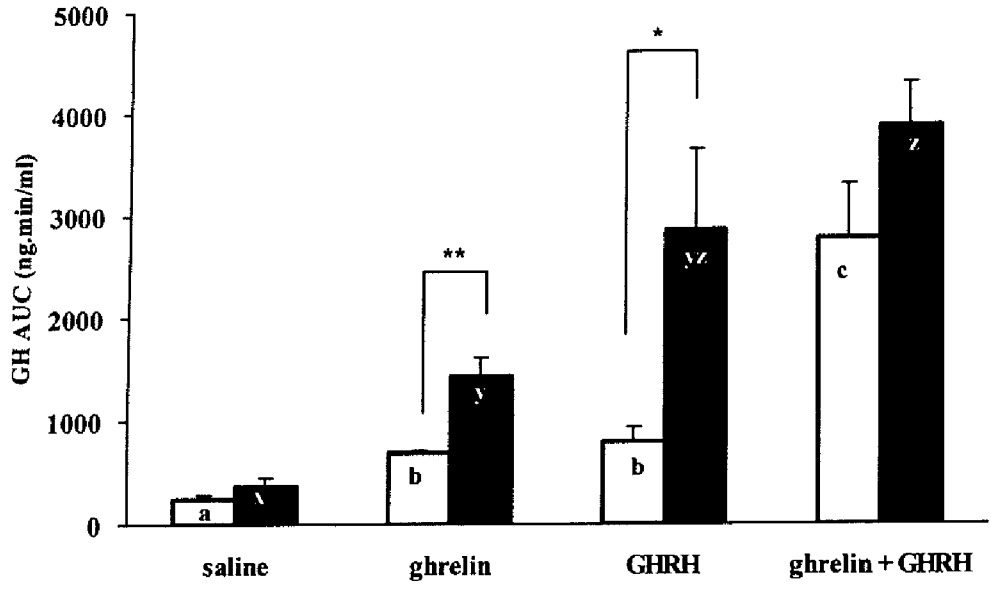

Fig. 5-2. The plasma GH area under the curve (AUC) for 120 min after acute intravenous injection of vehicle, n-octanoylated bovine ghrelin at 1 μg/kg, human GHRH amide (1-29) at 0.25 μg/kg and their combination in Holstein bull calves. Open columns represent the values of pre-weaning period (5 week of age) and closed columns represent the values of post-weaning period (10 week of age). Calves were weaned at the age of 6 week. The values shown are mean ± S.E.M for 6 animals.
* $P<0.05$, ** $P<0.01$ vs correspondent value between pre- and post-weaning periods.
$^{a, b, c}$ represent values that are significantly different within pre-weaning period ($P<0.05$)
$^{x, y, z}$ represent values that are significantly different within post-weaning period ($P<0.05$)

DISCUSSION

In the present study, we demonstrate that the coadministration of ghrelin and GHRH had a synergistic effect on GH secretion in preweaning calves and that this effect was lost after weaning. Previous *in vivo* studies have, too, reported a synergistic effect of ghrelin and GHRH on GH responses in humans (Arvat *et al.* 2001, Hataya *et al.* 2001). On the other hand, *in vitro* studies could not demonstrate that ghrelin has a synergistic or additive effect with GHRH on the GH release from bovine, porcine and rat anterior pituitary glands (Hashizume *et al.* 2003, Malagon *et al.* 2003, Yamazaki *et al.* 2002). These discrepancies in the results between *in vivo* and *in vitro* studies suggest that the effect of ghrelin on GH secretion is not only limited to the pituitary, but likely also involves effects on GHRH release from the hypothalamus. This assumption is based on the fact that ghrelin and GHS-R are both produced in the hypothalamus (Anderson *et al.* 2005). Recent *in vitro* and *in vivo* studies have reported that ghrelin stimulates GHRH release (Mogi *et al.* 2004, Wren *et al.* 2002). It is also possible that ghrelin and GHRH stimulate GH secretion from somatotrophs via different pathways. Indeed, ghrelin and GHRH regulate GH secretion through complex interdependent intracellular signaling pathways. It is generally accepted that the route of ghrelin to promote GH release is via GHS-R type 1a, which is coupled with the phospholipase C/inositol phosphate/ protein kinase C signaling pathway, while GHRH binds to GHRH-R which activates the adenylate cyclase / cAMP/ protein kinase A signaling pathway (Anderson *et al.* 2005). Moreover, it is noteworthy to consider the possibility of subpopulations of bovine somatotrophs that are responsive to ghrelin, GHRH or both stimulants since Mitani *et al* (1996) reported based on studies using reverse haemolytic plaque assay that there are at least three functionally distinct somatotroph subpopulations that responded to GHRP-6, GHRH and both stimulants in rat anterior pituitary cells. Further studies on expression and functional regulation of ghrelin in the

bovine anterior pituitary glands is needed.

We also observed that the effects of ghrelin and GHRH to elicit GH release became more potent after weaning when the peptides were injected independently. However, synergistic effect of ghrelin and GHRH on GH secretion seen in preweaning calves was no longer observed. It can be suggested that the greater GH responses to individual stimulants might mask the possible synergistic or additive effect of ghrelin with GHRH in weaned calves. Furthermore, it is possible that the effect of individual stimulants and their interaction on the hypothalamo-pituitary axis alter after weaning since several studies have reported that digestive and endocrine systems including the somatotropic axis are affected by weaning in ruminants (Katoh *et al.* 2004, Yonekura *et al.* 2002). Unfortunately, it is difficult to elucidate that weaning is the major influencing factor for these changes because we did not study age-matched un-weaned animals in the present experiment. However, based on the fact that basal plasma GH concentrations and the values of GH responses elicited by the combination of ghrelin plus GHRH were similar in pre- and post-weaning periods, it can be postulated that the basal GH releasing capacity of somatotrophs were similar in pre- and postweaning periods. It is possible that the proportions of subpopulations of somatotrophs that are responsive to ghrelin, GHRH, and to both secretagogues might change during the weaning.

Several studies have reported that the potency of ghrelin on GH stimulation is weaker when compared with GHRH in humans (Anderson *et al.* 2005, Hataya *et al.* 2001). In contrast, Seoane *et al* (2000) showed that ghrelin has similar or stronger potency on GH release compared with GHRH in humans. In the previously reported study in ruminants, the stimulatory effect of ghrelin was weaker than that of GHRH, but increased while GH secretory response to GHRH was blunted or relatively weak in early lactating cows (Itoh *et al.* 2005). We have shown in Holstein heifers that physiological doses of ghrelin could stimulate GH secretion while higher doses also enhanced plasma insulin and NEFA levels

(ThidarMyint *et al.* 2006). The present data also showed that ghrelin had comparable effect with GHRH on GH responses in calves. Therefore, ghrelin is an important regulator of GH secretion in ruminants.

The present data also showed that both active and total ghrelin concentrations between feeding times remained unchanged across the pre- and postweaning periods in bull calves. A recent study by Kobayashi *et al* (2006) also showed that plasma active ghrelin levels were not changed by feeding in young goats. As in our previous study, plasma total ghrelin concentration in Holstein heifers was higher (16 times) than active ghrelin levels (ThidarMyint *et al.* 2006). Again, we observed that the ratio of active and total ghrelin, independent of feeding time, is lower (1:7) in bull calves and that this ratio did not change after weaning. Moreover, plasma total ghrelin concentrations were similar in the two studies while plasma active ghrelin concentrations were higher in bull calves than heifers. Further studies on plasma active and total ghrelin levels by sex, physiological stage, feeding time and nutritional status may be helpful for a deeper understanding of the role of ghrelin in ruminant physiology.

On the other hand, significant changes in plasma IGF-1, glucose and NEFA concentrations were not observed over 2 h sampling period in all group. However, basal plasma insulin, glucose and NEFA concentrations were higher in the preweaning period compared with the values of postweaning period. Feeding time might not be the reason for these discrepancies because blood sampling procedures were done between meal times (started at 3 h after morning feeding and finished at 3 h before evening feeding) in both pre- and post weaning period. It can be suggested that differences in feed composition, developmental changes of gastrointestinal tract such as ruminal maturation around weaning, or by aging may influence the plasma insulin, glucose and NEFA levels but ghrelin might not be a major regulator in this process.

In conclusion, this study demonstrates that ghrelin synergistically stimulated GH

release with GHRH in the preweaning calves, but this effect was obviously lost after weaning. Basal plasma insulin, glucose and NEFA levels declined after weaning but acute intravenous administration of ghrelin, GHRH and their combination failed to induce any changes on these parameters. Although further investigation on the precise role of ghrelin on the GH-IGF-1 axis in ruminants in different physiological stages is needed, our study showed a major role of ghrelin on regulation of GH secretion in young ruminants.

SUMMARY

Ghrelin is a gut peptide which participates in growth regulation through its somatotropic, lipogenic and orexigenic effects. Synergism of ghrelin and GHRH on GH secretion has been reported in humans and rats, but not in domestic animals *in vivo*. In this study, effects of a combination of ghrelin and GHRH on plasma GH and other metabolic parameters, and changes in plasma active and total ghrelin levels were studied in Holstein bull calves before and after weaning. Six calves were intravenously injected with vehicle (0.1% BSA-saline), ghrelin (1 μg/kg BW), GHRH (0.25 μg/kg BW) or a combination of ghrelin plus GHRH at the age of 5 weeks and 10 weeks (weaning at 6 weeks of age). Ghrelin stimulated GH release with similar potency as GHRH and their combined administration synergistically stimulated GH release in preweaning calves. After weaning, GH responses to ghrelin and GHRH became greater compared with the values of preweaning calves, but a synergistic effect of ghrelin and GHRH was not observed. The GH areas under the concentration curves for 2 h post-injection were greater in weaned than in preweaning calves ($P<0.05$) if ghrelin or GHRH were injected alone, but were similar if ghrelin and GHRH were injected together. Basal plasma active and total ghrelin levels did not change around weaning, but transiently increased after ghrelin injection. Basal plasma insulin, glucose and non-esterified fatty acid levels were reduced after weaning, but no changes by treatments were observed. In conclusion, ghrelin and GHRH synergistically stimulated GH release in preweaning calves, but this effect was lost after weaning.

Chapter 6 (Experiment 5)

Effects of repeated administration of ghrelin on plasma hormones and metabolites in Holstein steers

INTRODUCTION

In the previous experiments, we have observed the potent stimulatory effect of ghrelin, gastric-derived GHS, on GH stimulation in Holstein cattle (Chapter 3: ThidarMyint *et al.* 2006, and chapter 4). In addition, intravenous administration of ghrelin has stimulatory effect on plasma insulin and NEFA levels indicating the important role of this peripheral hormone in ruminant physiology. Moreover, ghrelin and hypothalamic GHRH synergistically stimulate GH release when they were intravenously co-administered in pre-weaning calves (Chapter 5: ThidarMyint *et al.* 2008). The acyl-modification on its Ser^3 residue seems to be critical for ghrelin to activate its cognate G-protein coupled receptor, GHS-R1a, since the des-acyl ghrelin which is lack in octanyolation did not modify the endocrine or metabolic effects of ghrelin (Chapter 4).

Studies by radioreceptor assay and immunofluorescent histochemistry reported that GHS-R is detected in the numerous peripheral endocrine and nonendocrine tissues including gastrointestinal tract in humans, rats and ruminants (Papotti *et al.* 2000, Dass *et al.* 2003, Miller *et al.* 2005). Moreover, both ghrelin and GHS-R, at mRNA and peptide levels are present in the hypothalamus and pituitary (Korbonits *et al.* 2001, Miller *et al.* 2005) suggesting the endocrine and possible autocrine/paracrine modulatory effect of ghrelin. Although synergism between ghrelin and GHRH on GH secretion has been reported in humans (Arvat *et al.* 2001) and also in ruminants (ThidarMyint *et al.* 2008), the endocrine responses to ghrelin were not modified by the coadministration of hexarelin, a nonnatural peptidyl GHS (Arvat *et al.* 2001), and ghrelin was partially resistant to the homologus

desensitization exerted by GHRP-6 (Micic *et al.* 2002). These data suggest the existence of additional un-identified receptor rather than GHS-R1a for natural and nonnatural GHSs, or different intracellular mechanisms inserted by these compounds (Anderson *et al.* 2005). In rat, Yamazaki *et al.* (2002) have reported that serial ghrelin stimulation to the dispersed anterior pituitary cells at 1 h intervals decreased GH-response, but the response recovered with stimulation at 3 h intervals. The authors suggested the strong desensitization of ghrelin (receptor down-regulation) to the somatotrophs. It was supported by the results in human embryonic kidney (HEK) 293 cell line that stably expresses the human GHS-R1a in which ghrelin binding to these cells rapidly down-regulated the GHS-R (Camiña *et al.* 2004). In the present experiment, we studied the characteristic of the effect of ghrelin on plasma ghrelin, GH, insulin and metabolites in steers by using the serial intravenous injections of ghrelin.

MATERIALS AND METHODS

Animals and Experimental procedures

Five Holstein steers (257 ± 3 days old, 250 ± 10 kg BW) were housed under natural dark-light cycle. They were fed twice daily (0800 h & 1600 h) with commercially available concentrates (crude protein 14%, crude fat 1.5%, crude fiber 8%, and crude ash 7%, Hokuren, Japan) to meet the daily energy requirements according to the Japanese Nutritional Standard for beef cattle (2002). Animals finished eating concentrate within 20min. The timothy hay, mineral salt block and water were free-choice. Body weight was measured on the previous day of experiment. Steers were assigned to randomized cross-over study and were injected repeatedly with vehicle (5 ml 0.5% BAS-saline) or 1 μg bovine ghrelin/kg BW 2 h intervally for 4 times (0800, 1000, 1200 and 1400 h) and serial blood samplings were done from 0730 h to 1600 h. At the experimental day, water and mineral salt block was given continuously but concentrate and hay were supplied at the end of blood sampling. Plasma was treated as described in previous sections and experiments were carried-out with 2-day recovery period.

Measurement of plasma hormones and metabolites

Plasma acyl-ghrelin, ghrelin, GH and insulin were measured by RIA as previously described in Chapter 2. All samples were measured in single assay at 4℃. Sensitivity and intraassay CV were 17.42 pg/ml and 6.5% for acyl-ghrelin RIA, 0.05 ng/ml and 10.7% for ghrelin RIA, 0.14 ng/ml and 9.7% for GH RIA, 0.004ng/ml and 10.7% for insulin RIA respectively. Plasma glucose and NEFA levels were evaluated by commercially available kits (Code No. 439-90901 and 279-75401, respectively, Wako, Japan).

Statistical analysis

All data are presented as means ± S.E.M. The significant differences in the plasma concentrations at each time points were analyzed using repeated measures ANOVA. The values of AUCs for 120 min after each injection of vehicle or ghrelin were calculated by using the trapezoid method and compared with Student's paired *t*-test. All analyses were performed using SPSS for Windows version 10.0.0. $P < 0.05$ was considered significant.

RESULTS

Fig. 6.1 shows the changes in plasma acyl-ghrelin (**A**) and total ghrelin (**B**) concentrations followed by the repeated injections of vehicle and ghrelin every 2 h for 8 h in Holstein steers. The basal plasma acyl and total ghrelin levels for 8 h (average values of 0.54 ± 0.08 ng/ml and 2.06 ± 0.50 ng/ml respectively) were not modified by vehicle injection. When ghrelin was repeatedly administered at a dose of 1 μg/kg BW, both plasma acyl and total ghrelin levels were transiently elevated after each injection. The peak values of acyl-ghrelin tended to be increased by the repeated injections but the values of AUCs for 120 min post-injection of ghrelin (77.47 ± 15.76, 75.03 ± 13.41, 72.88 ± 10.77 and 72.98 ± 7.02 ng/ml/min for first, second, third and fourth injection respectively) were not different. On the other hand, both the peak values and AUCs for plasma total ghrelin followed by each injection were similar (AUCs; 582.47 ± 85.01, 636.72 ± 8.44, 675.90 ± 57.29 and 642.09 ± 60.59 ng/ml/min for first, second, third and fourth injection respectively).

The basal plasma GH concentrations in vehicle-injected group did not significantly change throughout the sampling period (5.42 ± 2.12 ng/ml; **Fig. 6.2A**). Each ghrelin injection significantly increased the plasma GH levels but the peak values responsive to second and third injections gradually decreased (23.60 ± 4.85 and 11.12 ± 4.56 ng/ml respectively) when compared with that to first injection (28.52 ± 8.86 ng/ml) and recovered after fourth administration (25.48 ±10.37 ng/ml). In accordance, GH AUC (ng/ml/min) for 120 min after first injection of ghrelin (1184.6 ± 249.1) was larger than those after second (727.3 ± 207.2; $P < 0.001$) and third (457.03 ± 154.4; $P < 0.01$) injections. The value of GH AUC after fourth injection (895.9 ± 234.5) was higher than that after third injection but still lower than that after first injection ($P < 0.05$). Basal plasma insulin (0.35 ± 0.06 ng/ml) levels were not modified by the repeated administration of vehicle (**Fig. 6.2B**). Plasma insulin levels significantly increased after each injection of ghrelin and peaked at 10-15 min

post-injection. No significant difference among the values of AUCs for insulin followed by each injection of ghrelin was observed (59.81 ± 16.49, 57.71 ± 12.15, 53.94 ± 8.65 and 51.89 ± 10.14 ng/ml/min). The repeated administration of ghrelin did not affect the plasma glucose levels throughout the experiment (average concentrations: 87.5 ± 2.3 mg/dl and 88.9 ± 3.3 mg/dl in vehicle- and ghrelin-injected group respectively, **Fig. 6.3A**). Plasma NEFA level (average basal value of 117.2 ± 20.8 μEq/l in vehicle-injected group) was significantly increased after the repeated administration of ghrelin, whereas the AUCs (mEq/l/min) followed by the each injection were not significantly differed (15.21 ± 1.77, 16.72 ± 1.98, 20.95 ±2.59 and 23.62 ± 2.20 respectively, **Fig. 6.3B**).

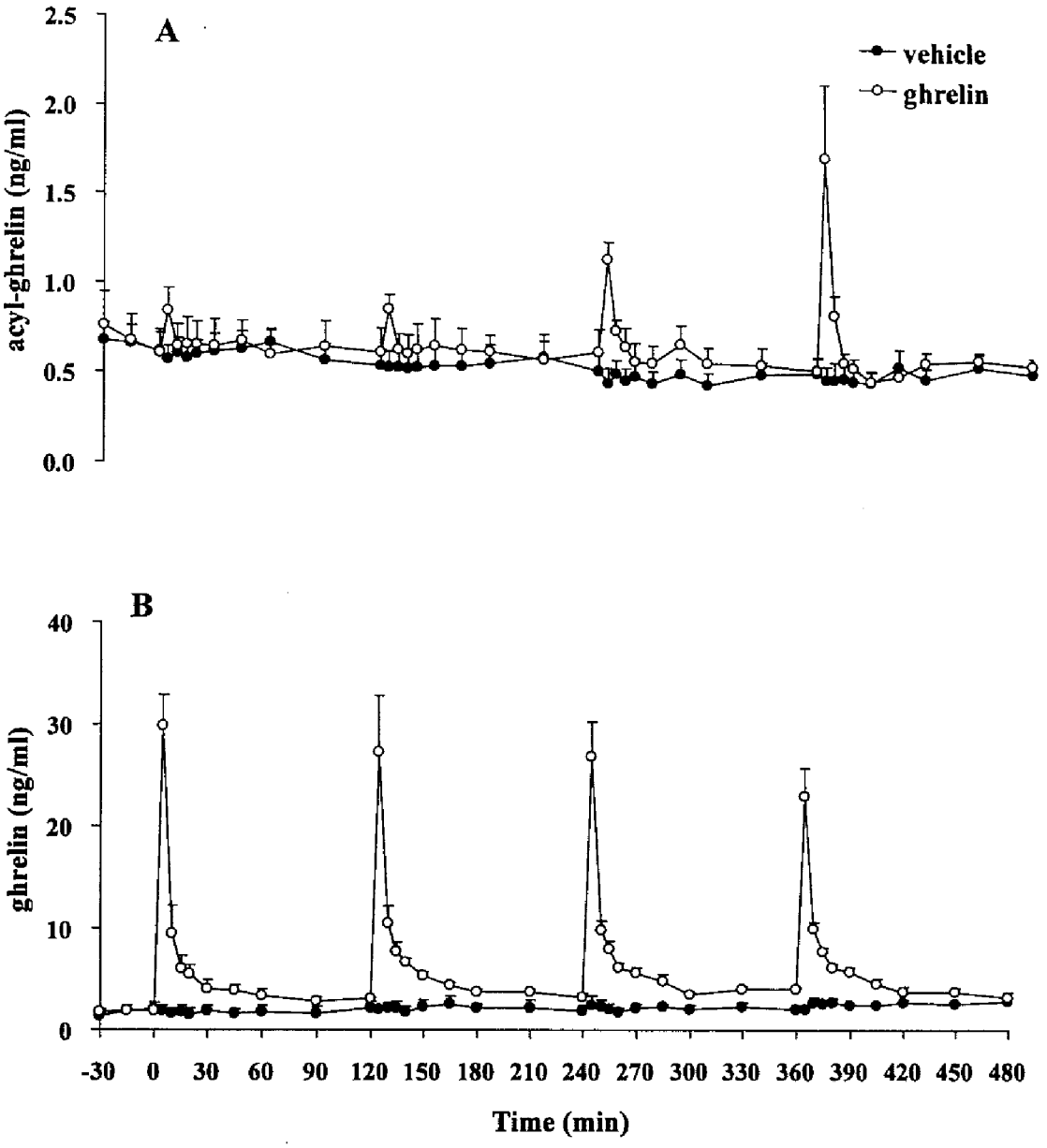

Fig. 6-1. The changes in plasma acyl-ghrelin (**A**) and total ghrelin (**B**) concentrations followed by ghrelin administration. Steers were intravenously injected with vehicle (5 ml 0.5% BSA-saline;●) or 1 μg acylated bovine ghrelin/kg BW (○) at 0, 120, 240 and 360 min. Blood sampling was started at 0730 h. Data are presented as mean ± S.E.M for 5 animals.

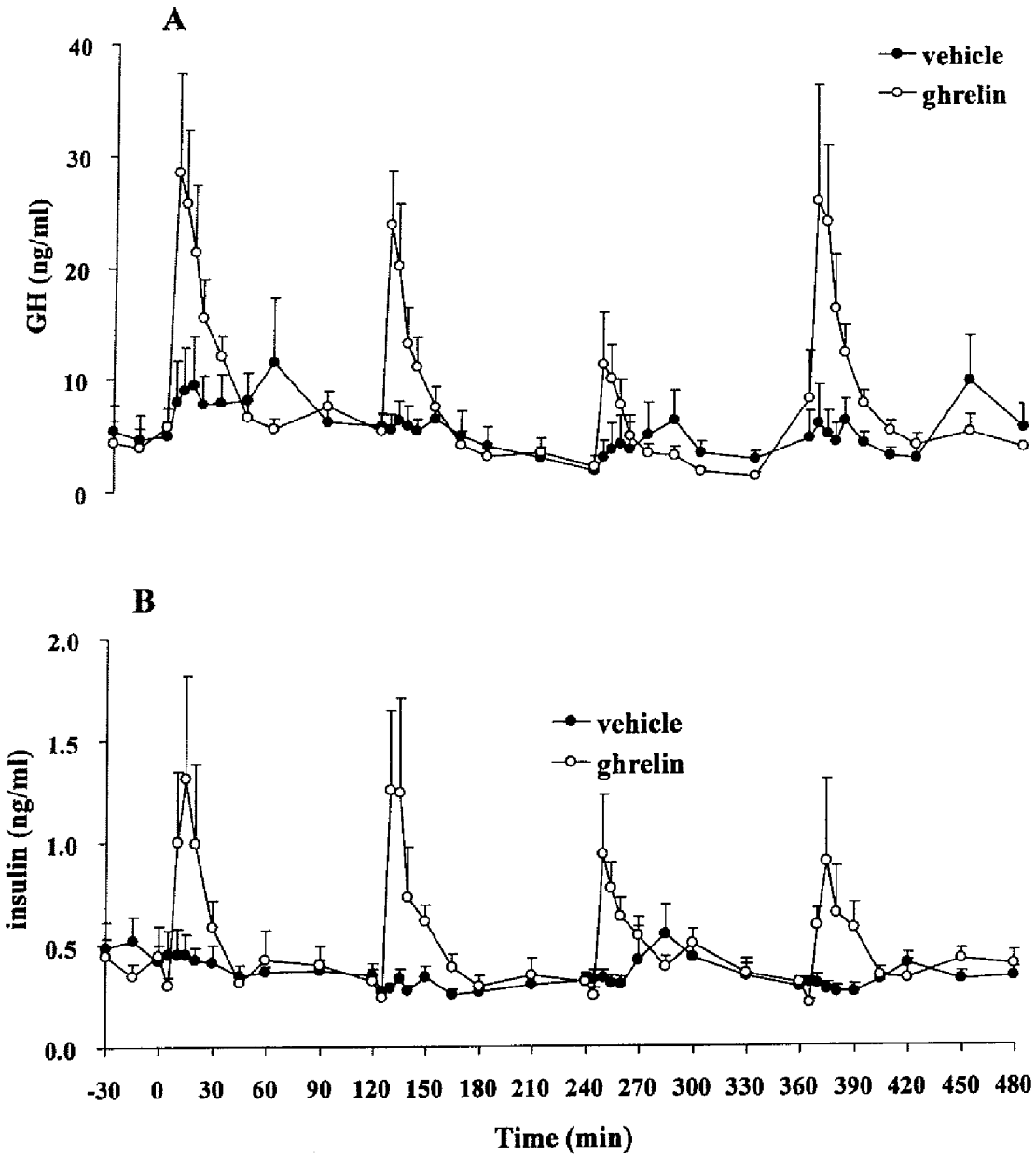

Fig. 6-2. The changes in plasma GH (**A**) and insulin (**B**) concentrations followed by ghrelin administration. Steers were intravenously injected with vehicle (5 ml 0.5% BSA-saline;●) or 1 μg acylated bovine ghrelin/kg BW (○) at 0, 120, 240 and 360 min. Blood sampling was started at 0730 h. Data are presented as mean ± S.E.M for 5 animals.

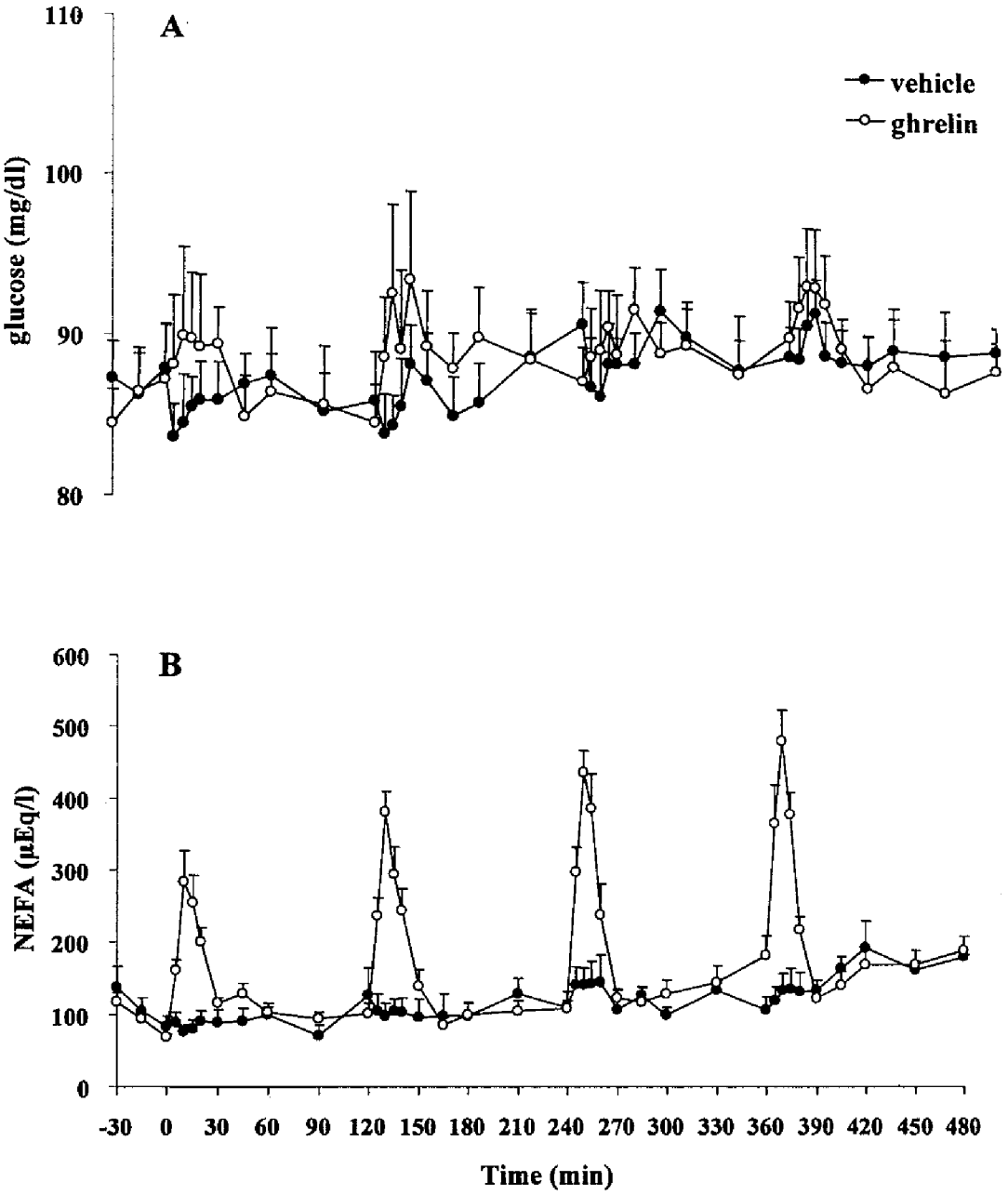

Fig. 6-3. The changes in plasma glucose (**A**) and NEFA (**B**) concentrations followed by ghrelin administration. Steers were intravenously injected with vehicle (5 ml 0.5% BSA-saline;●) or 1 μg acylated bovine ghrelin/kg BW (○) at 0, 120, 240 and 360 min. Blood sampling was started at 0730 h. Data are presented as mean ± S.E.M for 5 animals.

DISCUSSION

In the present study, we investigated the characteristic of the endocrine function of ghrelin in Holstein steers. The ratio of the basal plasma acyl-ghrelin and total ghrelin levels was approximately 1:4 in 10 month old Holstein steers. This ratio is different from the results of previous studies (approximately 1:16 in Holstein heifers in Experiment 2, 1:24 in Holstein steers in Experiment 3, 1:6 in Holstein pre- and post-weaning calves in Experiment 4). Therefore, it can be suggested that differences in the time and duration of sampling, age and sex of animlas and experimental conditions may affect the molecular structure of circulating ghrelin in ruminants. However, significant changes in both plasma acyl-ghrelin and total ghrelin levels were not seen in the vehicle-injected control group throughout the sampling time indicating the lack of the effect of short term fasting during sampling on plasma ghrelin levels in Holstein steers. More detail procedures are needed to investigate the regulatory factors that may influence the molecular forms of ghrelin in the plasma or tissue levels.

Intravenous administration of ghrelin every 2 h for 8 h transiently increased the basal plasma acyl-ghrelin and total ghrelin levels. Obviously, the peak value of plasma acyl-ghrelin increased after fourth injection of ghrelin while plasma total ghrelin showed similar response to each ghrelin injections. However, the values of AUCs for 120 min after post-injection were not different for both acyl-ghrelin and total ghrelin. We can not explain the reason for this finding in the present time but, at least in part, the residual effect of exogenous ghrelin is the rare case since elevated acyl or total ghrelin quickly declined to basal level after each injection. The rapid degradation of the plasma ghrelin has been reported in humans (Tschop *et al.* 2000, Gauna *et al.* 2004) and in pigs (Salfen *et al.* 2004). Our results in the previous studies also agree these findings. Therefore, we considered that physiological up-regulation of ghrelin can be induced by repeated administrations of

ghrelin but the precise evidence is not available yet.

In agreement with the results in ruminants (Hayashida *et al.* 2001, Itoh *et al.* 2006a, ThidarMyint *et al.* 2006 & 2008) and in monogastric animals (Seoane *et al.* 2000, Takaya *et al.* 2000), present study showed that ghrelin potently stimulated GH secretion even when it was repeatedly administered every 2 h for 8 h in Holstein steers. Moreover, the decrease in the response of GH in a peak value or in AUC by serial injections of ghrelin was observed. Roh *et al* (1996) also reported that GH responses were decreased by multiple injections of GHRP-2, one of the potent GH-secretagogues, in Holstein calves. A number of reasons can be considered. First, the capacity of somatotrophs to release GH might be reduced after repeated administration of agonist. However, we have reported that even 10 μg ghrelin/kg BW is not a saturation dose to stimulate GH secretion in Holstein heifers (ThidarMyint *et al.* 2006). Therefore, it can be considered that the depletion of pituitary GH content is not the reason for this reduction in plasma GH following the repeated injections of ghrelin at a dose of 1 μg/kg BW. Another possible factor is the homologus desensitization of GHS-R induced by the multiple injections of peptide (Luque *et al.* 2004). In the present result, the progressive reduction in the response of GH to the subsequent injections recovered after fourth injection which was done at 6 h after first injection. In fact, Camiña *et al* (2004) reported the slow recycling of ghrelin receptor which takes 360 min to recover from internalization after agonist treatment in the human embryonic kidney 293 cell line transfected with human GHS-R1a. The other possibility is that hypothalamic GHRH and SRIF may participate in this process (Kamegai *et al.* 2001, Thompson *et al.* 2003). However, we can not show direct evidences for these possibilities from our results, and advanced studies are necessary.

Plasma insulin level was also increased by ghrelin and every response to each injection was similar while plasma glucose levels were not changed. The numerous studies have been carried out on the effect of ghrelin on glucose mechanism in monogastric

animals including humans but the results are controversial. Some studies have shown the evidence of the stimulatory effect of ghrelin on pancreatic hormone secretion (Date *et al.* 2001; Lee *et al.* 2002) while some have not (Broglio *et al.* 2001; Reimer *et al.* 2003). According to the results from our previous study (Chapter 3), stimulatory effect of pharmacological doses of ghrelin on plasma insulin levels was observed while sub-pharmacological or physiological doses have no clear effect (ThidarMyint *et al.* 2006). Itoh *et al* (2006*b*) also reported that transient increase in plasma insulin and glucagon levels were observed in lactating cows in response to the ghrelin administration while plasma glucose levels were not changed. On the other hand, plasma NEFA level was also increased after each ghrelin injections. Differences in experimental conditions, nutritional status and physiological state of animals or sex may influence the effect of ghrelin on glucose and fatty acid metabolism. Furthermore, the present study shows the delay response in the changes of plasma insulin and NEFA levels after ghrelin administration compared with the response of plasma GH. This result was in consistence with our previous studies. It suggests the possibility that ghrelin indirectly affects the concentration of insulin and NEFA via stimulation of GH, not by modulating the glucose level.

In conclusion, the present study shows that the repeated administration of ghrelin promoted the increase in plasma acyl-ghrelin levels but the responses of plasma total ghrelin levels after each injection were similar. Moreover, the potent stimulatory effect of ghrelin on GH secretion was confirmed in Holstein steers while the GH response was decreased with repeated administration of ghrelin. Ghrelin also stimulated plasma insulin and NEFA concentration where plasma glucose levels were not changed. Although further experiments are necessary, the present data demonstrate the regulatory role of ghrelin in ruminant physiology.

SUMMARY

The present experiment was conducted to study the characteristic of the effects of ghrelin on plasma ghrelin, GH, insulin and metabolites in 10 month old Holstein steers. Animals were subjected to randomized cross-over study and serially injected 2 hourly for 8 h with vehicle (5 ml 0.5% BSA-saline) or synthesized acylated bovine ghrelin (1 μg/kg BW) via jugular vein catheters with 2 days recovery period. Blood sampling was done from 0700 h (30 min before first injection) to 1600 h. Vehicle injections did not modified the basal plasma acyl-ghrelin, total ghrelin, GH, insulin, glucose and NEFA levels throughout the sampling time. Plasma acyl-ghrelin and total ghrelin levels were transiently increased by ghrelin injections and rapidly eliminated thereafter. The peak value in plasma acyl-ghrelin reached highest after fourth injection of ghrelin but the ACUs values were not differ. Ghrelin injections promptly and significantly increased the plasma GH levels. The increase in plasma GH, in both peak value and AUCs level, after second injection of ghrelin was lower than that of first injection. That reduction still maintained after third injection and recovered after fourth administration. Plasma insulin and NEFA levels significantly, but less rapidly, increased after each injection of ghrelin while no difference in the response of peak values or AUCs levels was observed among injections. Serial administration of ghrelin did not affect the plasma glucose levels. These data indicates the possibility of strong desensitization and delayed recovery of GHS-R1a, a cognate receptor for endocrine effects of ghrelin in ruminants. Moreover, it can be suggested that the regulatory role of ghrelin in fatty acid metabolism and pancreatic peptide secretion is glucose-independent, rather indirect effect via GH response in Holstein steers.

Chapter 7 (Experiment 6)

Physiological and nutritional changes of plasma ghrelin concentrations in ruminants

INTRODUCTION

Ghrelin is a 27-28 amino-acid peptide predominantly produced by the stomach and other central or peripheral endocrine and nonendocrine tissues (Kojima *et al.* 1999, Korbonits *et al.* 2004). Ghrelin stimulates GH secretion from anterior pituitary gland via GH-secretagogue receptor type 1a (GHS-R1a) and acyl-modification of ghrelin at Ser^3 residue is essential for this endocrine effect. Another molecular form of ghrelin which is lack in acyl-modification (des-acyl ghrelin) also circulates in bloodstream with high concentrations (Hosoda *et al.* 2000a). Plasma ghrelin level is supposed to be under the control of nutritional state; ghrelin levels increase in negative energy balance such as fasting, anorexia and cachexia, and reduce in refeeding or obesity in human and laboratory animals (Cummings *et al.* 2001, Anderson *et al.* 2005). In rat, the concentrations of acyl-ghrelin and des-acyl ghrelin in plasma increased in response to fasting while they decreased in hypothalamus and stomach. On the other hand, ghrelin mRNA expression decreased in hypothalamus and increased in stomach (Sato *et al.* 2005). Recent studies also showed that plasma acyl-ghrelin levels were influenced by feeding pattern and energy balance in ruminants (Sugino *et al.* 2002*b*, Wertz-Lutz *et al.* 2006, Bradford *et al.* 2007) and in prepubertal gilts (Govoni *et al.* 2005). In deed, as shown in the previous chapters, ghrelin participates in the physiology of growth in ruminants by stimulating the GH release.

On the other hand, PYY, a 36 amino-acid peptide that isolated firstly from porcine intestine (Tatemoto & Mutt 1980) circulates in the bloodstream as two major molecular forms, PYY_{1-36} or PYY_{3-36} (Grandt *et al*, 1994). PYY_{3-36} is a 34-amino-acid peptide, which

is derived from PYY_{1-36} via cleavage of two amino acids from the N-terminal region by dipeptidyl peptidase IV. The anorexigenic effect of PYY_{3-36} has been reported in monogastric animals (Batterham *et al.* 2002) including pigs (Ito *et al.* 2006). The information on the role of PYY or the changes in plasma PYY levels in ruminants is still limited. The circulating levels of PYY which were measured by RIA using anti-porcine PYY serum were not altered over 48 h in both roughage and concentrate-fed sheep (Onaga *et al.* 2000). However, PYY immunoreactive cells are distributed in the mucosa of gastrointestinal tract (Onaga *et al.* 2000) and exogenous PYY altered the cycle of migrating motor complexes of the ovine intestine at doses in what is considered to be the physiological range (Onaga *et al.* 1997). We also have reported the inhibitory effect of PYY on feed intake in pigs while GH secretion was increased by PYY administration (Ito *et al.* 2006). These data indicate the peripheral or central effect of PYY in domestic animals. However, there is no information on how plasma ghrelin and PYY levels are regulated by the state of energy balance in ruminants. Therefore, the present experiment was designed to investigate the physiological changes in plasma acyl-ghrelin, total ghrelin and PYY concentrations followed by fasting and refeeding, and the effects of type of feedstuff on these changes in ruminants.

MATERIAL AND METHODS

Animals and experimental design

Protocol 1: To investigate the fasting-induced changes in plasma acyl-ghrelin and total ghrelin concentrations in ruminants, 11 sheep (2 years of age, 52 ± 2 kg BW) from Shintoku National Agricultural Research Center for Hokkaido Region, 5 steers (10 months of age, 248 ± 10 kg BW) and 6 sannen goats (12 months of age, 31 ± 2 kg BW) from laboratory of meat animal physiology, and 6 sheep (3 years of age, 58 ± 2 kg BW) from Laboratory of Nutrition, Obihiro University were used. Animals from Shintoku Research Center were raised in pasture while other animals were housed in groups and fed twice daily (0800 h and 1600 h) with commercially available concentrates/hay to meet the energy requirements for maintenance. Mineral salt block and water was available free choice. Animals were fasted for 48 h and blood sampling was done from jugular vein at 1100 h at day 1 (before fasting) and day 3 (after fasting). Plasma was isolated after centrifugation (3000 rpm × 20 min at 4°C) of blood and stored at -30°C until assay. For plasma ghrelin assay, 600 µl of plasma was separately acidified with 30 µl 1M HCl and stored at -30°C.

Protocol 2: To investigate the effect of diet-type on the changes of plasma acylated and total ghrelin concentrations in response to fasting time, five sannen goats (2 years of age, 37 ± 2 kg BW) were housed in large stalls without accessing to pasture. They were fed *ad libitum* Timothy hay (roughage-based diet; R-diet) twice a day at 0900 h and 1700 h. The initial blood sampling for measurement of basal plasma hormone concentrations was done via jugular venipuncture from each animal at 1300 h. After that, the residual feed was removed and animals were maintained under fasting condition for 48 h while mineral salt block and water was continued to give free choice. The additional blood samples were taken at day 2 (24 h fast) and 3 (48 h fast) at 1300 h. The animals were

then refed and the fourth sampling was done after 24 h refeeding. Body weight was measured before and after fasting. Then, animals were gradually shifted to the concentrate-based diet (C-diet) composed of 500g commercially available concentrate (crude protein 14%, crude fat 1.5%, crude fiber 8%, and crude ash 7%, Hokuren, Japan) and 1 kg timothy hay per day. Additional blood sampling and measurement of body weight were done within two weeks adaptation period. Thereafter, the fasting and sampling procedure was repeated. Plasma was treated as mentioned above and stored at -30°C.

Measurements

Plasma acyl-ghrelin, total ghrelin, insulin and PYY concentrations were quantified by RIA as described in chapter 2 & 3. Sensitivity and average intra-assay CV were 15.0 pg/ml & 11.0%, 0.44 ng/ml & 8.0%, 0.004 ng/ml & 10.7% and 0.20ng/ml & 11.0% for acyl-ghrelin, total ghrelin, insulin and PYY RIA respectively. Plasma GH concentration was measured by RIA (Chapter 3). Sensitivity and intra assay CV were 0.21 ng/ml and 7.0% respectively. Plasma glucose and NEFA levels were measured by commercially available kits (Code No. 439-90901 and 279-75401 respectively, Wako, Japan).

Statistics

Data are expressed as mean ± S.E.M. The differences between groups were compared with Student's paired *t*-test. Changes in plasma levels after fasting and refeeding were performed with one-way ANOVA with LSD *post hoc* analysis. The correlations between the changes in plasma hormone and metabolites levels for Trial 2 were analyzed by Pearson's 2-tailed correlation analysis. A P values less than 0.05 was considered significant. When P value was 0.01, it was considered that the values have tendency.

RESULTS

Protocol 1: The changes in plasma hormones and metabolites at fed and fasted condition in ruminants are presented in **Table 7-1**. In fed-state, the plasma acyl-ghrelin level was highest in sheep fed twice a day while plasma total ghrelin concentrations was lowest in steers. Both plasma acyl-ghrelin and total ghrelin levels, and the percentage of plasma acyl-ghrelin were increased by 48 h fasting in all groups. In sheep fed twice daily, the increase in plasma acyl ghrelin obviously reached to the value of plasma total ghrelin i.e., most of the measurable plasma ghrelin represent the acylated form in those animals. On the other hand, the significant changes in plasma PYY and GH levels after 48 h fasting were not observed. Both plasma insulin and glucose levels were significantly reduced in fast state in other animals except in goats. Plasma NEFA levels were significantly increased in all fasted animals.

Protocol 2: The goats showed similar pre-fasting BW in both diet. Fasting for 48 h induced 10% reduction in BW in all animals (37 ± 2 vs 33 ± 2 kg before and 48 h fasting, respectively). Basal plasma acyl-ghrelin concentration was not affected by type of diet (1.34 ± 0.34 vs 0.75 ± 0.17 ng/ml in R-diet and C-diet respectively, **Fig. 7-1**). The levels significantly increased by fasting (15.53 ± 3.35 and 7.16 ± 2.29 ng/ml at 24 h after fasting in R-diet and C-diet respectively) and maintained throughout the fasting period, then returned to pre-fasting value after realimentation (**Fig. 7-1A**).

The prefasting levels of plasma total ghrelin was significantly higher in R-diet than that of C-diet (8.24 ± 0.78 vs 3.29 ± 0.41 ng/ml respectively, $P < 0.05$) while the pattern in the response to fasting and refeeding was similar in both diets (**Fig. 7-1B**). The percentage of plasma acyl-ghrelin were increased by fasting and reduced by refeeding in both diet (**Fig. 7-1C**).

The basal plasma insulin levels were similar between diets (0.47 ± 0.13 vs 0.58 ± 0.018 ng/ml in R-diet and C-diet respectively, **Fig. 7-2A**). In R-diet, plasma insulin levels were significantly decreased at 24 h after fasting (0.09 ± 0.02 ng/ml, $P < 0.05$) and no further decrease was observed for 48 h fasting. Then, the level returned to prefasting level within 24 h after refeeding. On the other hand, plasma insulin levels were decreased only at 24 h after fasting in C-diet. The later concentrations at 48 h after fasting or 24 h after refeeding were not significantly differed to the prefasting values. The diet type did not affect the prefasting plasma PYY levels (0.51 ± 0.08 vs 0.46 ± 0.06 ng/ml in R-diet and C-diet respectively, $P = 0.69$). Plasma PYY concentration was significantly increased by fasting in R-diet (0.36 ± 0.04 ng/ml, $P < 0.05$, **Figure 7-2B**), and the level maintained until after refeeding. There were no changes in plasma PYY level in C-diet.

The basal plasma glucose level was higher in R-diet (74.73 ± 3.64 mg/dl) than C-diet (60.91 ± 1.84 mg/dl, $P < 0.05$). Plasma glucose levels were decreased by fasting in R-diet (57.07 ± 2.66 and 59.77 ± 1.94 mg/dl for 24 and 48 h after fasting, respectively, $P < 0.01$), and the levels remains in suppression until 24 h after refeeding (60.49 ± 1.85 mg/dl, $P < 0.01$). However, plasma glucose levels did not change by fasting or refeeding in C-diet (**Figure 7-3A**). In contrast, plasma NEFA concentrations increased during fasting in both diets (**Figure 7-3B**) but differences between prefasting values were not observed.

Pearson's correlation analysis showed that the changes in plasma acyl-ghrelin levels was positively correlated with plasma total ghrelin ($r = 0.594$, $P < 0.001$), PYY ($r = 0.331$, $P < 0.05$), and NEFA levels ($r = 0.615$, $P < 0.001$), and negatively correlated with plasma insulin levels ($r = -0.307$, $P < 0.05$). Plasma total ghrelin levels also showed strong positive correlation with PYY ($r = 0.345$, $P < 0.05$) and NEFA ($r = 0.356$, $P < 0.05$) and negative correlation with insulin ($r = -0.464$, $P < 0.001$). Plasma insulin positively correlated with plasma glucose levels ($r = 0.358$, $P < 0.05$) while plasma PYY has no significant correlation with plasma glucose and insulin levels.

Table 7-1. The changes in plasma hormone and metabolite levels at fed and fasted conditions in ruminants.

	pasture	2-times per day		
	Sheep (n=11)	Sheep (n=6)	Steer (n=5)	Goat (n=5)
Acyl-ghrelin (ng/ml)				
Fed	0.97 ± 0.14	8.41 ± 1.20	0.95 ± 0.12	1.25 ± 0.20
Fast	2.19 ± 0.33**	33.02 ± 7.50**	1.25 ± 0.09†	1.65 ± 0.09†
Total ghrelin (ng/ml)				
Fed	19.43 ± 1.73	19.20 ± 1.18	1.17 ± 0.17	6.12 ± 0.85
Fast	28.47 ± 2.51*	25.23 ± 1.68*	8.55 ± 0.6**	9.80 ± 1.06**
% acyl-ghrelin				
Fed	5.41 ± 0.93	43.87 ± 5.88	88.33 ± 16.75	22.13 ± 2.55
Fast	9.87 ± 3.07	127.28 ± 25.53*	15.19 ± 2.06*	17.49 ± 2.11
PYY (ng/ml)				
Fed	0.84 ± 0.07	0.56 ± 0.05	0.84 ± 0.07	0.47 ± 0.04
Fast	0.81 ± 0.06	0.51 ± 0.04	0.96 ± 0.13	0.47 ± 0.05
GH (ng/ml)				
Fed	2.59 ± 0.64	2.70 ± 0.73	5.43 ± 2.47	6.45 ± 2.78
Fast	3.43 ± 0.31	3.60 ± 1.11	1.41 ±0.26	3.90 ± 1.26
Insulin (ng/ml)				
Fed	0.34 ± 0.06	0.46 ± 0.07	0.34 ± 0.05	0.13 ± 0.02
Fast	0.09 ± 0.02**	0.23 ± 0.05*	0.18 ± 0.03*	0.08 ± 0.02
Glucose (mg/dl)				
Fed	61.65 ± 1.62	80.20 ± 6.66	77.09 ± 1.61	55.87 ± 4.15
Fast	42.88 ± 1.90**	56.46 ± 1.60**	68.87 ± 1.61*	47.13 ± 1.67**
NEFA (μEq/l)				
Fed	67.87 ± 34.57	192.02 ± 23.44	127.33 ± 18.81	85.62 ± 13.77
Fast	503.68 ± 137.93**	1050.69 ±113.23**	919.31 ± 57.49**	906.91 ± 186.22**

* $P < 0.05$, ** $P < 0.01$, † $P < 0.1$ vs values at fed-state

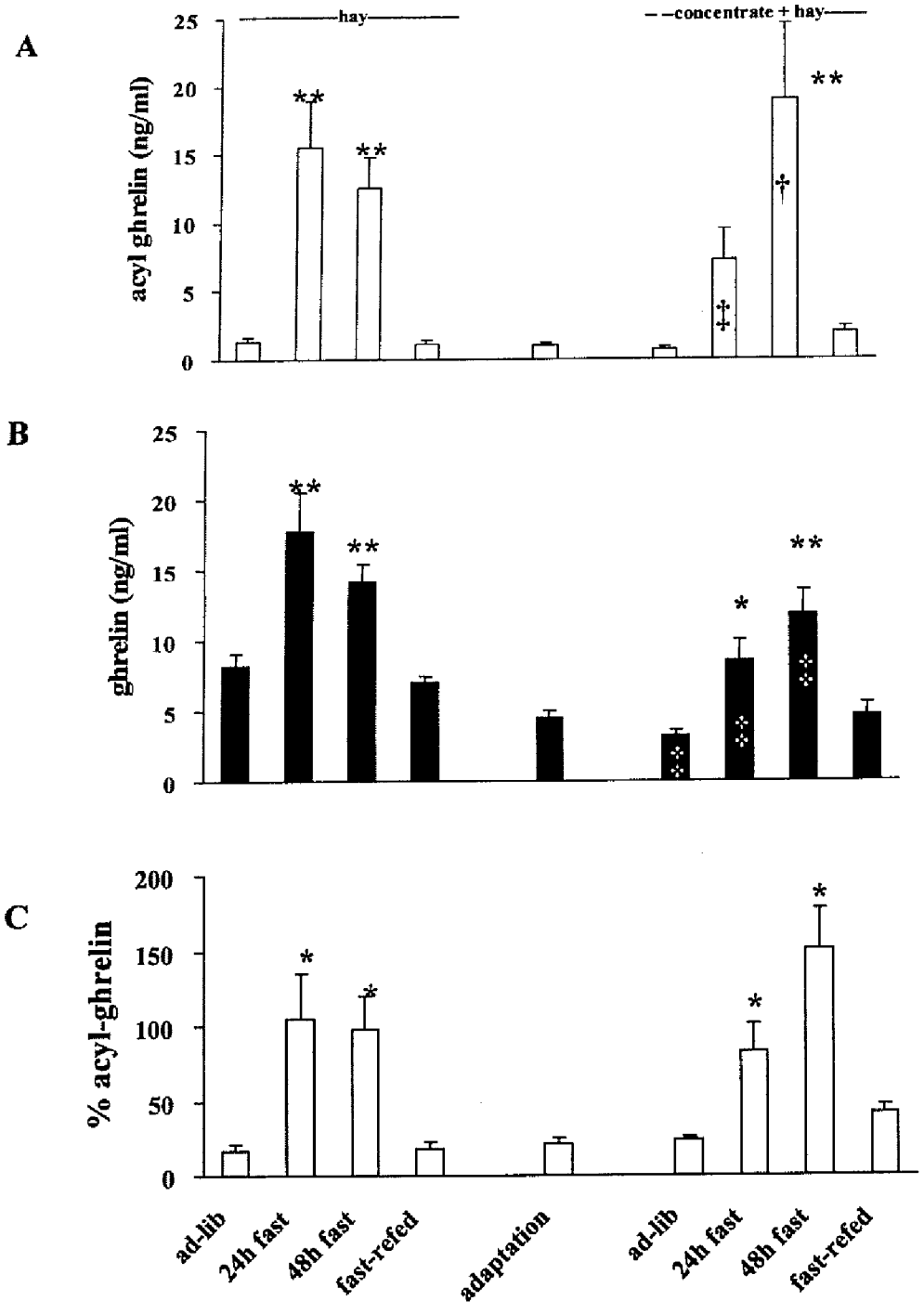

Fig. 7-1. The effects of type of feed and fasting on plasma ghrelin concentrations in Sennen goats. Animals were maintained on *ad libitum* hay or concentrate + hay diet before 48 h fasting. **A**; the values of acyl-ghrelin, **B**; the values of total ghrelin, **C**; percentage of acyl-ghrelin to total ghrelin levels. Data are expressed as means ± S.E.M for 5 animals. * $P<0.05$, ** $P<0.01$, † $P<0.1$ vs. prefasting values; ‡ $P<0.05$ vs. correspondent value in hay-feeding group.

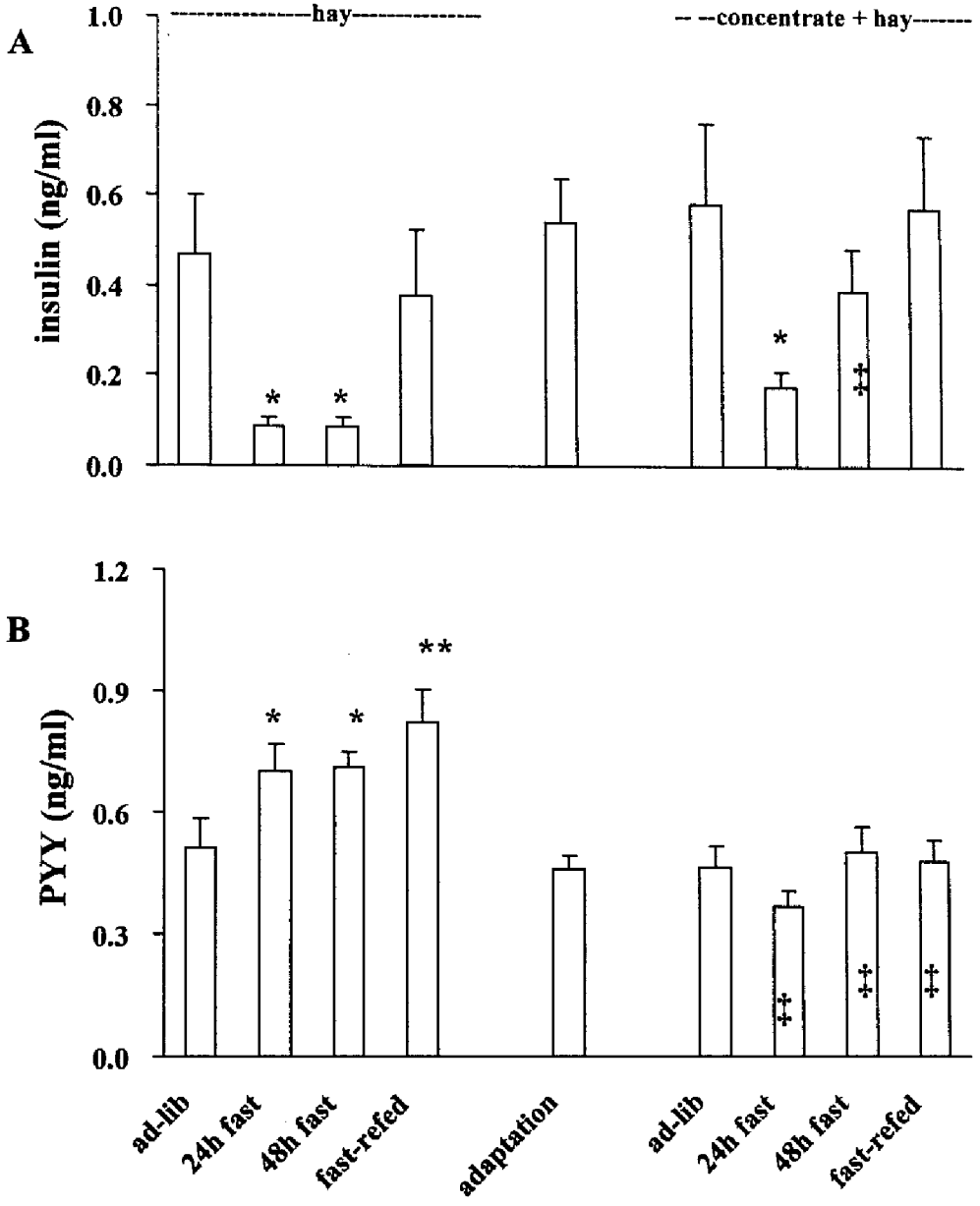

Fig. 7-2. The effects of type of diet and fasting on plasma insulin (**A**) and PYY (**B**) concentrations in Sennen goats. Animals were maintained on *ad libitum* hay or concentrate + hay diet before 48 h fasting. Data are expressed as means ± S.E.M for 5 animals. * $P<0.05$, ** $P<0.01$ vs. prefasting values; ‡ $P<0.05$ vs. correspondent value in hay-feeding group.

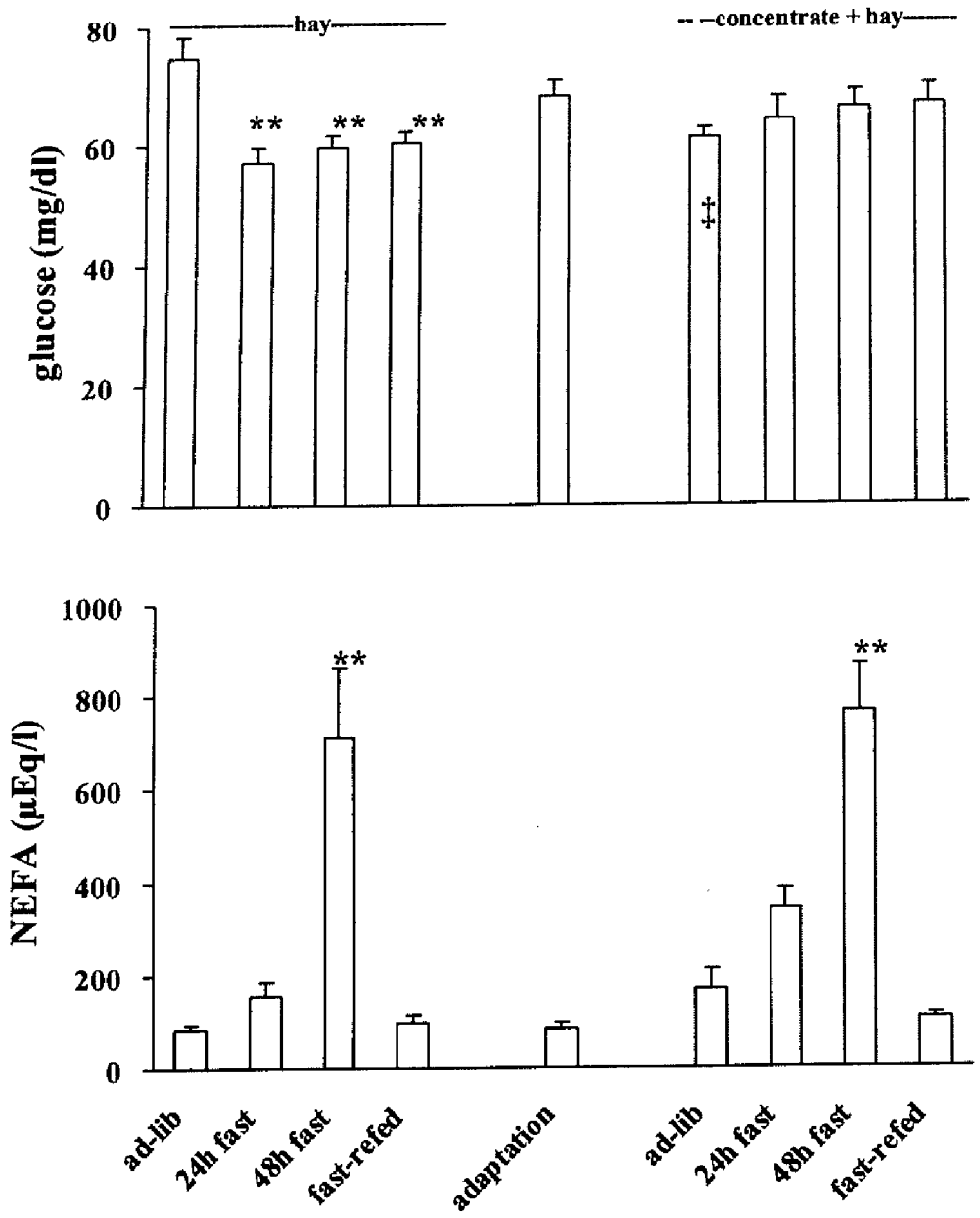

Fig. 7-3. The effects of type of diet and fasting on the plasma glucose (**A**) and non-esterified fatty acid (NEFA, **B**) concentrations in Sennen goats. Animals were maintained on *ad libitum* hay or concentrate + hay diet before 48 h fasting. Data are expressed as means ± S.E.M for 5 animals. ** $P<0.01$ vs. prefeeding values; ‡ $P<0.05$ vs. correspondent value in hay-feeding group.

DISCUSSION

The influence of the nutritional status, and differential effects of macronutrients on plasma ghrelin levels has been reported in monogastric animals (Murakami *et al.* 2002, Erdmann *et al.* 2003, Sánchez *et al.* 2004, Govoni *et al.* 2005) whereas both acylated and des-acyl ghrelin levels increased upon fasting and reduced after refeeding. In ruminants, it has been reported that plasma acyl-ghrelin levels fluctuated periprandially (Sugino *et al.* 2001*a*), and increased in fasting (Bradford *et al.* 2007) and the state of negative energy balance such as lactation (Wertz-Lutz *et al.* 2006). However, little is known about the regulatory mechanism of des-acyl ghrelin secretion in ruminants. In the present study, we firstly report the effect of fasting and refeeding on plasma total ghrelin levels in sheep, goats and steers. Both plasma acyl-ghrelin and total ghrelin levels increased followed by 48 h fasting in all animals. Interestingly, the elevation of plasma acyl-ghrelin levels in the sheep fed with twice a day feeding regimen reached to the values of total ghrelin, i.e., it is likely that most of the circulating ghrelin becomes octanoylated after 48 h fasting in twice-daily-fed sheep. However, this elevation pattern was not seen when sheep were maintained on pasture feeding. Moreover, this obvious increase in plasma acyl-ghrelin levels were not seen in steers or goats maintained at twice-daily feeding regimen.

To understand more precisely, we studied the effect of diet-type on fasting-induced plasma acyl- and total ghrelin concentrations in Sannen goats. The diet-type did not influence the pre-fasting acyl-ghrelin levels but the total ghrelin levels were lowered when animals were maintained with concentrate-based C-diet. As expected, both plasma acyl-ghrelin and total ghrelin levels were increased during fasting and reduced by refeeding. Again, increase in plasma acyl-ghrelin concentrations during fasting reached to the similar levels of plasma total ghrelin in goats. These data clearly indicates that feeding pattern (Sugino *et al.* 2002*b*) and nutrient-related mechanisms (Bradford *et al.* 2007) influence the

plasma acyl-ghrelin levels because gastric distension alone has no effect (Sugino *et al.* 2002*a*). In rat, ghrelin mRNA expression in stomach of fasted rats after food intake was clearly dependent on the type of ingested macronutrient (Sánchez *et al.* 2004) whereas feeding of fat-rich diet induced quick recovery of ghrelin mRNA to pre-fasting levels compared with the ingestion of carbohydrate diet. Erdmann *et al* (2003) also showed the different time-pattern in postprandial total ghrelin secretion between the ingestions of carbohydrates and fats in human. Very recent microdialysis study by Dornonville de la Cour *et al* (2007) has been reported that secretion of total ghrelin from rat stomach is stimulated by adrenaline, noradrenaline, endothelin and secretin whereas somatostatin and gastrin-releasing peptides inhibited. However, these results in monogastric animals is in deed not directly interpretable for the release of ghrelin in fasting and/or refeeding state in ruminants since nutrients are constantly being absorbed from the forestomach in ruminants and passing from the forestomach down the remainder of their gastrointestinal tract (Frandson *et al.* 2003). Although the effect of different macronutrients on the secretion of ghrelin is needed to be addressed in future studies, our present result suggests that the regulatory mechanism of ghrelin in ruminants is critically complex, and the different diet and many endocrine or paracrine factors may influence the plasma ghrelin levels and/or its molecular form especially in the adaptation to energy deficit.

We hypothesized that fasting might suppressed the plasma PYY levels in ruminants since plasma PYY levels increased after feeding (Adrian *et al.* 1987) and reduced in fasting (Ito *et al.* 2006) in monogastric animals. However, the present study showed that plasma PYY levels were not significantly changed by fasting for 48 h in all small and large ruminants that were maintained with different environment and different diet. Obviously, when goats were intensively fed roughage diet, the plasma PYY levels increased during 48 h fasting state and the level remained unchanged until 24 h after refeeding. In deed, Onga *et al* (2000) has reported that the plasma concentrations of

immunoreactive PYY did not significantly fluctuate by ingestion of roughage and concentrate. Therefore, it is suggested that periprandial changes in plasma PYY concentrations are not prominent in ruminants but the levels during energy deficit can be partly influenced by the type of maintenance diet and consequence effects such as rumen fermentation rate. Moreover, plasma PYY levels were positively correlated with both acyl- and total ghrelin levels in the present study, and both ghrelin and PYY immunoreactive cells are present in the gastrointestinal tract of ruminants (Hayashida *et al.* 2001, Onaga *et al.* 1997) indicating the possible counterregulation between these two peptides to maintain the energy homeostasis. In contrast, recent studies have been reported that intravenously injected PYY did not affect the plasma ghrelin levels *in vitro* (Dornonville de la Cour *et al.* 2007) and *in vivo* (Ito *et al.* 2006).

On the other hand, plasma GH levels showed tendency to decrease in steers but not in small ruminants. It is believed that the state of energy deficit suppressed the plasma GH levels in ruminants (Rule *et al.* 1985) but the discrepancy results has been reported by others (Bradford *et al.* 2007). It is suggested that experimental design, duration of sampling time and individual differences may explained these discrepancy results. In addition to the well-documented stimulatory effects of ghrelin on GH secretion in monogastric animals (Korbonitis *et al.* 2004) and also in ruminants (ThidarMyint *et al.* 2006 & 2008), the negative feedback regulation of GH on ghrelin release from gastric tissues explants in rats has been reported (Seoane *et al.* 2007a). However, the lack of correlation between physiological concentrations of circulating ghrelin and GH has been reported in humans (Barkan *et al.* 2003) partly explained the fact that increase in plasma ghrelin levels in response to fasting does not affect the plasma GH levels during short-term fasting in ruminants.

The present study also showed that both plasma insulin and glucose levels decreased while plasma NEFA levels increased by fasting in ruminants. Moreover, the

reversed correlation of insulin with acyl- and total ghrelin levels were observed. Hypoglycemia and hyperlipidemia are the characteristics for the negative energy balance in ruminants. It can be suggested that plasma insulin levels decreases to prevent the greater hypoglycemia and the mobilization of stored fatty acids increases to adapt the energy deficit in fasting state. Ghrelin may participate in these processes. The reduced insulin level is associated with increase in ghrelin levels since insulin and ghrelin reciprocally affect each other (Broglio *et al.* 2001, Saad *et al.* 2002, Reimer *et al.* 2003). Therefore, it is suggested that insulin, but not glucose, modulates the fasting-induced ghrelin release in ruminants. However, it can not be excluded that additional gastrointestinal tract derived hormones and neurotransmitters (Dornonville de la Cour *et al.* 2007) are responsible for the meal-related changes in ghrelin concentrations.

In conclusion, our data demonstrated that plasma ghrelin levels were elevated by fasting and reduced after refeeding regardless of the type of maintenance diet. Plasma PYY, insulin and NEFA levels might participate in the fasting-induced changes in plasma ghrelin concentrations. Obviously, it is likely that most of the circulating ghrelin becomes acylated during fasting in Sannen goats. Our present data clearly demonstrated that the evaluation of both circulating acyl-and total ghrelin levels is important to understand the regulatory mechanism of ghrelin secretion in ruminants.

SUMMARY

Gastric-derived orexigenic hormone ghrelin circulates in the bloodstream as at least two different molecular forms; acyl-ghrelin which is acylated with octanoic acid on its Ser^3 residue and des-acyl ghrelin. In ruminants, it has been reported that the concentration of acyl-ghrelin increased preprandially and at the energy deficit, while the levels decreased after feeding. On the other hand, plasma PYY levels show the reverse changes in plasma with acyl-ghrelin to maintain the energy homeostasis in monogastric animals. However, the changes in plasma total ghrelin and PYY levels have not been studied in ruminants at the time of energy deficit. We have conducted two protocols for ruminants to investigate the physiological changes of these peptide hormones in short-term fasting and refeeding conditions. In Protocol 1, 11 sheep raised in pasture, and 6 sheep, 5 steers and 6 goats raised with 2-times a day feeding regimen were fasted for 48 h. Plasma samples that were taken at before and after fasting showed the increase in both plasma acyl- and total ghrelin levels in all animals. Plasma PYY and GH levels did not change while plasma insulin and glucose levels decreased by fasting. There was an increase in plasma NEFA levels in fasted animals. Obviously, percentage of plasma acyl-ghrelin to total ghrelin levels were increased after 48 h fasting to 127.28 ± 25.53 % in sheep primarily maintained with 2-times per day feeding regimen. To understand more precisely, in Protocol 2, five goats were fasted for 48 h under 2 different types of diet (roughage only: R-diet and concentrate-based diet: C-diet) and single blood samplings were done at day 1 (prefasting), day 2 (24 h after fasting), day 3 (48 h after fasting) and day 4 (24 h after refeeding). Basal plasma acyl-ghrelin levels were not different between diets while plasma total ghrelin levels were lower in goats fed with C-diet. Both plasma acyl-ghrelin and total ghrelin levels increased during fasting and the percentage of plasma acyl-ghrelin were increased by fasting time and reduced by refeeding in both diet. Plasma GH levels were not affected by fasting or refeeding in both diets. There

was a decrease in plasma insulin and an increase in plasma NEFA levels in fast-state and these levels retuned to the prefasting values after refeeding. Plasma glucose levels lowered while plasma PYY levels increased in fast-state in goats maintained with R-diet. These data indicates that both animal species and the type of maintenance feed differentially affect the plasma acyl-ghrelin, total ghrelin and their ratio in ruminants, however, the changes in plasma PYY levels in fasted- or fed-condition is not clear. The plasma insulin declined probably to maintain the glucose homeostasis in fasted state and indirectly affected the plasma acyl-and total ghrelin levels in the condition of energy deficit. Obvious finding is that most of the circulating ghrelin was likely to become acylated form during fasting in Sannen goats. Overall, our present data clearly demonstrate that the evaluation of both circulating acyl- and total ghrelin levels is more preferable than measuring only type of molecular form to understand the regulatory mechanism of ghrelin secretion in ruminants.

Chapter 8 (General discussion)

Growth hormone (GH) which is secreted from the somatotrophs of anterior pituitary gland participates in the postnatal growth, energy partition and fatty acid metabolism in dairy cattle (Etherton TD & Bauman DE. 1998). It is generally accepted that GH secretion is stimulated by hypothalamic GH-stimulating hormone (GHRH) and is inhibited by somatostatin (McMahon *et al.* 2001*b*). Synthetic peptidyl or nonpeptidyl GH-secretagogues (GHSs) also stimulate GH secretion by acting directly to pituitary or indirectly through hypothalamus via activating G-protein coupled receptors GHS-R (Bowers CY. 1998). In 1999, endogenous ligand of GHS-R was firstly purified from rat and human stomach tissues by Dr. Masayasu Kojima and colleagues, and the authors designated the ligand "ghrelin" since it stimulates GH release (Kojima *et al.* 1999). Apart from its potent stimulatory effect on GH secretion, ghrelin has also been implicated in the regulation of other endocrine and exocrine metabolisms including stimulation of the lactotroph and corticortroph secretions, appetite stimulation, glucose metabolism and control of gastric motility and acid secretion (Korbonits *et al.* 2004).

Ruminant ghrelin is a 27 amino acid peptide with acyl-modification on its Ser^3 residue (Dickin *et al.* 2004, Kita *et al.* 2005). This acyl-modification is supposed to be essential to activate the GHS-R type 1a resulting the endocrine effects of ghrelin in monogastric animals (Kojima *et al.* 1999, Hosoda *et al.* 2000*a*). Stomach is the major source of ghrelin (Date *et al.* 2000, Hayashida *et al.* 2001) and ghrelin circulates in bloodstream with two different molecular forms, acylated and non-acylated (des-acyl) forms. In monogastric animals, it has been reported that des-acyl ghrelin is the major portion of circulating ghrelin (Hosoda *et al.* 2000*a*). In agreement with the results from human studies (Cummings *et al.* 2001), circulating concentration of acylated ghrelin transiently increased preprandially in scheduled meal-fed sheep and decreased after feeding

(Sugino *et al.* 2002a) and those changes can be modified by different feeding regimen (Sugino *et al.* 2002b) indicating that ghrelin participates in feed-regulation in ruminants. However, the information about des-acyl ghrelin had not been available in ruminant species. The differences in amino acid sequences of ghrelin between monogastric and ruminant species did not allow the researchers to evaluate the total ghrelin concentrations in ruminants by using commercially available ghrelin radioimmunoassay (RIA) kits of monogastric origin. Moreover, it is natural that plenty amount of ghrelin becomes necessary to perform the *in vivo* studies in large animals such as dairy cattle. For these reasons, our first attempt was to synthesize the desired peptides (Table 2-1) and to evaluate the specific RIA systems for measurement of plasma hormone levels in ruminant species (Chapter 2). Subsequently, the specific antibodies for n-terminal acylated ghrelin, intact C-terminal bovine ghrelin peptides, bovine PYY and bovine leptin were successfully raised. By using these peptides and antibodies, we have reported, for the first time as my own knowledge, the plasma concentration of plasma total ghrelin including acylated ghrelin, des-acyl ghrelin and intact C-terminal ghrelin peptide in Holstein cattle. Moreover, we have also evaluated the plasma PYY and leptin levels in ruminants.

In general, our results showed that des-acyl ghrelin is the dominant portion of circulating ghrelin in ruminants in consistent with the results of others in monogastric animals (Hosoda *et al.* 2000*a*). Moreover, the ratio of acylated ghrelin to total ghrelin differs among experiments i.e., 1:16 in 6 month old Holstein heifer (Experiment 2), 1:7 in Holstein bull calves (Experiment 4), 1:24 in 10 month old Holstein steers fed *ad libitum* (Experiment 3) and 1:4 in 10 month old Holstein steers in fasting condition (Experiment 5). Therefore, it is suggested that sex, age, physiological state, nutritional status and probably the experimental conditions influence the plasma ghrelin levels and their ratio in ruminants. The interesting point is that plasma acylated ghrelin, rather total ghrelin concentrations fluctuate among these animals. However, weaning did not affect the concentrations of

acylated ghrelin and total ghrelin and their ratio in plasma (Experiment 4). The more detailed studies were done in Experiment 6 in which the plasma hormone concentrations were measured in sheep, goat and steers that were maintained in different environment and different diet. Moreover, effects of fasting and refeeding on plasma ghrelin concentrations were also studied in goats fed with different maintenance diets. Fasting increased, while refeeding reduced both plasma acylated ghrelin and total ghrelin levels in all animals. The effects of feeding pattern (Sugino *et al.* 2002b) and fasting (Bradford *et al.* 2007) on plasma acylated ghrelin levels have been reported in ruminants and their results were in consistence with our present study. Obviously, when both molecular forms of circulating ghrelin were measured, the elevated plasma acylated ghrelin reached to the similar values of plasma total ghrelin during fasting. It is likely that almost all the circulating ghrelin becomes acylated in energy deficit indicating that acyl-modification is critical for the ghrelin to implicate the adaptation with energy deficit in ruminants. Moreover, there was a positive correlation of plasma ghrelin with plasma PYY and NEFA levels while plasma ghrelin negatively correlated with plasma insulin and glucose during the nutritional changes in goats. However, the significant correlation between plasma ghrelin and GH levels were not observed. Therefore, it can be suggested that the mechanisms of ghrelin secretion is critically complex and at least in a part, gastrointestinal tract derived hormones and neurotransmitters (de la Cour *et al.* 2007) are responsible for the meal-related changes in ghrelin concentrations. Overall, our data clearly demonstrate that the evaluation of both circulating acylated and total ghrelin levels favors us to understand the physiology of ghrelin in ruminants.

Although the results from our series of experiments did not show any relations between plasma ghrelin and GH concentrations in fasting or refeeding conditions in goats, the stimulatory effect of ghrelin on GH release has widely been reported in all species studied to-date (Korbonits *et al.* 2004) including ruminants (Hayashida *et al.* 2001,

Hashizume *et al.* 2003 & 2005, Itoh *et al.* 2005). All of the studies in ruminants utilized the supraphysiological doses of ghrelin peptide of monogastric origin since the ruminant ghrelin has not been available commercially yet. Therefore, we investigated the dose-dependent effect of ghrelin on GH secretion in Holstein heifers by injecting 5 different doses of bovine ghrelin ranging from 0.1 to 10.0 μg/kg BW and the results were compared with those from vehicle-injected control group (Experiment 2). All doses of intravenously administered ghrelin significantly stimulated GH release. The GH response to the highest dose of ghrelin (10.0 μg/kg BW) was greater than the response to 5.0 μg/kg BW ghrelin indicating that ghrelin is a potent stimulator of GH release in ruminants. In addition, we observed that plasma insulin and NEFA levels were transiently stimulated by higher doses of ghrelin while plasma glucose and leptin levels were not affected. It is reported that ghrelin activates its cognate receptor, GHS-R1a, to stimulate GH release, and the acyl-modification on its Ser3 residue is supposed to be indispensable for the binding of ghrelin to GHS-R1a (Kojima *et al.* 1999, Bednarek *et al.* 2000). However, it is noteworthy that truncated ghrelin analogs encompassing the first 5 amino acids are ineffective in the stimulation of GH release in neonatal rats (Torsello *et al.* 2002). To clarify these discrepancies, we studied the effects of the pretreatment of des-acyl ghrelin on ghrelin-induced hormonal changes in Holstein steers (Experiment 3). Intravenous injections of bovine acylated ghrelin at a dose of 1.0 μg/kg BW significantly stimulated GH, insulin, glucose and NEFA levels while plasma PYY levels were not changed. The pretreatment of bovine des-acyl ghrelin at a dose of 10.0 μg/kg BW did not affect these changes. Furthermore, the responses of plasma GH to the intravenous administrations of bovine and porcine acylated ghrelin were similar in calves. Therefore, it is suggested that acyl-modification is essential for ghrelin-induced endocrine changes in ruminants.

In consistant with our results showing ghrelin as an important regulator of the somatotropic axis in ruminants, Itoh *et al* (2005) reported that ghrelin-induced GH

secretion is higher in early lactating cows than in calves and late-lactating cows. Therefore, we hypothesized that the effect of ghrelin might be altered by the physiological status of animals. On the other hand, there was no report on associations between ghrelin and GHRH to release GH *in vivo* in ruminant species. Weaning induced the prominent changes in endocrine responses in ruminants (Katoh *et al.* 2004). Therefore we designed the Experiment 4 to study the possible changes of interactions between ghrelin and GHRH around weaning in Holstein calves. Ghrelin stimulated GH release with similar potency as GHRH and their combined administration synergistically stimulated GH release in preweaning calves but this effect was lost after weaning. On the other hand, *in vitro* studies could not demonstrate the synergistic or additive effect of ghrelin and GHRH on the GH release from bovine (Hashizume *et al.* 2003), porcine (Malagon *et al.* 2003) and rat (Yamazaki *et al.* 2002) anterior pituitary glands. Therefore, it can be suggested that the effect of ghrelin on GH secretion is not only limited to the pituitary, but likely also involves in GHRH release from the hypothalamus (Mogi *et al.* 2004, Wren *et al.* 2002). Moreover, complex interdependent intracellular signaling pathways induced by ghrelin and GHRH (Anderson et al. 2005), and the possible changes in the proportions of subpopulations of somatotrophs that are responsive to ghrelin, GHRH and to both secretagogues might be responsible for the different results in pre- and post-weaning calves. In addition, different from the results of previously described studies, ghrelin or GHRH injections did not affect the basal plasma insulin, glucose and NEFA levels in these calves.

In the experiment 5, we studied the characteristic of ghrelin to stimulate GH release from the results of serial intravenous injections of 1.0 μg ghrelin/kg BW every 2 h for 8 h in steers. The plasma GH concentration was significantly increased by each injection of ghrelin, and the decrease in the responses of GH in a peak value or in AUC by repeated injections of ghrelin was observed. These reductions recovered after fourth injection of ghrelin. It can be considered that the depletion of pituitary GH content is not

the reason since even the 10.0 μg/kg BW is not a saturation dose for ghrelin to stimulate GH secretion in Holstein heifers (Experiment 2). It can be explained by the fact that ghrelin strongly desensitized to the somatotrophs in dispersed anterior pituitary cells (Yamazaki *et al.* 2002, Luque *et al.* 2004). Moreover, Camiña *et al* (2004) reported the slow recycling of ghrelin receptor which takes 360 min to recover from internalization after agonist treatment in the human embryonic kidney 293 cell line transfected with human GHS-R1a. Although we can not show the direct evidences, it is suggested that repeated administrations of ghrelin induce homologous desensitization of GHS-R, and slow recycling of GHS-R may reduce the effects of subsequent injections of ghrelin on GH release. Moreover, it can not be excluded the possible implications of hypothalamic GHRH and SRIF in this process (Kamegai *et al.* 2001).

The repeated administration of ghrelin every 2 h for 8 h stimulated plasma insulin and NEFA concentrations while plasma glucose levels were not changed. These data are in consistence with the results of previous studies. It is noteworthy that the responses of plasma insulin and NEFA were less rapid when compared with the prompt response of plasma GH in all studies. Therefore, it can be suggested that ghrelin might not directly participate in the glucose and fatty acid metabolism rather it affects plasma insulin and NEFA levels via stimulation of GH (McMahon *et al.* 2001*a*).

Finally, we would like to focus on the responses of plasma acyl-ghrelin and total ghrelin concentrations induced by intravenous administrations of ghrelin and des-acyl ghrelin from our series of experiments. Plasma acyl-ghrelin levels were transiently elevated after ghrelin injections and were rapidly returned to the pre-injected values in all studies. Similarly, plasma total ghrelin levels temporally elevated in plasma after injection of bovine acylated ghrelin and were degraded/eliminated in the bloodstream with 15 min. On the other hand, the degradation time of plasma total ghrelin levels were relatively delayed (within 60 min post-injection) when bovine ghrelin was injected as des-acyl form

(Experiment 3). It has been reported that the half life of plasma ghrelin was supposed to be very short (Tschöp *et al.* 2000, Gauna *et al.* 2004, Salfen *et al.* 2004). Therefore, it can be suggested that the rate of degradation of ghrelin in circulation is also rapid in ruminants and perhaps the structure of ghrelin (acylated or des-acyl form) may affect the degradation rate of ghrelin. Another interesting finding is that the peak values of plasma acyl-ghrelin to ghrelin injections become higher when ghrelin was repeatedly administered (Experiment 5). Although the information to explain the reasons for this finding is not available at present time, the residual effect of exogenous ghrelin is the rare case since elevated acylated or total ghrelin quickly declined to basal level after each injection. Further studies are necessary to broaden this scope.

In conclusion, our studies demonstrate that both acylated and des-acyl ghrelin are circulating in the bloodstream in considerable quantities in ruminant species and the degradation/elimination rate of ghrelin is rapid. The ratio of plasma acyl-ghrelin and total ghrelin levels are affected by sex, age, species and nutritional state. Moreover, their plasma levels are elevated in energy deficit and reduced after refeeding whereas acyl-ghrelin levels seems to be more susceptible to the physiological and nutritional changes. Our data also report that and intravenous injection of ghrelin at physiological dose is sufficient to induce endogenous GH secretion and confirm the potent stimulatory effect of ghrelin on plasma GH secretion in Holstein cattle. Furthermore, ghrelin synergistically stimulated GH release with GHRH in preweaning calves and this effect was lost after weaning indicating that the effect of ghrelin on somatotropic axis is partly influenced by the physiological stages of ruminants. Ghrelin also has considerable effect on plasma insulin and NEFA levels indicating that ghrelin plays an important role in ruminant physiology and acyl-modification at Ser^3 residue is critical for the ghrelin's endocrine effects in ruminants.

Chapter 9

General summary

We have conducted six experiments to investigate the effects of ghrelin on GH secretion mechanisms in Holstein cattle. We also studied the influences of the physiological and nutritional factors on plasma ghrelin concentrations in ruminants.

Experiment 1

Since ruminant ghrelin for *in vivo* experiments and the specific radioimmunoassay (RIA) kits to measure the concentrations of feeding-regulating hormones in ruminants were not commercially available, we not only synthesized pure peptide but also established the specific RIA systems. We have synthesized the n-acylated bovine ghrelin [1-27], [Cys12]-ghrelin [1-11] with acylation, [Cys-0]-bovine ghrelin [11-27], bovine PYY-NH_2 [1-36], bovine PYY-NH_2 [3-36], bovine [Cys0]-PYY-NH_2 [4-36] and human GHRH-NH_2 [1-29]. And we also have raised the antibody against acyl-ghrelin in rabbit (final dilution of 1:240000), antibodies against bovine total ghrelin (final dilution of 1:15000), bovine PYY (final dilution of 1:50000) and bovine leptin (final dilution of 1:200000) in guinea-pigs. By using these antibodies, we have established the RIA systems specific for acyl-ghrelin, bovine total ghrelin, bovine PYY and bovine leptin. These RIA systems are also applicable for the measurements of the plasma concentrations in sheep and goats. These specific RIA systems will provide new information on the counterregulation among appetite-regulating hormones and will lead to the deeper understanding of ruminant digestive physiology.

Experiment 2

In this experiment, we studied the dose-dependent effect of ghrelin on the GH secretion in six Holstein heifers. All doses of intravenously injected ghrelin ranging from 0.1 to 10.0 μg/kg BW significantly stimulate GH release in dose-dependent manner. Moreover, higher doses of ghrelin stimulate plasma insulin and NEFA concentrations. However, ghrelin injection does not affect the plasma leptin levels. Plasma acyl-ghrelin, and total ghrelin levels responding acyl-ghrelin, des-acyl ghrelin and intact C-terminal fragments are transiently elevated after the ghrelin administration and rapidly disappear from the plasma. This is the first report on the plasma total ghrelin concentrations in ruminants (Journal of Endocrinology 2006 Jun;189:655-664).

Experiment 3

This study was conducted to clarify the role of acylation on Ser^3 residue of ghrelin sequence on the endocrine effects of ghrelin. The pre-injection of full-length bovine des-acyl ghrelin (10.0μg/kg BW) was done to Holstein steers 5 min before the administration of acylated full-length bovine ghrelin at a dose of 1.0 μg/kg BW. The increased in plasma acyl-ghrelin followed by the injection of acyl-ghrelin rapidly returns to the basal levels. On the other hand, the degradation time of total ghrelin in plasma after the des-acyl ghrelin injection is relatively delayed when compared with the correspondent time after the injection of the same dose of acylated ghrelin in experiment 2. The acyl-ghrelin injection significantly stimulates the plasma GH, insulin, glucose and NEFA levels but plasma PYY levels are not changed. Pre-injection of des-acyl ghrelin fail to modify the acyl-ghrelin-induced hormonal changes in steers. Moreover, the GH response to the intravenous administrations of porcine or bovine ghrelin is similar. Although we can not exclude the possible metabolic effect of des-acyl ghrelin which has been reported in the

monogastric animals, the results from the present study indicate the critical role of acylation on its Ser^3 residue in the endocrine effects of ghrelin in ruminants.

Experiment 4

We studied the influence of weaning on the effects of the exogenous administration of ghrelin (1.0 μg/kg BW) and GHRH (0.25 μg/kg BW), in single or in combination, to the GH secretion in the Holstein male calves. Basal concentrations of plasma acy-ghrelin, total ghrelin, GH and IGF-1 concentrations are not changed over the weaning period. On the other hand, reduction in plasma insulin, glucose and NEFA concentrations are observed in post-weaning calves. Both ghrelin and GHRH significantly stimulate GH release in both pre- and post-weaning calves. Combined administration of ghrelin and GHRH synergistically stimulates the GH release in the preweaning calves, and this effect is obviously lost after weaning. However, the value of GH response to the combined administration of ghrelin and GHRH between pre- and post-weaning period did not significantly differ. The plasma IGF-1, insulin, glucose and NEFA levels are not changed by the ghrelin and/or GHRH administration in both pre- and post-weaning calves. All together, it is suggested that the GH-releasing ability of somatotrophs may not alter over the weaning period but the ratio of these cells responsive to ghrelin, GHRH and/or both may alter around weaning.

Experiment 5

In this experiment, we studied the effects of repeated administrations of ghrelin on plasma GH secretion in Holstein steers. Both plasma acyl-ghrelin and total ghrelin levels transiently increase after each ghrelin injection. The peak values in plasma acyl-ghrelin

become higher in accordance with the repeated injection but the values of area under the curves (AUCs) for 120 min after each injection are not differed. The intravenous administrations of 1.0 μg ghrelin/kg BW at 2 h intervals significantly stimulate GH release. GH AUCs 120 min after each injection decreases until after the third injection, and recover after forth injection to the similar values of the second injection. Plasma insulin and NEFA levels are also increased followed by ghrelin injections while the significant changes of plasma glucose concentration are not found.

Experiment 6

To study the influences of physiological and nutritional factors upon the changes in plasma ghrelin concentrations in the ruminants, sheep maintained with or without grazing system, and goats and steers in intensive systems are fasted for 48 h and the pre- and post-fasting plasma ghrelin concentrations are measured. The plasma acylated and total ghrelin concentrations, and the plasma NEFA levels increase after 48 h fasting while glucose levels decrease. The fasting for 48 h fails to affect the plasma GH and PYY levels in all animals. To understand the effects of the types of feed and the duration of fasting on plasma ghrelin levels, goats are raised in intensive system and fed two times a day with hay *ad libitum* or 500 g concentrate + 1 kg hay per head per day regimen. They are fasted for 48 h and more frequent samplings are done. The plasma acylated and total ghrelin levels increase 24 h after fasting and plasma acylated ghrelin levels reach to similar values of plasma total ghrelin levels. These levels are returned to the pre-fasting values 24 h after refeeding. It is unlikely that the types of feed affect the changes of plasma acylated and total ghrelin levels by fasting, however, basal total ghrelin levels are affected by type of feed. Plasma glucose levels decrease, while plasma NEFA levels increase by fasting, and return to the pre-fasting values after refeeding. No changes of the plasma PYY levels are

observed by fasting in goats maintained with high-grain feed whereas the levels increased when goats were maintained with roughage diet.

In conclusion, the series of the present studies clearly demonstrate that:

1. Ghrelin is the potent GH releaser in ruminants, and the acyl modification at Ser^3 residue is essential for the endocrine effects of ghrelin in ruminant physiology.
2. Ghrelin has synergistic effect with GHRH on GH release *in vivo* in pre-weaning calves, and the physiological stage of animal influences the effect of ghrelin upon GH axis in ruminants.
3. Nutritional balance not only affects the plasma ghrelin levels but also influcenced on the structure of plasma ghrelin (acylated or des-acyl form) in the ruminants.
4. Ghrelin rapidly degradates/eliminates in plasma, and the structure of ghrelin may influence the rate of degradation.

All together, it is considered that ghrelin plays an important role in growth physiology of ruminants by increasing GH release and by inserting some effects on glucose metabolism. Moreover, changes in plasma ghrelin levels by fasting and refeeding suggest the participation of ghrelin in appetite regulation in ruminants.

Chapter 9

Japanese general summary

脳下垂体細胞から放出される成長ホルモン（GH）は乳牛の成長・エネルギー分配や脂肪代謝など様々な代謝に関与している。一般にGH分泌は視床下部から放出されたGH促進ホルモン（GHRH）とGH分泌抑制ホルモン（SRIF）により制御されていると受けとめられている。一方、人工的に合成されたGH分泌促進因子（GHS）は直接下垂体に作用するか、または視床下部でのGHRH経路を通してGH分泌を促進している。それらの作用はオーファン受容体であるGHS-R1aの活性化を介して起こることが知られていた。1999年、GHS-R1aの内因性リガンドは小島博士らによりラットやヒトの胃から精製され、GH分泌を促進する機能を持つことからグレリン「ghrelin (ghre (growth) + release)」と名付けられた。グレリンはGH・乳腺刺激ホルモン・副腎皮質刺激ホルモン分泌を促進し、さらに食欲や糖代謝に関与し、胃運動と胃酸分泌を制御すると報告されている。グレリンは反芻動物においても成長や乳生産に関与する主な制御ホルモンと考えられる。

反芻動物のグレリンは27個のアミノ酸からなり、3番目のSerアミノ酸残基がオクタン酸によりアシル化される（Dickin *et al.* 2004, Kita *et al.* 2005）。そのアシル化はグレリンのGHS-R1a活性化に重要であることが単胃動物で示されている（Kojima *et al.* 1999, Hosoda *et al.* 2000*a*）。グレリンは胃から主に分泌され（Date *et al.* 2000, Hayashida *et al.* 2001）血中ではアシル化されているグレリン（アシルグレリン）とアシル化されていないグレリン（デスグレリン）として異なる分子構造で循環し、デスグレリンが主な分子種である（Hosoda *et al.* 2000*a*）。反芻動物において血漿アシルグレリンは決められた給餌時間の給餌前に上昇し、給餌後に低下する（Sugino et al. 2002*a*）。そのようなグレリン濃度の変化は給餌回数に依存する（Sugino et al. 2002*b*）。これらの結果からグレリンは反芻動物の摂食調整メカニズムに関与していると考えられる。一方、反芻動物でのデスアシルグレリンについての情報はない。単胃動物と反芻動物のグレリンのアミノ酸配列は異なり、市販試薬として利用できるヒト・ラット用ラジオイムノアッセイキット（RIA Kit）で反芻動物の血漿デスグレリン濃

度を測定することは不可能である。また、ウシのような大動物においてin vivoの実験を行うためには高価なペプチドを大量に必要とするため市販品を利用することは実際上不可能である。

我々はホルスタイン牛における成長ホルモン（GH）分泌メカニズムに対するグレリンの役割を明らかにすることを目的として6つの実験を行い、反芻動物での生理・栄養による血漿グレリン濃度の変化を検討した。

実験1－固相ペプチド合成とラジオイムノアッセイ

反芻動物の*In vivo*研究で利用できるグレリンおよび反芻動物の食欲制御ホルモン濃度が測定できるラジオイムノアッセイ系（RIA）の市販品を入手することは不可能であることから我々はペプチドを合成して免疫作成を行い、RIA法を構築することを試みた。グレリンについてはウシの胃のmRNAを抽出し、cDNA作成を行った。得られたcDNAライブラリーからウシグレリン遺伝子を得たが、N末端側のSer^3残基にオクタン酸をアシル化導入することが困難なため遺伝子技術でウシアシルグレリンを合成することはできなかった。そこで固相合成法でペプチドを合成し、高速液体クロマトグラフィーで精製を行った。固相法で合成したペプチドはウシアシルグレリン[1-27]、[Cys12]-アシルグレリン[1-11]、[Cys-0]-ウシグレリン [11-27]、ウシPYY-NH_2 [1-36], ウシPYY-NH_2 [3-36], ウシ[Cys0]-PYY-NH_2 [4-36]とヒトGHRH-NH_2 [1-29]である。作成した免疫は抗アシルグレリンウサギ血清（希釈率1:240000）、抗ウシグレリンモルモット血清（1:15000）、抗ウシPYYモルモット血清（1:50000）と抗ウシレプチンモルモット血清（1:200000）である。これらの免疫血清を用い、アシルグレリンRIA、ウシグレリンRIA、ウシPYY RIAとウシレプチンRIA法を確立した。これらのRIA法は山羊や羊など反芻動物の各血漿ホルモン濃度も測定できる。従って、反芻動物の消化管ホルモン間の調節制御に対する新たな知見を得て、反芻動物の栄養生理学をより深く理解することが可能となった。

実験2－ホルスタイン雌仔牛における血漿GH濃度に対するグレリンの用量依存的影響

反芻動物でのグレリンによるGH分泌促進はいくつか報告されているがどの研究でも単胃動物由来グレリンの薬理学的投与量が用いられている。本研究ではホルスタイン雌仔牛におけるグレリンによるGH分泌反応を生理的投与量から薬理学的投与量まで用量依存的に検討した。6ヶ月齢の雌牛6頭を用い合成ウシアシルグレリン[1-27]を0.1から10.0 μg/kg BWで頸静脈内カテーテルよりそれぞれ注入し、継続的に採血を行い、血漿ホルモン濃度を測定した。血漿GH濃度はグレリン投与後用量依存的に上昇した。また、薬理学的投与量のグレリン投与により血漿インスリンやNEFA濃度は上昇したが血漿レプチン濃度は変化しなかった。血漿アシルグレリン・全グレリン（アシル・デスアシル・C末端グレリン断片を含む）はそれぞれグレリン投与後一時的に上昇した。本研究では反芻動物の血漿全グレリン濃度を世界で初めて報告できた。またグレリンの生理的投与量によるGH分泌促進効果を反芻動物で初めて報告した（ThidarMyint *et al.* 2006）。

実験3－ホルスタイン去勢牛におけるグレリン投与によるホルモン濃度の変化に対するデスグレリン前処理の影響

反芻動物においてグレリンのN末端側のSer^3残基におけるオクタン酸によるアシル化がグレリンレセプター（GHS-R1a）を活性化するために必要であるかどうかを検討した。ホルスタイン雄牛に頸静脈内カテーテルを装着し、前処理としてウシデスグレリン[1-27]を10.0 μg/kg BW条件で投与し、5分後にウシアシルグレリン[1-27]を1.0 μg/kg BW条件で投与した。アシルグレリン投与により血漿アシルグレリンと全グレリンは共に上昇したが、10分以内に基礎レベルまで急速に低下した。その際、デスグレリン投与で上昇した血漿全グレリン濃度の低下の速度は実験２のそれらの結果と比較して遅かった。アシルグレリン投

与後血漿GH、インスリン、グルコースおよびNEFA濃度は上昇したが血漿PYY濃度に変化はなかった。アシルグレリン投与によるこれらのホルモンや代謝産物濃度の変化にデスグレリン前処理の影響はみられなかった。また、子牛においてウシアシルグレリンとC末端側のアミノ酸配列が異なるブタアシルグレリンによる血漿GH濃度の上昇に違いはなかった。従って、グレリンの内分泌的機能にSer^3残基のオクタン酸によるアシル化が重要であることが反芻動物で確認できた。

実験4－子牛におけるグレリンとGHRHの併用投与によるGH分泌に対する離乳の影響

離乳によって反芻動物の内分泌経路が変化することは以前から知られている。一方、グレリンとGHRHの相乗作用は単胃動物で報告されているが反芻動物では不明である。そこで子牛の離乳前後におけるグレリン(1.0 μg/kg BW)とGHRH (0.25 μg/kg BW)の単独あるいは併用投与によるGH分泌反応の変化を検討することを試みた。ホルスタイン雄子牛を6週齢に離乳し、5週齢（哺乳期）と10週齢（離乳期）で実験を行った。血漿アシルグレリン・全グレリン・GHとIGF-1の基礎濃度は離乳前後で同程度だった。一方、血漿インスリン・グルコース・NEFA濃度は離乳後低下した。グレリンとGHRHの単独投与は哺乳期と離乳後共に血漿GH濃度を有意に増加させた。グレリンとGHRHのGH分泌に対する相乗作用は哺乳期のみ認められたが、併用投与によるGH濃度の上昇は離乳前後で同程度だった。離乳前後共にグレリンとGHRHの単独あるいは併用投与は血漿IGF-1、インスリン、グルコース、NEFA濃度に影響しなかった。従って、前下垂体細胞のGH分泌機能は離乳前後で変化はなく、グレリンとGHRHに応答する細胞の割合が変化する可能性が考えられる（ThidarMyint *et al.* 2008）。

実験5－ホルスタイン去勢牛におけるグレリン反復投与による血漿ホルモンや代謝産物濃度の変化

本研究では反芻動物におけるグレリンの反復投与によるGH分泌を検討した。去勢牛5頭を用い、合成ウシグレリン(1.0 μg/kg BW)を頸静脈内カテーテルより2時間間隔で4回反復投与し、継続的採血を行い、血漿ホルモン濃度を測定した。グレリン投与後血漿アシルグレリン・全グレリン濃度の上昇と急速な低下がみられた。血漿アシルグレリンのピークはグレリン反復投与によってより上昇したが投与後120分間のAUC値の間に差はなかった。グレリンの反復投与により血漿GH濃度の上昇が確認できた。2回目と3回目グレリン投与後のGH AUC値は1回目後の値と比べて低下したが4回目グレリン投与後GH AUC値は2回目投与後のGH AUC値と同程度まで回復した。血漿インスリンとNEFA濃度はグレリンの反復投与後上昇したが血漿グルコース濃度は変化しなかった。反復投与により下垂体でのグレリン受容体（GHS-R1a）の発現低下が起き、GHS-R1aのスローリサイクリングがGH反応反応の低下に繋がったと考えられる。グレリンによる糖代謝と脂肪代謝への影響は部分的にGHS-R1a活性化経路と異なるかもしれない。なぜなら本研究でのグレリンによる血漿インスリンとNEFA濃度の上昇は血漿GH濃度の上昇と比較してやや遅く、グレリンはそれらのメカニズムに直接影響を与えるよりGH経路を通して関与していることが示唆される。

実験6－反芻動物における代謝・栄養による血漿グレリン濃度の変化

代謝や栄養状態による血漿アシルグレリン濃度の変化についていくつかの報告があるが血漿デスグレリンについての情報はない。本実験では血漿デスグレリン濃度の変化を検討し血漿 PYY 濃度や代謝産物濃度との関係を調べた。プロトコル１：羊 11 頭（道立試験場、放牧下）または帯広畜産大学の羊 6 頭・去勢牛 5 頭と去勢山羊 6 頭（1 日 2 回 0800 と 1600 時に給餌）を 48 時間絶食させ、絶食前後に頸静脈より単独採血を行った。プロトコル２：山羊 5 頭を乾草のみで自由給餌下（R-diet）または配合飼料 500g＋乾草 1kg/head/day を 1

日2回給餌下（C-diet）でおき、それぞれ48時間絶食させた。絶食前（1300h）・中（24時間後）・後（48時間後）と再給餌後24時間で採血を行い、血漿ホルモンや代謝産物濃度を測定した。すべての実験動物で絶食による血漿アシルグレリン・全グレリン・NEFA 濃度の上昇が認められたが血漿GHやPYY濃度の変化はなかった。血漿グルコースやインスリン濃度は絶食によって低下した。アシルグレリン濃度は絶食後全グレリン濃度と同程度まで上昇した。再給餌によりそれらホルモンや代謝産物濃度は絶食前の値に戻った。全グレリンの基礎濃度はR-dietの方がC-dietより高かったが栄養状態によるグレリン濃度の変化パターンは両dietの間に差はなかった。従って、血漿アシルグレリン・全グレリン共に絶食時に上昇し、再給餌によって低下すること、また循環しているグレリンは絶食時にアシル化されていたことからグレリンは分子構造の相違によって反芻動物のエネルギーバランスを調節する可能性が示唆される。

まとめ

1. 反芻動物においてグレリンはGH分泌を強力に促進し、3番目のアミノ酸残基（Ser）でのオクタン酸によるアシル化はグレリンの内分泌機能のため必要である。
2. グレリンとGHRHのGH分泌に対する相乗作用は哺乳期の子牛でみられた。また反芻動物の生理状態によってグレリンのGH分泌機能が変化する。
3. 栄養状態は血漿グレリン濃度だけではなくグレリンの構造（アシルあるいはデスアシル）に影響する。
4. 血漿グレリンの分解は急速であり、グレリンの分子構造は分解速度を制御している可能性がある。

従って、グレリンは反芻動物の生理状態によってGH分泌を促進し、糖代謝や脂肪代謝に影響を与えて成長メカニズムに対する主な役割を持っていると示唆される。また、栄養状態によってグレリンが変化することからグレリンは反芻動物の食欲調整メカニズムに関わっていることが考えられる。それらの役割を果たすにはグレリンの分子構造が重要かもしれない。

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References

Adrian TE, Bacarese-Hamilton AJ, Smith HA, Chohan P, Manolas KJ & Bloom SR. 1987. Distribution and postprandial release of porcine peptide YY. *Journal of Endocrinology*. 113:11-14.

Anderson LL, Jeftinija S, Scanes CG, Stromer MH, Lee JS, Jeftinija K & Glavaski-Joksimovic A. 2005. Physiology of ghrelin and related peptides. *Domestic Animal Endocrinology* 29:111-144

Anderson LL. 1977. Development in calves and heifers after hypophysial stalk transection or hypophysectomy. *American Journal of Physiology* 232:E497-503

Ariyasu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, Suda M, Koh T, Natsui K, Toyooka S, Shirakami G, Usui T, Shimatsu A, Doi K, Hosoda H, Kojima M, Kangawa K & Nakao K. 2001. Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. *Journal of Clinical Endocrinology and Metabolism* 86:4753-4758.

Arvat E, Maccario M, Di Vito L, Broglio F, Benso A, Gottero C, Papotti M, uccioli G, Dieguez C, Casanueva FF, Deghenghi R, Camanni F & Ghigo E. 2001. Endocrine activities of ghrelin, a natural growth hormone secretagogue (GHS), in humans: comparison and interactions with hexarelin, a nonnatural peptidyl GHS, and GH-releasing hormone. *Journal of Clinical Endocrinology and Metabolism* 86:1169-1174.

Asakawa A, Inui A, Fujimiya M, Sakamaki R, Shinfuku N, Ueta Y, Meguid MM & Kasuga M. 2005. Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. *Gut* 54:18-24

Asakawa A, Inui A, Kaga T, Katsuura G, Fujimiya M, Fujino MA & Kasuga M. 2003. Antagonism of ghrelin receptor reduces food intake and body weight gain in mice.

Gut 52:947-952.

Asakawa A, Inui A, Kaga T, Yuzuriha H, Nagata T, Ueno N, Makino S, Fujimiya M, Niijima A, Fujino MA & Kasuga M. 2001. Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. *Gastroenterology* 120:337-345.

Badger TM, Millard WJ, McCormick GF, Bowers CY & Martin JB. 1984. The effects of growth hormone (GH)-releasing peptides on GH secretion in perifused pituitary cells of adult male rats. *Endocrinology* 115:1432-1438.

Banks WA, Tschop M, Robinson SM & Heiman ML. 2002. Extent and direction of ghrelin transport across the blood-brain barrier is determined by its unique primary structure. *Journal of Pharmacology and Experimental Therapeutics* 302:822-827.

Barinaga M, Yamonoto G, Rivier C, Vale W, Evans R & Rosenfeld MG. 1983. Transcriptional regulation of growth hormone gene expression by growth hormone-releasing factor. *Nature* 306:84-85.

Barkan AL, Dimaraki EV, Jessup SK, Symons KV, Ermolenko M, Jaffe CA. 2003. Ghrelin secretion in humans is sexually dimorphic, suppressed by somatostatin, and not affected by the ambient growth hormone levels. *Journal of Clinical Endocrinology and Metabolism*. 88:2180-2184.

Batterham RL, Cowley MA, Small CJ, Herzog H, Cohen MA, Dakin CL, Wren AM, Brynes AE, Low MJ, Ghatei MA, Cone RD & Bloom SR. 2002. Gut hormone PYY(3-36) physiologically inhibits food intake. *Nature* 418:650-654.

Bednarek MA, Feighner SD, Pong SS, McKee KK, Hreniuk DL, Silva MV, Warren VA, Howard AD, Van Der Ploeg LH & Heck JV. 2000. Structure-function studies on the new growth hormone-releasing peptide, ghrelin: minimal sequence of ghrelin necessary for activation of growth hormone secretagogue receptor 1a. *Journal of Medicinal Chemistry* 43:4370-4376.

Bloch B, Brazeau P, Ling N, Bohlen P, Esch F, Wehrenberg WB, Benoit R, Bloom F &

Guillemin R. 1983. Immunohistochemical detection of growth hormone-releasing factor in brain. *Nature* 301:607-608.

Bohlen P, Esch F, Brazeau P, Ling N & Guillemin R. 1983. Isolation and characterization of the porcine hypothalamic growth hormone releasing factor. *Biochemical and Biophysical Research Communications* 116:726-734.

Bowers CY, Chang J, Momany F & Folkers K. 1977. Effect of the enkephalins and enkephalin analogs on the release of pituitary hormones *in vitro*. In: MacIntyre I and Szelke M, eds. *Proceedings of 6th International Conference on Endocrinology, Molecular Endocrinology. Amsterdam:* Elsevier/North-Holland Biomedical Press 287-292.

Bowers CY, Momany F, Reynolds GA, Chang D, Hong A & Chang K. 1980. Structure-activity relationships of a synthetic pentapeptide that specifically releases growth hormone *in vitro*. *Endocrinology* 106:663-667.

Bowers CY, Momany FA, Reynolds GA & Hong A. 1984. On the *in vitro* and *in vivo* activity of a new synthetic hexapeptide that acts on the pituitary to specifically release growth hormone. *Endocrinology* 114:1537-1545.

Bowers CY. 1998. Growth hormone-releasing peptide (GHRP). *Cellular and Molecular Life Sciences* 54:1316-1329.

Bradford BJ, Allen MS. 2008. Negative energy balance increases periprandial ghrelin and growth hormone concentrations in lactating dairy cows. *Domestic Animal Endocrinology* 34:196-203.

Brazeau P, Bohlen P, Esch F, Ling N, Wehrenberg WB & Guillemin R. 1984. Growth hormone-releasing factor from ovine and caprine hypothalamus: isolation, sequence analysis and total synthesis. *Biochemical and Biophysical Research Communications* 125:606-614.

Brazeau P, Vale W, Burgus R, Ling N, Butcher M, Rivier J & Guillemin R. 1973.

Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. *Science* 179:77-79.

Broglio F, Arvat E, Benso A, Gottero C, Muccioli G, Papotti M, van der Lely AJ, Dehenghi R & Ghigo E. 2001. Ghrelin, a natural GH secretagogue produced by the stomach, induces hyperglycemia and reduces insulin secretion in humans. *Journal of Clinical Endocrinology and Metabolism* 86:5083-5086.

Broglio F, Gottero C, Van Koetsveld P, Prodam F, Destefanis S, Benso A, Gauna C, Hofland L, Arvat E, van der Lely AJ & Ghigo E. 2004 Acetylcholine regulates ghrelin secretion in humans. *Journal of Clinical Endocrinology and Metabolism* 89:2429-2433.

Camina JP, Carreira MC, El Messari S, Llorens-Cortes C, Smith RG & Casanueva FF. 2004. Desensitization and endocytosis mechanisms of ghrelin-activated growth hormone secretagogue receptor 1a. *Endocrinology* 145:930-40.

Campbell RG, Steele NC, Caperna TJ, McMurtry JP, Solomon MB, Mitchell AD. 1988. Interrelationships between energy intake and endogenous porcine growth hormone administration on the performance, body composition and protein and energy metabolism of growing pigs weighing 25 to 55 kilograms live weight. *Journal of Animal Science* 66:1643-1655.

Cataldi M, Magnan E, Guillaume V, Dutour A, Conte-Devolx B, Lombardi G & Oliver C. 1994. Relationship between hypophyseal portal GHRH and somatostatin and peripheral GH levels in the conscious sheep. *Journal of Endocrinological Investigation* 17:717-722.

Chartrel N, Alvear-Perez R, Leprince J, Iturrioz X, Reaux-Le Goazigo A, Audinot V, Chomarat P, Coge F, Nosjean O, Rodriguez M, Galizzi JP, Boutin JA, Vaudry H & Llorens-Cortes C. 2007. Comment on "Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake". *Science* 315:766c.

Chelikani PK, Haver AC & Reidelberger RD. 2006. Ghrelin attenuates the inhibitory effects of glucagon-like peptide-1 and peptide YY(3-36) on food intake and gastric emptying in rats. *Diabetes* 55:3038-3046.

Chen CY, Chao Y, Chang FY, Chien EJ, Lee SD & Doong ML. 2005. Intracisternal des-acyl ghrelin inhibits food intake and non-nutrient gastric emptying in conscious rats. *International Journal of Molecular Medicine* 16:695-699.

Chilliard Y, Ferlay A, Faulconnier Y, Bonnet M, Rouel J & Bocquier F. 2000. Adipose tissue metabolism and its role in adaptations to undernutrition in ruminants. *Proceeding of the Nutrition Society* 59:127-134.

Connor EE, Ashwell MS & Dahl GE. 2002. Characterization and expression of the bovine growth hormone-releasing hormone (GHRH) receptor. *Domestic Animal Endocrinology* 22:189-200.

Cortelazzi D, Cappiello V, Morpurgo PS, Ronzoni S, Nobile De Santis MS, Cetin I, Beck-Peccoz P & Spada A. 2003. Circulating levels of ghrelin in human fetuses. *European Journal of Endocrinology.* 149:111-116.

Cummings DE & Overduin J. 2004. Circulating ghrelin levels in pathophysiological conditions. 207-223. from *Ghrelin*. Edited by Ghigo E. Kluwer Academic Publishers.

Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE & Weigle DS. 2001. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes* 50:1714-1719.

Dass NB, Munonyara M, Bassil AK, Hervieu GJ, Osbourne S, Corcoran S, Morgan M & Sanger GJ. 2003. Growth hormone secretagogue receptors in rat and human gastrointestinal tract and the effects of ghrelin. *Neuroscience* 120:443-453.

Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K & Nakazato M. 2000. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal

tracts of rats and humans. *Endocrinology* 141:4255-4261.

Date Y, Murakami N, Toshinai K, Matsukura S, Niijima A, Matsuo H, Kangawa K & Nakazato M. 2002a. The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. *Gastroenterology* 123:1120-1128.

Date Y, Nakazato M, Hashiguchi S, Dezaki K, Mondal MS, Hosoda H, Kojima M, Kangawa K, Arima T, Matsuo H, Yada T & Matsukura S. 2002b. Ghrelin is present in pancreatic alpha-cells of humans and rats and stimulates insulin secretion. *Diabetes* 51:124-129.

Date Y, Nakazato M, Murakami N, Kojima M, Kangawa K & Matsukura S. 2001. Ghrelin acts in the central nervous system to stimulate gastric acid secretion. *Biochemical and Biophysical Research Communications* 280:904-907.

Daughaday WH, Mariz IK & Blethen SL. 1980. Inhibition of access of bound somatomedin to membrane receptor and immunobinding sites: a comparison of radioreceptor and radioimmunoassay of somatomedin in native and acid-ethanol-extracted serum. *Journal of Clinical Endocrinology and Metabolism* 51:781-788.

De la Cour CD, Norlen P & Hakanson R. 2007. Secretion of ghrelin from rat stomach ghrelin cells in response to local microinfusion of candidate messenger compounds: a microdialysis study. *Regulatory Peptides* 143:118-26.

Di Vito L, Broglio F, Benso A, Gottero C, Prodam F, Papotti M, Muccioli G, Dieguez C, Casanueva FF, Deghenghi R, Ghigo E & Arvat E. 2002. The GH-releasing effect of ghrelin, a natural GH secretagogue, is only blunted by the infusion of exogenous somatostatin in humans. *Clinical Endocrinology (Oxford)*. 56:643-648.

Dickin JC, Thue TD & Buchanan FC. 2004. An alternative splice site in ghrelin is missing in ruminants. *Animal Genetics* 35:411-412.

Dornonville de la Cour C, Bjorkqvist M, Sandvik AK, Bakke I, Zhao CM, Chen D &

Hakanson R. 2001 A-like cells in the rat stomach contain ghrelin and do not operate under gastrin control. *Regulatory Peptides* 99:141–150.

Drisko JE, Faidley TD, Chang CH, Zhang D, Nicolich S, Hora DF Jr, McNamara L, Rickes E, Abribat T, Smith RG & Hickey GJ. 1998. Hypophyseal-portal concentrations of growth hormone-releasing factor and somatostatin in conscious pigs: relationship to production of spontaneous growth hormone pulses. *Proceeding of the Society for Experimental Biology and Medicine* 217:188-196.

Erdmann J, Lippl F & Schusdziarra V. 2003. Differential effect of protein and fat on plasma ghrelin levels in man. *Regulatory Peptides.* 116:101-107.

Esch F, Bohlen P, Ling N, Brazeau P & Guillemin R. 1983. Isolation and characterization of the bovine hypothalamic growth hormone releasing factor. *Biochemical and Biophysical Research Communications* 117:772-779.

Etherton TD & Bauman DE. 1998. Biology of somatotropin in growth and lactation of domestic animals. *Physiological Reviews* 78:745-761.

Evans HM & Simpson ME. 1931. Hormones of the anterior hypophysis. *American Journal of Physiology* 98:511-546.

Frandson RD, Wilke WL & Fails AD. 2003. Nutrition and Metabolism 343-349 in Anatomy and Physiology of Farm Animals (sixth edition). Lippincott Williams & Wilkins.

Frohman LA & Jansson JO. 1986. Growth hormone-releasing hormone. *Endocrine Reviews* 7:223-253.

Frohman LA, Downs TR, Clarke IJ & Thomas GB. 1990. Measurement of growth hormone-releasing hormone and somatostatin in hypothalamic-portal plasma of unanesthetized sheep. Spontaneous secretion and response to insulin-induced hypoglycemia. *Journal of Clinical Investications* 86:17-24.

Gauna C, Delhanty PJ, Hofland LJ, Janssen JA, Broglio F, Ross RJ, Ghigo E & van der

Lely AJ. 2005. Ghrelin stimulates, whereas des-octanoyl ghrelin inhibits, glucose output by primary hepatocytes. *Journal of Clinical Endocrinology and Metabolism* 90:1055-1060.

Gauna C, Meyler FM, Janssen JA, Delhanty PJ, Abribat T, van Koetsveld P, Hofland LJ, Broglio F, Ghigo E & van der Lely AJ. 2004. Administration of acylated ghrelin reduces insulin sensitivity, whereas the combination of acylated plus unacylated ghrelin strongly improves insulin sensitivity. *Journal of Clinical Endocrinology and Metabolism* 89:5035-5042.

Gourcerol G, St-Pierre DH & Taché Y. 2007. Lack of obestatin effects on food intake: should obestatin be renamed ghrelin-associated peptide (GAP)? *Regulatory Peptides* 141:1-7.

Govoni N, De Iasio R, Cocco C, Parmeggiani A, Galeati G, Pagotto U, Brancia C, Spinaci M, Tamanini C, Pasquali R, Ferri GL & Seren E. 2005. Gastric immunolocalization and plasma profiles of acyl-ghrelin in fasted and fasted-refed prepuberal gilts. *Journal of Endocrinology* 186:505-513.

Grandt D, Schimiczek M, Beglinger C, Layer P, Goebell H, Eysselein VE & Reeve JR Jr. 1994. Two molecular forms of peptide YY (PYY) are abundant in human blood: characterization of a radioimmunoassay recognizing PYY 1-36 and PYY 3-36. *Regulatory Peptides* 51:151-159.

Guillemin R, Brazeau P, Böhlen P, Esch F, Ling N & Wehrenberg WB. 1982. Growth hormone-releasing factor from a human pancreatic tumor that caused acromegaly *Science* 218:585-587.

Harrison JL, Adam CL, Brown YA, Wallace JM, Aitken RP, Lea RG & Miller DW. 2007. An immunohistochemical study of the localization and developmental expression of ghrelin and its functional receptor in the ovine placenta. *Reproductive Biology and Endocrinology* 5:25.

Hashizume T, Horiuchi M, Nonaka S, Kasuya E, Kojima M, Hosoda H & Kangawa K. 2005. Effects of ghrelin on growth hormone secretion in vivo in ruminants. *Regulatory Peptides* 126:61-65.

Hashizume T, Horiuchi M, Tate N, Nonaka S, Kojima M, Hosoda H & Kangawa K. 2003. Effects of ghrelin on growth hormone secretion from cultured adenohypophysial cells in cattle. *Endocrine Journal* 50:289-295.

Hataya Y, Akamizu T, Takaya k, Kanamoto N, Ariyasu H, Saijo M, Moriyama K, Shimatsu A, Kojima M, Kangawa K & Nakao K. 2001. A low dose of ghrelin stimulates growth hormone (GH) release synergistically with GH-releasing hormone in humans. *Journal of Clinical Endocrinology and Metabolism* 86:4552-4555.

Hayashida T, Murakami K, Mogi K, Nishihara M, Nakazato M, Mondal MS, Horii Y, Kojima M, Kangawa K & Murakami N. 2001. Ghrelin in domestic animals: distribution in stomach and its possible role. *Domestic Animal Endocrinology* 21:17-24.

Hayashida T, Nakahara K, Mondal MS, Date Y, Nakazato M, Kojima M, Kangawa K & Murakami N. 2002. Ghrelin in neonatal rats: distribution in stomach and its possible role. *Journal of Endocrinology* 173:239-245.

Heijboer AC, van den Hoek AM, Parlevliet ET, Havekes LM, Romijn JA, Pijl H & Corssmit EP. 2006. Ghrelin differentially affects hepatic and peripheral insulin sensitivity in mice. *Diabetologia.* 49:732-738.

Hodate K, Johke T & Ohashi S. 1986. Response of growth hormone release to human growth hormone-releasing factor and its analogs in the bovine. *Endocrinologia Japonica* 33:519-525.

Hosoda H, Kojima M, Matsuo H & Kangawa K. 2000*a*. Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. *Biochemical and Biophysic Research Communication* 279:909-913.

Hosoda H, Kojima M, Matsuo H & Kangawa K. 2000*b*. Purification and characterization of rat des-Gln14-Ghrelin, a second endogenous ligand for the growth hormone secretagogue receptor. *The Journal of Biological Chemistry* 275:21995-22000.

Hosoda H, Kojima M, Mizushima T, Shimizu S & Kangawa K. 2003. Structural divergence of human ghrelin. Identification of multiple ghrelin-derived molecules produced by post-translational processing. *The Journal of Biological Chemistry* 278:64-70.

Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, Hamelin M, Hreniuk DL, Palyha OC, Anderson J, Paress PS, Diaz C, Chou M, Liu KK, McKee KK, Pong SS, Chaung LY, Elbrecht A, Dashkevicz M, Heavens R, Rigby M, Sirinathsinghji DJ, Dean DC, Melillo DG, Patchett AA, Nargund R, Griffin PR, DeMartino JA, Gupta SK, Schaeffer JM, Smith RG & Van der Ploeg LH. 1996. A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* 273:974-977.

Hughes J, Smith TW, Kosterlitz HW, Fothergill LA, Morgan BA & Morris HR. 1975. Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature* 258:577-580.

Inoue H, Watanuki M, Hnin ThidarMyint, Ito T, Kuwayama H & Hidari H. 2005. Effects of fasting and refeeding on plasma concentrations of leptin, ghrelin, insulin, growth hormone and metabolites in swine. *Animal Science Journal* 76:367-374.

Iqbal J, Kurose Y, Canny B & Clarke IJ. 2006. Effects of central infusion of ghrelin on food intake and plasma levels of growth hormone, luteinizing hormone, prolactin, and cortisol secretion in sheep. *Endocrinology* 147:510-519.

Ito T, Thidarmyint H, Murata T, Inoue H, Neyra RM & Kuwayama H. 2006. Effects of peripheral administration of PYY3-36 on feed intake and plasma acyl-ghrelin levels

in pigs. *Journal of Endocrinology* 191:113-119. Erratum in: *Journal of Endocrinology* 2006;191(2):513.

Itoh F, Komatsu T, Kushibiki S & Hodate K. 2006. Effects of ghrelin injection on plasma concentrations of glucose, pancreatic hormones and cortisol in Holstein dairy cattle. *Comparative Biochemistry and Physiology. Part A Molecular & Integrative Physiology* 143:97-102.

Itoh F, Komatsu T, Yonai M, Sugino T, Kojima M, Kangawa K, Hasegawa Y, Terashima Y & Hodate K. 2005. GH secretory responses to ghrelin and GHRH in growing and lactating dairy cattle. *Domestic Animal Endocrinology* 28:34-35.

Kaiser E, Colescott R. L, Bossinger C. D & Cook P. I. 1970. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Analytical Biochemistry* 34:595-598.

Kaiya H, Kojima M, Hosoda H, Koda A, Yamamoto K, Kitajima Y, Matsumoto M, Minamitake Y, Kikuyama S & Kangawa K. 2001. Bullfrog ghrelin is modified by n-octanoic acid at its third threonine residue. *Journal of Biological Chemistry.* 276:40441-40448

Kaiya H, Kojima M, Hosoda H, Riley LG, Hirano T, Grau EG & Kangawa K. 2003. Amidated fish ghrelin: purification, cDNA cloning in the Japanese eel and its biological activity. *Journal of Endocrinology.* 176:415-423.

Kaiya H, Van Der Geyten S, Kojima M, Hosoda H, Kitajima Y, Matsumoto M, Geelissen S, Darras VM & Kangawa K. 2002. Chicken ghrelin: purification, cDNA cloning, and biological activity. *Endocrinology.* 143:3454-3463.

Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H & Oikawa S. 2001. Regulation of the ghrelin gene: growth hormone-releasing hormone upregulates ghrelin mRNA in the pituitary. *Endocrinology* 142:4154-4157.

Kato Y, Shimokawa N, Kato T, Hirai T, Yoshihama K, Kawai H, Hattori M, Ezashi T,

Shimogori Y & Wakabayashi K. 1990. Porcine growth hormone: molecular cloning of cDNA and expression in bacterial and mammalian cells. *Biochimica et Biophysica Acta.* 1048:290-293.

Katoh K, Furukawa G, Kitade K, Katsumata N, Kobayashi Y & Obara Y. 2004. Postprandial changes in plasma GH and insulin concentrations, and responses to stimulation with GH-releasing hormone (GHRH) and GHRP-6 in calves around weaning. *Journal of Endocrinology* 183:497-505.

Kita K, Harada K, Nagao K & Yokota H. 2005. Characteristics of gene structure of bovine ghrelin and influence of aging on plasma ghrelin. *Asian-Australian Journal of Animal Science* 18:723-727.

Klindt J, Ford JJ, Berardinelli JG & Anderson LL. 1983. Growth hormone secretion after hypophysial stalk transection in pigs *Proceeding of the Society of Experimental Biology and Medicine* 172:508-513.

Kobayashi Y, Onodera M, Yoshida M, Sugino T, Katoh K & Obara Y. 2006. Postprandial changes in plasma concentrations of growth hormone and ghrelin around weaning in the goat. *General and Comparative Endocrinology* 148:368-374.

Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H & Kangawa K. 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 402:656-660

Korbonits M, Bustin SA, Kojima M, Jordan S, Adams EF, Lowe DG, Kangawa K & Grossman AB. 2001. The expression of the growth hormone secretagogue receptor ligand ghrelin in normal and abnormal human pituitary and other neuroendocrine tumors. *Journal of Clinical Endocrinology and Metabolism* 86:881-887.

Korbonits M, Goldstone AP, Gueorguiev M & Grossman AB. 2004. Ghrelin-a hormone with multiple functions. *Frontiers in Neuroendocrinology* 25:27-68.

Lee HG, Vega RA, Phung LT, Matsunaga N, Kuwayama H & Hidari H. 2000. The effect of growth hormone-releasing peptide-2 (KP102) administration on plasma

insulin-like growth factor (IGF)-1 and IGF-binding proteins in Holstein steers on different planes of nutrition. *Domestic Animal Endocrinology* 18:293-308.

Lee HM, Wang G, Englander EW, Kojima M & Greeley GH Jr. 2002. Ghrelin, a new gastrointestinal endocrine peptide that stimulates insulin secretion: enteric distribution, ontogeny, influence of endocrine, and dietary manipulations. *Endocrinology* 143:185-190.

Lindholm J. 2006. Growth hormone: Historical notes. *Pituitary* 9:5-10

Ling N, Baird A, Wehrenberg WB, Ueno N, Munegumi T & Brazeau P. 1984. Synthesis and in vitro bioactivity of C-terminal deleted analogs of human growth hormone-releasing factor. *Biochemical and Biophysical Research Communications* 123:854-861.

Lucidi P, Murdolo G, Di Loreto C, Parlanti N, De Cicco A, Fantone C, Taglioni C, Fanelli C, Broglio F, Ghigo E, Bolli GB, Santeusanio F & De Feo P. 2005. Metabolic and endocrine effects of physiological increments in plasma ghrelin concentrations. *Nutrition, Metabolism and Cardiovascular Diseases* 15:410-417.

Luque RM, Kineman RD, Park S, Peng XD, Gracia-Navarro F, Castano JP & Malagon MM. 2004. Homologous and heterologous regulation of pituitary receptors for ghrelin and growth hormone-releasing hormone. *Endocrinology* 145:3182-3189.

Malagón MM, Luque RM, Ruiz-Guerrero E, Rodríguez-Pacheco F, García-Navarro S, Casanueva FF, Gracia-Navarro F & Castaño JP. 2003. Intracellular signaling mechanisms mediating ghrelin-stimulated growth hormone release in Somatotropes. *Endocrinology* 144:5372-5380.

Martial JA, Hallewell RA, Baxter JD & Goodman HM. 1979. Human growth hormone: complementary DNA cloning and expression in bacteria. *Science* 205:602-607.

Mayo KE. 1992. Molecular cloning and expression of a pituitary-specific receptor for

growth hormone-releasing hormone. *Molecular Endocrinology* 6:1734-1744.

McKee KK, Palyha OC, Feighner SD, Hreniuk DL, Tan CP, Phillips MS, Smith RG, Van der Ploeg LH & Howard AD. 1997. Molecular analysis of rat pituitary and hypothalamic growth hormone secretagogue receptors. *Molecular Endocrinology* 11:415-423.

McMahon CD, Chapin LT, Radcliff RP, Lookingland KJ & Tucker HA. 2001*a*. GH-releasing peptide-6 overcomes refractoriness of somatotropes to GHRH after feeding. *Journal of Endocrinology* 170:235-241.

McMahon CD, Radcliff RP, Lookingland KJ & Tucker HA. 2001*b*. Neuroregulation of growth hormone secretion in domestic animals. *Domestic Animal Endocrinology* 20:65-87.

Melendez P, Krueger T, White J, Badinga L, Verstegen J, Donovan GA & Archbald LF. 2006. Effect of ghrelin in dry matter intake and energy metabolism in prepartum sheep: a preliminary study. *Theriogenology* 66:1961-1968.

Micic D, Macut D, Sumarac-Dumanovic M, Kendereski A, Popovic V, Deghenghi R, Dieguez C & Casanueva FF. 2002. Ghrelin-induced GH secretion in normal subjects is partially resistant to homologous desensitization by GH-releasing peptide-6. *European Journal of Endocrinology* 147:761-766.

Miller DW, Harrison JL, Brown YA, Doyle U, Lindsay A, Adam CL & Lea RG. 2005. Immunohistochemical evidence for an endocrine/paracrine role for ghrelin in the reproductive tissues of sheep. *Reproductive and Biological Endocrinology* 3:60.

Miller WL, Martial JA & Baxter JD. 1980. Molecular cloning of DNA complementary to bovine growth hormone mRNA. *Journal of Biological Chemistry* 255:7521-7524.

Mitani M, Kaji H, Abe H & Chihara K. J. 1996. Growth hormone (GH)-releasing peptide and GH releasing hormone stimulate GH release from subpopulations of somatotrophs in rats. *Journal of Neuroendocrinology* 8:825-830.

Miura H, Tsuchiya N, Sasaki I, Kikuchi M, Kojima M, Kangawa K, Hasegawa Y & Ohnami Y. 2004. Changes in plasma ghrelin and growth hormone concentrations in mature Holstein cows and three-month-old calves. *Journal of Animal Science* 82:1329-1933.

Mogi K, Yonezawa T, Chen DS, Li JY, Suzuki M, Yamanouchi K, Sawasaki T & Nishihara M. 2004. Relationship between growth hormone (GH) pulses in the peripheral circulation and GH-releasing hormone and somatostatin profiles in the cerebrospinal fluid of goats. The *Journal of Veterinary Medical Science* 66:1071-1078.

Murakami N, Hayashida T, Kuroiwa T, Nakahara K, Ida T, Mondal MS, Nakazato M, Kojima M & Kangawa K. 2002. Role for central ghrelin in food intake and secretion profile of stomach ghrelin in rats. *Journal of Endocrinology* 174:283-288.

Nair RM, deVillier C, Barnes M, Antalis J & Wilbur DL. 1978. A bovine hypothalamic peptide possessing immunoreactive growth hormone-releasing activity. *Endocrinology* 103:112-120.

Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K & Matsukura S. 2001. A role for ghrelin in the central regulation of feeding. *Nature* 409 194-198.

Nishi Y, Hiejima H, Mifune H, Sato T, Kangawa K & Kojima M. 2005. Developmental changes in the pattern of ghrelin's acyl modification and the levels of acyl-modified ghrelins in murine stomach. *Endocrinology* 146:2709-2715.

Onaga T, Kubagawa S, Lee S, Mineo H, Kato S, Naruse S & Ozaki T. 1997. Role of peptide YY in regulation of duodenal motility during the interdigestive period in sheep. *Journal of Comparative Physiology* [B]. 167:352-360.

Onaga T, Yoshida M, Inoue H, Yokota H. 2000. Regional distribution and plasma concentration of peptide YY in sheep. *Peptides* 21:655-667.

Papotti M, Ghe C, Cassoni P, Catapano F, Deghenghi R, Ghigo E & Muccioli G. 2000

Growth hormone secretagogue binding sites in peripheral human tissues. *Journal of Clinical Endocrinology and Metabolism* 85:3803-3807.

Patchett AA, Nargund RP, Tata JR, Chen MH, Barakat KJ, Johnston DB, Cheng K, Chan WW, Butler B, Hickey G, et al. 1995. Design and biological activities of L-163,191 (MK-0677): a potent, orally active growth hormone secretagogue. *Proceeding of the National Academy of Sciences of the United States of America* 92:7001-7005.

Patel YC & O'Neil W. 1988. Peptides derived from cleavage of prosomatostatin at carboxyl- and amino-terminal segments. Characterization of tissue and secreted forms in the rat. *Journal of Biological Chemistry* 263:745-751.

Peel CJ, Bauman DE, Gorewit RC & Sniffen CJ. 1981. Effect of exogenous growth hormone on lactational performance in high yielding dairy cows. *Journal of Nutrition* 111:1662-1671.

Reimer MK, Pacini G & Ahren B. 2003. Dose-dependent inhibition by ghrelin of insulin secretion in the mouse. *Endocrinology* 144:916-921.

Rindi G, Necchi V, Savio A, Torsello A, Zoli M, Locatelli V, Raimondo F, Cocchi D & Solcia E. 2002. Characterisation of gastric ghrelin cells in man and other mammals: studies in adult and fetal tissues. *Histochemistry and Cell Biology* 117(6):511-519.

Rivier J, Spiess J, Thorner M & Vale W. 1982. Characterization of a growth hormone-releasing factor from a human pancreatic islet tumour. *Nature* 300:276-278.

Roh SG, Matsunaga N, Hidaka S & Hidari H. 1996. Characteristic of Growth Hormone Secretion Responsiveness to Growth Hormone-Releasing Peptide-2 (GHRP-2 or KP-102) in calves. *Endocrine Journal* 43:291-298.

Rule DC, Beitz DC, de Boer G, Lyle RR, Trenkle AH & Young JW. 1985. Changes in hormone and metabolite concentrations in plasma of steers during a prolonged fast. *Journal of Animal Science*. 61:868-875.

Saad MF, Bernaba B, Hwu CM, Jinagouda S, Fahmi S, Kogosov E & Boyadjian R.

2002. Insulin regulates plasma ghrelin concentration. *Journal of Clinical Endocrinology and Metabolism.* 87:3997-4000.

Sakata I, Nakamura K, Yamazaki M, Matsubara M, Hayashi Y, Kangawa K & Sakai T. 2002. Ghrelin-producing cells exist as two types of cells, closed- and opened-type cells, in the rat gastrointestinal tract. *Peptides* 23:531-536.

Salehi A, Dornonville de la Cour C, Hakanson R & Lundquist I. 2004. Effects of ghrelin on insulin and glucagon secretion: a study of isolated pancreatic islets and intact mice. *Regulatory Peptides* 118:143-150.

Salfen BE、Carroll JA, Keisler DH & Strauch TA. 2004. Effects of exogenous ghrelin on feed intake, weight gain, behavior, and endocrine responses in weanling pigs. *Journal of Animal Science* 82:1957-1966.

Sartin JL, Coleman ES & Steele B. 1996. Interaction of cyclic AMP- and calcium-dependent mechanisms in the regulation of growth hormone-releasing hormone-stimulated growth hormone release from ovine pituitary cells. *Domestic Animal Endocrinology* 13:229-238.

Sato T, Fukue Y, Teranishi H, Yoshida Y & Kojima M. 2005. Molecular forms of hypothalamic ghrelin and its regulation by fasting and 2-deoxy-d-glucose administration. *Endocrinology* 146:2510-2516.

Scanes CG, Glavaski-Joksimovic A, Johannsen SA, Jeftinija S & Anderson LL. 2007. Subpopulations of Somatotropes with Differing Intracellular Calcium Concentration Responses to Secretagogues. *Neuroendocrinology* 85:221-231

Seeburg PH, Shine J, Martial JA, Baxter JD & Goodman HM. 1977. Nucleotide sequence and amplification in bacteria of structural gene for rat growth hormone. *Nature* 270:486-494.

Seoane LM, Al-Massadi O, Barreiro F, Dieguez C & Casanueva FF. 2007a. Growth hormone and somatostatin directly inhibit gastric ghrelin secretion. An in vitro organ

culture system. *Journal of Endocrinological Investication.* 30:RC22-25

Seoane LM, Al-Massadi O, Caminos JE, Tovar SA, Dieguez C & Casanueva FF. 2007b Sensory stimuli directly acting at the central nervous system regulate gastric ghrelin secretion. An ex-vivo organ culture study. *Endocrinology* 148:3998-4006.

Seoane LM, Tovar S, Baldelli R, Arvat E, Ghigo E, Casanueva FF & Dieguez C. 2000. Ghrelin elicits a marked stimulatory effect on GH secretion in freely-moving rats. *European Journal of Endocrinology* 143:R7-9.

Smith RG, Cheng K, Schoen WR, Pong S, Hickey G, Jacks T, Butler B, Chan W, Chaung L, Judith F, Taylor J, Wyvratt MJ & Fisher MH. 1993. A nonpeptidyl growth hormone secretagogue. *Science* 260:1640-1643.

Spiess J, Rivier J & Vale W. 1983. Characterization of rat hypothalamic growth hormone-releasing factor *Nature* 303:532-535.

Sugino T, Hasegawa Y, Kikkawa Y, Yamaura J, Yamagishi M, Kurose Y, Kojima M, Kangawa K & Terashima Y. 2002a. A transient ghrelin surge occurs just before feeding in a scheduled meal-fed sheep. *Biochemistry and Biophysics Research Communications* 295:255-260.

Sugino T, Hasegawa Y, Kurose Y, Kojima M, Kangawa K & Terashima Y. 2004. Effects of ghrelin on food intake and neuroendocrine function in sheep. *Animal Reproduction Science* 82-83:183-194.

Sugino T, Yamaura J, Yamagishi M, Ogura A, Hayashi R, Kurose Y, Kojima M, Kangawa K, Hasegawa Y & Terashima Y. 2002b. A transient surge of ghrelin secretion before feeding is modified by different feeding regimens in sheep. *Biochemistry and Biophysics Research Communications* 298:785-788.

Suhr ST, Rahal JO & Mayo KE. 1989. Mouse growth-hormone-releasing hormone: precursor structure and expression in brain and placenta. *Molecular Endocrinology* 11:1693-1700.

Sun Y, Ahmed S & Smith RG. 2003. Deletion of ghrelin impairs neither growth nor appetite. *Molecular Cell Biology* 23:7973-7981.

Sánchez J, Oliver P, Palou A & Picó C. 2004. The inhibition of gastric ghrelin production by food intake in rats is dependent on the type of macronutrient. *Endocrinology*. 145:5049-5055.

Tai HH, Korsch B & Chey WY. 1975. Preparation of 125I-labeled secretin of high specific radioactivity. *Analytical Biochemistry* 69:34-42.

Takaya K, Ariyasu H, Kanamoto N, Iwakura H, Yoshimoto A, Harada M, Mori K, Komatsu Y, Usui T, Shimatsu A, Ogawa Y, Hosoda K, Akamizu T, Kojima M, Kangawa K & Nakao K. 2000. Ghrelin strongly stimulates growth hormone release in humans. *Journal of Clinical Endocrinology and Metabolism* 85:4908-4911.

Takeno R, Okimura Y, Iguchi G, Kishimoto M, Kudo T, Takahashi K, Takahashi Y, Kaji H, Ohno M, Ikuta H, Kuroda Y, Obara T, Hosoda H, Kangawa K & Chihara K. 2004. Intravenous administration of ghrelin stimulates growth hormone secretion in vagotomized patients as well as normal subjects. *European Journal of Endocrinology* 151:447-450.

Tannenbaum GS, Epelbaum J & Bowers CY. 2003. Interrelationship between the novel peptide ghrelin and somatostatin/growth hormone-releasing hormone in regulation of pulsatile growth hormone secretion. *Endocrinology* 144:967-974.

Tatemoto K & Mutt V. 1980. Isolation of two novel candidate hormones using a chemical method for finding naturally occurring polypeptides. *Nature*. 285:417-418.

ThidarMyint H, Yoshida H, Ito T & Kuwayama H. 2006. Dose-dependent response of plasma ghrelin and growth hormone concentrations to bovine ghrelin in Holstein heifers. *Journal of Endocrinology* 189:655-664.

Thidarmyint H, Yoshida H, Ito T, He M, Inoue H & Kuwayama H. 2008. Combined administration of ghrelin and GHRH synergistically stimulates GH release in

Holstein preweaning calves. *Domestic Animal Endocrinology* 34:118-123.

Thompson NM, Davies JS, Mode A, Houston PA & Wells T. 2003. Pattern-dependent suppression of growth hormone (GH) pulsatility by ghrelin and GH-releasing peptide-6 in moderately GH-deficient rats. *Endocrinology* 144:4859-4867.

Thompson NM, Gill DA, Davies R, Loveridge N, Houston PA, Robinson IC & Wells T. 2004. Ghrelin and des-octanoyl ghrelin promote adipogenesis directly in *vivo* by a mechanism independent of the type 1a growth hormone secretagogue receptor. *Endocrinology* 145:234-242.

Tolle V, Zizzari P, Tomasetto C, Rio MC, Epelbaum J & Bluet-Pajot MT. 2001. *In vivo* and *in vitro* effects of ghrelin/motilin-related peptide on growth hormone secretion in the rat. *Neuroendocrinology* 73:54-61.

Torsello A, Ghe' C, Bresciani E, Catapano F, Ghigo E, Deghenghi R, Locatelli V & Muccioli G. 2002. Short ghrelin peptides neither displace ghrelin binding *in vitro* nor stimulate GH release *in vivo*. *Endocrinology* 143:1968-1971.

Toshinai K, Yamaguchi H, Sun Y, Smith RG, Yamanaka A, Sakurai T, Date Y, Mondal MS, Shimbara T, Kawagoe T, Murakami N, Miyazato M, Kangawa K & Nakazato M. 2006. Des-acyl ghrelin induces food intake by a mechanism independent of the growth hormone secretagogue receptor. *Endocrinology* 147:2306-2314

Toullec R, Chayvialle JA, Guilloteau P & Bernard C. 1992. Early-life patterns of plasma gut regulatory peptide levels in calves. Effects of age, weaning and feeding. *Comparative Biochemistry and Physiology. Comparative Physiology* 102:203-209.

Tschöp M, Smiley DL & Heiman ML. 2000. Ghrelin induces adiposity in rodents. *Nature* 407:908-913.

Warwick JM, Wallis OC & Wallis M. 1989. Cloning, sequence and expression in *Escherichia coli* of cDNA for ovine pregrowth hormone. *Biochimica et Biophysica Acta*. 1008:247-250.

Watanuki Masashi. 2004. Effect of intravenous injection of recombinant bovine leptin on feed intake, plasma concentrations of GH, and metabolites in fattening Holstein cull cows. *Master thesis*: The Course of Animal Production and Agricultural Economics, Graduate School of Obihiro University of Agriculture and Veterinary Medicine.

Wertz-Lutz AE, Knight TJ, Pritchard RH, Daniel JA, Clapper JA, Smart AJ, Trenkle A & Beitz DC. 2006. Circulating ghrelin concentrations fluctuate relative to nutritional status and influence feeding behavior in cattle. *Journal of Animal Science* 84:3285-3300.

Wortley KE, Anderson KD, Garcia K, Murray JD, Malinova L, Liu R, Moncrieffe M, Thabet K, Cox HJ, Yancopoulos GD, Wiegand SJ & Sleeman MW. 2004 Genetic deletion of ghrelin does not decrease food intake but influences metabolic fuel preference. *Proceedings of the National Academy of Sciences of the United States of America* 101:8227-8232.

Wren AM, Small CJ, Fribbens CV, Neary NM, Ward HL, Seal LJ & Ghatei MA, Bloom SR. 2002. The hypothalamic mechanisms of the hypophysiotropic action of ghrelin. *Neuroendocrinology* 76:316-324.

Yamazaki M, Nakamura K, Kobayashi H, Matsubara M, Hayashi Y, Kangawa K & Sakai T. 2002. Regulational effect of ghrelin on growth hormone secretion from perifused rat anterior pituitary cells. *Journal of Neuroendocrinology* 14:156-162.

Yonekura S, Kitade K, Furukawa G, Takahashi K, Katsumata N, Katoh K & Obara Y. 2002. Effects of aging and weaning on mRNA expression of leptin and CCK receptors in the calf rumen and abomasum. *Domestic Animal Endocrinology* 22:25-35.

Zhang JV, Ren PG, Avsian-Kretchmer O, Zhang JV, Ren PG, Avsian-Kretchmer O, Luo CW, Rauch R, Klein C, Hsueh AJ.Luo CW, Rauch R, Klein C & Hsueh AJ. 2005.

Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. *Science* 310:996-999.

Zhu X, Cao Y, Voogd K & Steiner DF. 2006. On the processing of proghrelin to ghrelin. *Journal of Biological Chemistry* 281:38867-38870.