Regulatory Mechanisms of Endothelin, Gastrin and Gastrin-releasing Peptide on the Secretion of Ghrelin and Growth Hormone in Cattle

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Abstract

Ghrelin secretory capacity was found mainly in the stomach of monogastric animals as well as the abomasum of ruminants. The dominant putative roles of ghrelin include functioning as an endogenous growth hormone (GH) secretagogue (GHS) and in the regulation of appetite. The regulatory mechanisms for ghrelin synthesis and secretion are of interest in domestic animals, as the two putative roles of ghrelin are critical for animal growth and productivity. The studies in this thesis were designed to investigate the regulatory mechanisms of three brain-gut peptide hormones, endothelin (ET), gastrin, and gastrin-releasing peptide (GRP) on the secretion of ghrelin, GH, and insulin as well as glucose metabolism in cattle.

In the study reported in **Chapter 2**, 23 peptides, such as bovine sulfated gastrin-34, N-GRP-EE (an antagonist for GRP-preferring receptors), [D-Lys^3]-GHRP-6 (an antagonist for GHS receptor type 1a [GHS-R1a]) and IRL 1620 (a high selective agonist for endothelin B receptor [ET_B]), were synthesized by solid phase peptide synthesis (SPPS) and used as haptens for generating the first antibodies, cold standards and radioligands for radioimmunoassay (RIA), or administered to animals, since bovine peptide hormones and related first antibodies are not always available commercially. Radioimmunoassay systems using the raised specific first antibodies from guinea pigs and/or hens were established for measurements of sulfated gastrin, total gastrin, GRP, N-GRP-EE and [D-Lys^3]-GHRP-6 in plasma.

In studies described in Chapters 3 to 6, a series of animal experiments were carried out using pre-weaned Holstein male calves and post-weaned steers ranging in age between 4 and 9 mo. The study in **Chapter 3** was aimed to characterize the receptors involved in the effects of endothelin-3 (ET-3) on the secretion of ghrelin and GH, and the response for glucose metabolism. Concentrations of acyl ghrelin, total ghrelin and glucose were significantly increased by ET-3 in a dose-dependent manner. Concentrations of GH were markedly elevated by administration of 0.4, 0.7 and 1.0 μg/kg body weight of ET-3, and the effect of 0.7 μg/kg was greater than that of 1.0 μg/kg. The agonist for ET_B receptors, IRL 1620, mimicked the effects of ET-3 on the elevation of ghrelin, GH and glucose in plasma, indicating the involvement of ET_B receptors in this effect. The ET-3-induced increase of plasma GH was blocked by [D-Lys^3]-GHRP-6, suggesting the increase of GH

was mediated by GHS-R1a.

The study in **Chapter 4** aimed to determine the effects of sulfated gastrin-34 on the circulating levels of ghrelin, GH, insulin, glucagon and glucose in steers. Concentrations of acyl and total ghrelin in response to gastrin injection were significantly increased ($P< 0.05$) in a dose-dependent manner. Concentrations of GH were also markedly elevated ($P< 0.05$) by gastrin injection; however, the effect of 20.0 μg/kg of injected gastrin was weaker than that of 4.0 μg/kg. The three doses of gastrin equally decreased ($P< 0.05$) insulin levels within 15 min and maintained the level until the last sampling time point. Gastrin had no effect ($P > 0.05$) on the levels of glucagon and glucose.

The objective of the study described in **Chapter 5** was to delineate the effects of different forms of gastrin on the circulating levels of ghrelin and GH, and whether the pre-weaning and post-weaning periods changed their effects. Secondly, the study was designed to determine whether endogenous ghrelin in response to gastrin stimulated GH secretion. Plasma GH level significantly increased ($P < 0.05$) at 15 and 20 min after sulfated gastrin-17 injection compared with vehicle injection in pre-weaned calves; however, the changes for acyl ghrelin could not be analyzed due to the great variation of acyl ghrelin levels among animals and time points in both vehicle and gastrin injection groups. Both sulfated and non-sulfated gastrin-17 increased plasma ghrelin and GH levels; although, the duration of total gastrin increase in response to sulfated gastrin-17 injection (10 min) was greater than that of non-sulfated gastrin-17 injection (5 min) in post-weaned steers. Sulfated gastrin-9 injection did not change the levels of any hormones assayed. Injection of [D-Lys^3]-GHRP-6 did not block the gastrin-induced GH increase, indicating the regulatory effect of gastrin on GH may be independent on the ghrelin/GHS-R system.

The study in **Chapter 6** aimed to explore the effects of bombesin-like peptides on the circulating levels of ghrelin, GH, insulin and glucose in cattle during pre-weaning and post-weaning, and to characterize the receptor subtypes mediating these effects. Both GRP and neuromedin C (NMC) injection increased GH and insulin levels from 5 min, and the minimal effective doses of NMC were 1.0 μg/kg for GH and 0.2 μg/kg for insulin in post-weaned steers. The plasma glucose response occurred with two distinct phases; the first being a significant rise in concentration followed by the second phase in which there was a drastic reduction in plasma glucose after NMC or GRP administration. The antagonist for GRP-preferring receptors, N-GRP-EE, completely blocked the

NMC-induced changes of GH, insulin and glucose, indicating GRP-preferring receptors mediating these effects; however, [D-Lys^3]-GHRP-6 did not block any of these changes. Administration of neuromedin B (NMB) or N-GRP-EE alone did not change the circulating levels of hormones assayed and glucose during all the sampling time points. Effects of NMC on GH and insulin in pre-weaned calves were approximately identical to those in post-weaned steers. But the duration of increase for both GH and insulin levels was longer in pre-weaned calves than in post-weaned steers though peak levels of immunoreactive GRP at 5 min in response to NMC injection in the pre-weaned group (0.23 ± 0.03 ng/mL) was significantly lower ($P < 0.01$) than those in the post-weaned group (0.70 ± 0.12 ng/mL). Ghrelin levels were not changed by any treatments in this study.

The studies in this thesis demonstrated that (1) endogenous ghrelin in response to ET-3 injection increases GH secretion, noting that ET_B receptors are involved in this process in steers; (2) the two main forms of gastrin, gastrin-34 and gastrin-17, regardless if they were in the sulfate form or not, stimulate both ghrelin and GH secretion, and that the gastrin-induced secretion of GH was independent to the ghrelin/GHS-R1a system; and (3) both GRP and NMC enhanced GH secretion which was mediated by GRP-preferring receptors.

Key words: ghrelin, growth hormone (GH), insulin, endothelin, gastrin, gastrin-releasing peptide (GRP), calf, steer, cattle, radioimmunoassay (RIA), immunoglobulin Y (IgY)

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Charpter 1

General Introduction

1. Introduction

In 1999, ghrelin, an endogenous ligand for growth hormone (GH) secretagogue receptors, was first discovered in rat stomach with its GH releasing function by Kojima et al. [55, 84]. Growth hormone secretagogue receptor type 1a (GHS-R1a) has been shown to mediate the GH-releasing action of ghrelin in vitro and in vivo [57, 103]. Acyl modification on Ser^3 is essential for the binding of ghrelin to GHS-R1a. However, des-acyl ghrelin does not interact with the receptors. Over the last ten years, many studies have been condected to investigate the physiological roles of ghrelin. Ghrelin stimulates GH secretion not only in monogastric species but also in ruminants [48, 64, 154, 159, 160]. The amino acid sequence of bovine pre-pro-ghrelin consisted of 116 amino acids, which contained the 27-amino acid ghrelin. Although rats can produce 28 amino acid-ghrelin and 27 amino acid-des-Gln^{14}-ghrelin by alternative splicing, ruminant species, including cattle, might be able to produce only one type of ghrelin peptide, des-Gln^{14}-ghrelin [80]. Immunohistochemical studies revealed that ghrelin immunostained cells were in oxyntic gland of cow [49]. In addition, ghrelin was more than endogenous GH secretagogue and defined as an important appetite regulator, energy conservator, and sympathetic nerve suppressor [115]. At present, ghrelin is the only circulating orexigenic hormone secreted from the peripheral organ and acts on the central nervous system.

Over the years, the physiological roles of ghrelin have been elucidated. However, the regulatory mechanisms of the release of ghrelin have not fully clarified. To understand the mechanism of ghrelin release, neural regulation, humoral regulation and autoregulation are usually considered. In this thesis studies, we have focused on the humoral regulation of ghrelin secretion, especially, the regulatory effects of endothelin, gastrin and gastrin-releasing pepetide on ghrelin secretion in ruminants. Therefore, ET, gastrin and gastrin-releasing pepetide will be mainly reviewed in the following literature review

sections. The discovery of the three hormones and their receptors as well as their location in the organs and tissues will be presented. Differences in amino acid sequences of each hormone will be compared among their family peptides in cattle and among specifics. Especially, the sequence difference between cattle and other species will be aligned since we will choose animals, which sequence is more different from cattle, to produce specific antibody against bovine peptide hormones mentioned above. Moreover, the selective agonist and/or antagonist of the related receptors will be listed since we will use the specific agonist and/or antagonist to stimulate or block the effect of the three hormones on ghrelin or other hormones or metabolites investigated in chapter 3-6. In addition, the effect of these three hormones on ghrelin secretion will be reviewed from the published papers. The objective of this thesis studies are stated at the end of this chapter.

2. Endothelin

2.1. Discovery of endothelin and endothelin receptors

Furchgott and Zawadzki first demonstrated that relaxation of isolated preparations of rabbit thoracic aorta and other blood vessels by acetylcholine required the presence of endothelial cells (EC) in 1980 [36]. Following the discovery of endothelium-dependent vasodilation, the existence of an endothelium-derived polypeptide vasoconstrictor from cultured bovine aortic EC was proposed by Hickey et al. in 1985 [52]. However, the structure of the vasoconstrictor was not determined. In 1988, Yanagisawa et al. identified a 21-residue vasoconstrictor peptide, endothelin (ET), with free amino- and carboxy-termini from the culture supernatant of porcine aortic EC. Four cysteine residues of ET were found to form two intrachain disulphide bonds (Fig. 1-1) [175]. Three distinct human ET-related genes were cloned by screening a genomic DNA library under a low hybidization stringency with a synthetic oligonucleotide probe encoding a portion of the ET sequence. Each of the human genes predicted a putative 21-residue peptide, similar to but distinct from each other: the "classical" endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3). Fig. 1-2 shows that the differences of amino acid sequence in bovine ET-1, ET-2 and ET-3. BLAST results from Uniprot (http://www.uniprot.org) display that the 21-amino acid sequence of ET-1 are identical in the bovine, sheep, horse, pig, human, monkey, dog, rabbit, rat, mouse, guinea pig and frog (http://www.uniprot.org); Fig. 1-3 and 1-4 show the differences of amino acid sequence of ET-2 and ET-3 in species.

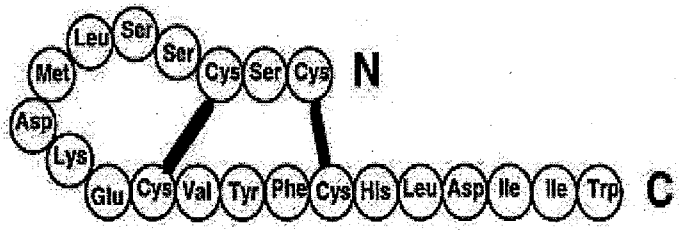

Fig. 1-1. Structure of endothelin (modified from Yanagisawa et al., 1988 [175])

CSCSSLMDKECVYFCHLDIIW	Bovine ET-1
CSCSSWLDKECVYFCHLDIIW	Bovine ET-2
CTCFTYKDRECVYYCHLDIIW	Bovine ET-3

Fig. 1-2. Comparison of amino acid sequence of bovine ET-1(Uniprot accession number: P17322), ET-2(Q867A9) and ET-3(A6QLQ7). The amino acids shaded the background show differences in ET-2 and ET-3 compared to ET-1.

CSCSSWLDKECVYFCHLDIIW	Bovine, rabbit, mustela furo, human, horse, cat, dog
CSCNSWLDKECVYFCHLDIIW	Rat, mouse
CSCNSWLDKECTYFCHLDIIW	Chicken

Fig. 1-3. Comparison of ET-2 amino acid sequence among species: cattle (Uniprot accession number: Q867A9), rabbit(Q765Z5), mustela furo(Q8MJW9), human(P20800), horse(Q867D0), cat(Q5NRQ0), dog(P12064), rat(P23943), mouse(P22389) and chicken(P22389). The amino acids shaded the background show differences in rat, mouse and chicken compared to bovine.

CTCFTYKDRECVYYCHLDIIW	Bovine
CTCFTYKDKECVYYCHLDIIW	Human, rat, mouse, rabbit, pig, dog, goat, horse
CTCYTYKDKECVYYCHLDIIW	Chicken, frog

Fig. 1-4. Comparison of ET-3 amino acid sequence among species: cattle (Uniprot accession number: A6QLQ7), human (P14138), rat (P13207), mouse (P48299), rabbit (P19998), pig (A5A752), dog (Q765Z4), goat (B4XR11), horse (Q3L1G9), chicken (Q3MU75) and frog (A0PGA0). The amino acids shaded the background indicate differences compared to bovine.

The vasoconstrictor activity of synthetic ET-1, ET-2 and ET-3 peptides was ET-2 > ET-1 > ET-3, indicating the possible existence of ET receptor subtypes [60]. In 1990, Arai et al. reported that the cloning of a complementary DNA encoding a bovine ET receptor had a transmembrane topology similar to that of other G protein-coupled receptors. Displacement experiments of ^{125}I-labelled ET-1 binding indicated that ET-1 is 10 times more potent than ET-2 inhibition of radioligand binding, but ET-3 is much less effective than either ET-1 or ET-2 in animal cells transfected with the cloned ET receptor cDNA [6].

Furthermore, binding of ^{125}I-labelled ET-3 to bovine carotid artery EC was more effective displaced by ET-3 than by ET-1[31]. These findings support that additional receptor subtypes was present. At the same year, Sakurai et al. demonstrated that the existence of a subtype of receptor that was non-selective among the three ET peptides and called the ET_B endothelin receptor; whereas the ET-1/ET-2-selective subtype was called the ET_A endothelin receptor [141]. The structures of ET_A and ET_B receptors have been deduced from the nucleotide sequences of the cDNAs. Both receptor subtypes are belong to the seven-transmembrane (7 TM) domains, the superfamily of G protein-coupled receptor with approximately 400 amino acids. Both receptor subtypes have an N-terminal signal sequence, which is rare among heptahelical receptors. The extracellular loops, particularly between TM 4 to 6, determine the selectivity of the receptor for ligands [22]. Amino acid sequences of the bovine ET_A and ET_B receptors are shown in Fig. 1-5.

```
ETA  1 ---------METFWLRLSEWVALVGGVISDNPESYSTN-LSIHVDSVATFHGTELSFVVT  50
ETB  1 MQPLPSLCGRALVALILACGVAGIQAEEREFPPAGATQPLPGTGEMMETPTETSWPGRSN  60

ETA 51  THQPTN---LALPSNGSMHNY-------CPQQTKITSAFKYINTVISCTIFIVGMVGNAT 100
ETB 61  ASDPRSSATPQIPRGGRMAGIPPRTPPPCDGPIEIKETFKYINTVVSCLVFVLGIIGNST 120
                                                    (TM 1)
ETA 101 LLRIIYQNKCMRNGPNALIASLALGDLIYVVIDLPINVFKLLAGRWPFEQNDFGVFLCKL 160
ETB 121 LLRIIYKNKCMRNGPNILIASLALGDLLHIIIDIPINTYKLLAKDWPF-----GVEMCKL 175
                                  (TM 2)
ETA 161 FPFLQKSSVGITVLNLCALSVDRYRAVASWSRVQGIGIPLVTAIEIVSIWILSFILAIPE 220
ETB 176 VPFIQKASVGITVLSLCALSIDRYRAVASWSRIKGIGVPKWTAVEIVLIWVVSVVLAVPE 235
          (TM 3)                                          (TM 4)
ETA 221 AIGFVMVPFEYKGAQHRTCMLNATSK--FMEFYQDVKDWWLFGFYFCMPLVCTAIFYTLM 278
ETB 236 AVGFDIITSDHIGNKLRICLLHPTQKTAFMQFYKTAKDWWLFSFYFCLPLAITALFYTLM 295
                                                    (TM 5)
ETA 279 TCEMLNRRNGSLRIALSEHLKQRREVAKTVFCLVVIFALCWFPLHLSRILKKTVYDEMDT 338
ETB 296 TCEMLRKKSG-MQIALNDHLKQRREVAKTVFCLVLVFALCWLPLHLSRILKLTLYDQHDP 354
                                              (TM 6)
ETA 339 NRCELLSFLLLMDYIGINLATMNSCINPIALYFVSKKFKNCFQSCLCCCCYQSKSLMTSV 398
ETB 355 RRCEFLSFLLVLDYIGINMASLNSCINPIALYLVSKRFKNCFKSCLCCWC-QSFEEKQSL 413
                        (TM 7)
ETA 399 PMNGTSIQWKNHEQNNHNTERSSHKDSIN 427
ETB 414 EEKQSCLKFKANDHG-YDNFRSSNKYSSS 441
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Fig. 1-5. Amino acid sequence alignment of bovine ET_A and ET_B receptors. Domains of transmembrane are shown underline. N-terminal signals are indicated by shading the background of amino acids. Uniprot accession number is P21450 and P28088 for ET_A and ET_B receptors, respectively.

2.2 Agonists and Antagonists for ET receptors

The two ET receptor subtypes were originally distinguished by their different affinity

for the endogenous peptides. ET_A receptors bind ET-1 and ET-2 with higher affinity than ET-3 (ET-1 = ET-2 >> ET-3); whereas ET_B receptors equally bind the three isoform peptides (ET-1 = ET-2= ET-3) [22]. A selective ET_A agonist with comparable potency to ET-1 has not yet been discovered. Sarafotoxin S6c, a peptide extracted from the venom of *Atractaspis engaddenensis*, is one of the most widely used ET_B selective agonist [172]. The truncated linear synthetic analogs BQ3020 and IRL 1620 [156] are the most widely used selective agonists to characterize ET_B receptors. Antagonists of ET receptors include ET_A selective, ET_B selective and non-selective ET antagonists. The most highly selective peptide antagonists for ET_A receptors are the cyclic pentapeptide BQ123 and FR139317. ET_B antagonists, such as peptide BQ788 and non-peptide A192621, have been developed; however, ET_B antagonists are less potent than ET_A antagonist and display lower selectivity.

2.3. Localization of ET and ET receptors

In general, ET_A receptors are present in vascular smooth muscle, and activation of ET_A receptors by ET-1 and ET-2 mediates vasoconstriction. Eendothelin B receptors exist in endothelial cells in blood vessels, and activation of ET_B receptors by ET-1, ET-2 and ET-3 causes release of nitric oxide and relaxation of vascular smooth muscle. Rat ET_A mRNA is predominantly expressed in vascular smooth muscle cells of a variety of tissues; ET_A mRNA prominent expression is seen in glial cells throughout the brain regions [54]. It was found that ET_B receptors present in canine spleen were different from those present in lung by binding studies [115]. Only ET-l is produced by endothelial cells. Endothelin-like immunoreactivity had been demonstrated by immunohistochemistry in the hypothalamus and pituitary of the pig and the rat [178].

2.4. Roles of ET and ET receptors

2.4.1 Effect on contraction of smooth muscle

Eendothelin-1 was found to contract the isolated bovine coronary artery by stimulating ET_A receptors on smooth muscle cells, and that ET_B receptors might suppress the ET-1-induced contractions [63]. In ex vivo perfusions of isolated mouse islets showed a dose-dependent constriction of vasculature in response to ET-1 mediated by ET_A receptors [89]. Acute hypotension increased the coronary vasoconstriction in response to ET-1 related to inhibition of nitric oxide release in anesthetized goats [34]. Rosselli et al. reported that bovine oviduct epithelial cells produce ET and, therefore, may be locally

involved in the muscular oviduct wall contractility and play an important role in the transport of gametes and embryos [139]. Studies in the human saphenous vein indicated pharmacological heterogeneity of both ET_A and ET_B receptors-mediated contractions, although contractions mediated by ET_B receptors have smaller maximal responses than those mediated by the ET_A receptors [116]. Studies from ET_B receptor-deficient rats showed that ET_B receptors in the brain appeared to play an important role in the control of extracelllular ET-1 levels and in counter-balancing ET_A-mediated vasoconstriction [30]. Endothelin B receptors seem to play an important role in the clearance of ET-l.

2.4.1 Effects on regulation of hormone secretion and circulating glucose levels

In a microdialysis study, human ET-1 increased the microdialysate ghrelin concentration in a dose-dependent manner and the stimulatory effect was maintained throughout the period of infusion in rats [23]. Our laboratory previous study demonstrated that intravenous (IV) injections of both bovine ET-1 and -3 stimulated ghrelin secretion in steers [157]. Moreover, bovine ET-1 and -3 also were found to increase the levels of GH in plasma, and that ET-3 was more effective than ET-1 [157]. Therefore, we suggest that the effects of ET-1 or ET-3 on the release of ghrelin and GH may be mediated by ET_B receptors, as ET_A receptors have lower affinity for ET-3 than ET-1, while ET_B receptors associate equally with these two peptides [6, 141]. The effect of ET on ghrelin and GH can be explain by that ET-1 inhibited the release of somatostatin (SS), which is one of the putative inhibitor of ghrelin and GH release, from the rat stomach in vitro[109]. However, in healthy men, IV ET-1 suppressed the growth hormone-releasing hormone (GHRH)-stimulated increase in GH and prolactin (PRL) and augmented corticotrophin-releasing factor (CRF)-stimulated secretion of corticotrophin [166].

Infusion of ET-1 or ET-3 into the portal vein increased glucose and lactate output, and both ET_A and ET_B receptors were suggested to be involved in the metabolic effects of circulating ET in rat liver [20]. Intraperitoneally (IP) administered ET-1 increased plasma glucose and induced insulin resistance in conscious rats [70]. In contrast, ET-1 infusion was found to decrease glucose initially associated with a decrease in glucagon and subsequently associated with enhanced insulin release in anesthetized rats [182]. Eendothelin-1 inhibited histamine-elicited ^{3}H-cyclic AMP generation in brain vessels isolated from bovine brain, and ETs modulated the actions of histamine on the blood-brain barrier, probably by ET_A receptors [51]. Both ET-1 and ET-3 were found to diminish

neuronal norepinephrine release through a nitric oxide pathway mediated by ET_B receptors in the rat anterior hypothalamus [66].

3. Gastrin

3.1 The discovery of gastrin and its receptors

In the 1820s, William Beaumont, the Father of Gastric Physiology, discovered that secretion of gastric acid from human stomach was important for food digestion [10]. After that, the predominant ideas of regulation of gastric secretion by vagal innervation had been developed by the Russian neurophysiologist Pavlov [111]. In 1902, Bayliss and Starling overturned Pavlov's doctrine of the nervous regulation of gastrointestinal function by discovering the pancreatic secretagogue secretin, the first identifiable chemical messenger [9]. This discovery provided clear evidence of the role of hormone in the regulation of secretion. In 1905, it was John Edkins from London who presented that jugular venous injection of a pyloric mucous membrane extract, active by hydrochloric acid or boiling, resulted in gastric acid. This active agent was called "gastrin" [28]. Although Edkins' ideas were initially accepted, the discovery of histamine in 1910 and the identification that extracts from other tissues had a similar physiologic effect raised serious questions regarding the validity of the existence of gastrin [111]. In 1964, Gregory R.A. and Tracy described they had purified two almost identical peptides: gastrin I and gastrin II, from porcine antral mucosa, and both were many times more potent than histamine in stimulating gastric acid secretion in conscious dogs [42]. Their studies allowed final validation of Edikins' original hypothesis.

Then, Gregory H. et al using the natural gastrin obtained from Gregory R.A. carried out chemical structure studies on both gastrins. Gastrin II (G-17-II) has heptadecapeptide amide structure and a tyrosyl residue in position six, as counted from the C-terminus, that is sulfated (Fig. 1-6). Gastrin I (G-17-I) differs from gastrin II only in lacking the tyrosyl sulfated ester group [40]. The C-terminal tetrapeptide amide is essential for the physiological activities, which are possessed by the natural gastrin [163]. In 1970, Yalow and Berson firstly reported that the larger form of gastrin mainly presented in the blood of Zollinger-Ellison patients and called this "big gastrin" [174]. It is a molecule of 34 amino acids (G-34) of which the C-terminal heptadecapeptide is identical to G-17, and like G-17 it exists in both non-sulfated (G-34-I) and sulfated (G-34-II) form [41]. Other molecular

forms of gastrin have also been identified in the blood in physiological conditions, including component I as gastrin-71[129], gastrin-14. The amino acid sequences of gastrin-34 different species are shown in Fig. 1-7.

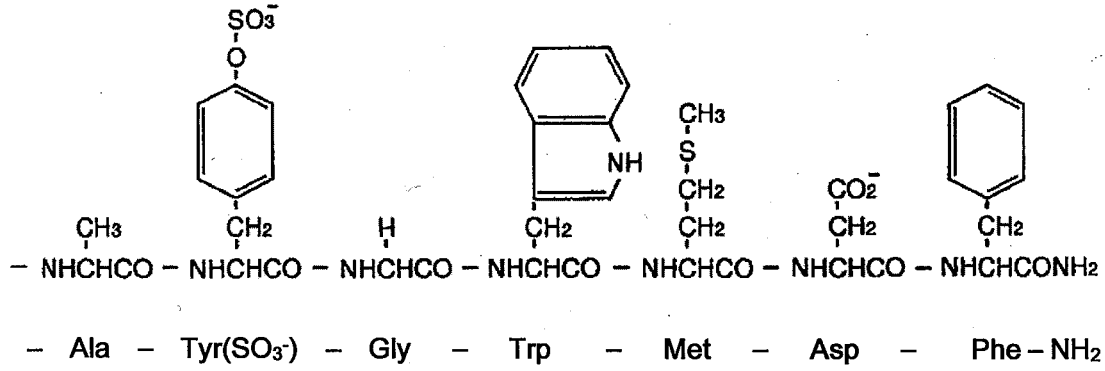

Fig. 1-6. C-terminal amide sequence of sulfated gastrin (modified from Rehfeld JF, 1998 [132])

Fig. 1-7. Comparison of gastrin amino acid sequence among species: bovine(Uniprot accession number: P01352), sheep (O02686), goat(P04564), rat (P04563), mouse(Q6GSF5), cat (P01354), horse (P55885), pig(P01351), dog(P01353), human (P01350), guinea pig (P06885), North American opossum (P33713) and chicken (reference [26]). The amino acids shaded the background show differences compared to bovine gastrin.

The C-terminal terapeptide amide sequence of gastrin and that of another gastrointestinal hormone, cholecystokinin (CCK), are identical and constitute the active site of both hormones. This sequence has been preserved during evolution in the gastrin-CCK family (Fig. 1-8). It is hypothesized that a dityrosyl-sulfated peptide coin, isolated from protochordean neurons, is a common ancestor [68]. Caerulein and phyllocaerulein, isolated from the frog skin [2, 4], are also members of the gastrin-CCK family. From the present knowledge, only gastrin and CCK in the family are present in

mammals. Phylogenetically speaking [69], early members of the family look more CCK- than gastrin-like; and it is first at the level of elasmobranch that separate CCK and gastrin genes are expressed [127].

Receptors for the gastrin-CCK family have been pharmacologically classified on the basis of their affinity for the endogenous peptide gastrin and CCK. The first receptor subtype for the gastrin-CCK family was originally characterized on isolated rat pancreatic acini [142], therefore was named cholecystokinin-A (CCK_A, A means "alimentary"). The second receptor subtype was distinguished from the CCK_A receptor using guinea pig brain and rat pancreas membranes [59]. In brain, gastrin and pentagastrin displayed nanomolar affinity for binding sites, whereas in pancreas these two peptides were virtually inactive. Both pancreas and brain receptors showed greater sensitivity to sulfated than to non-sulfated octapeptide CCK (CCK-8) [59]. These results indicate that brain possess distinct CCK receptors, which was initially named cholecystokinin-B（CCK_B, B means "brain"）. Gastrin receptors mediating acid secretion in the stomach were initially thought to constitute the third subtype of receptor on the basis of their location and small differences in affinity for CCK and gastrin-like peptides. However, subsequent cloning of gastrin and CCK_B receptors proved their molecular identity [25,86], therefore the CCK_B receptor was also named gastrin/CCK_B receptor. Gastrin/CCK_B receptors bind gastrin and CCK, irrespective of the degree of tyrosin-O-sulfation [118]. The two receptor subtypes are 7 TM G-protein coupled receptors and the amino acid sequences of bovine CCK_A and gasrin/CCK_B are shown in Fig.1-9.

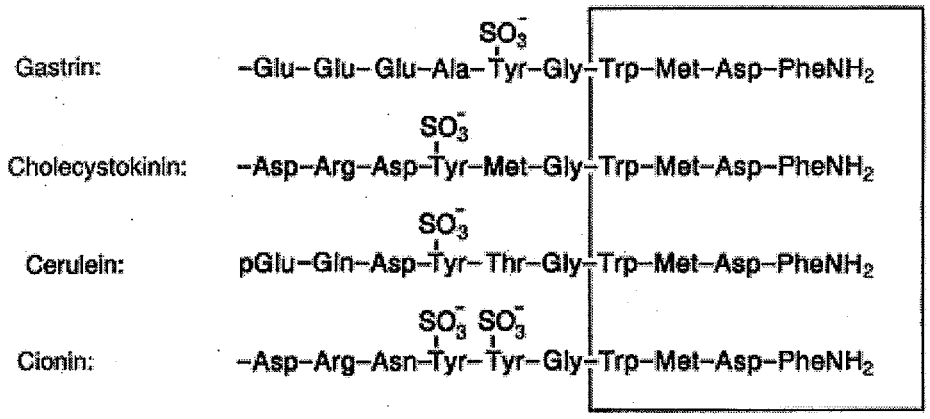

Fig. 1-8. Structural homology of members of gastrin family: gastrin, cholecystokinin (CCK), cerulein, and cionin. The common C-terminal tetrapeptide amide in box constitutes minimal structure necessary for bioactivity. Note position of characteristic sulfated tyrosine in the 4 peptides (modified from Rehfeld JF, 1998 [136].

```
CCK_A 1 MDVVD---SLLMNE----SNLTP---PCELGIENETLFCLDQPHP---SKEWQPAVQILL 47
CCK_B 1 MELLKPNRSVLGSGPGPGASLCRSGGPLLNGSGTGNLSCEPPRIRGAGTRELELAIRVTL 60

CCK_A 48 YSLIFLLSVLGNTLVITVLIRNKRMRTVTNIFLLSLAVSDLMLCLFCMPFNLIPNLLKDF 107
CCK_B 61 YAVIFLMSVGGNVLIIVVLGLSRRLRTVTNAFLLSLAVSDLLLAVACMPFTLLPNLMGTF 120
              (TM 1)                          (TM 2)
CCK_A 108 IFGSAVCKTTTYFMGTSVSVSTFNLVAISLERYGAICKPLQSRVWQTKSHALKVIAATWC 167
CCK_B 121 IFGTVVCKAVSYFMGVSVSVSTLSLVAIALERYSAICRPLQARVWQTRSHAARVIVATWM 180
                                (TM 3)                          (TM 4)
CCK_A 168 LSFTIMTPYPIYSNLVPFTKNNNQTANMCRFLLPSDVMQQSWHTFLLLILFLIPGIVMMV 227
CCK_B 181 LSGLLMVPYPVYTAVQPAG----PRVLQCMHRWPSARVRQTWSVLLLLLLFFVPGVVMAV 236
                                                                (TM 5)
CCK_A 228 AYGLISLELYQGIKFDASQKKSARERKRS-----------SASSGRY--------ADSA 267
CCK_B 237 AYGLISRELYLGLRFDGDSDSESQSRVGSQGGLPGGTGQGPAQANGRCRSETRLAGEDGD 296

CCK_A 268 GCCLQRPKHPRKLELRQLSTGSAGRADRIRSSSPAASLMAKKRVIRMLMVIVVLFFLCWM 327
CCK_B 297 GCYVQLPRSRPALEMSALTAPTPGPGSGTR--PAQAKLLAKKRVVRMLLVIVVLFFLCWL 354
                                                          (TM 6)
CCK_A 328 PIFSANAWRAFDTASAERRLSGTPIAFILLLSYTSSCVNPIIYCFMNKRIVEAALRLRSP 387
CCK_B 355 PVYSANTWRAFDGPGAHRALSGAPISFIHLLTYASACVNPLVYCFMHRRFRQACLDTCTR 414
                                          (TM 7)
CCK_A 388 SLFQEHSVTTHLTMTTDGNRKQTLFWPFSVLQTSRSKGEL 427
CCK_B 415 CCPRPPRARPRPLPDEDPPTPSIASLSRLSYTTISTLGPG 454
```

Fig. 1-9. Amino acid sequences of the bovine CCK_A and gastrin/CCK_B receptors. Ttransmembrane domains of CCK_B receptors are shown underline. Uniprot accession number is A6QLH2 and P79266 for CCK_A and CCK_B receptors, respectively.

3.2. Distribution of gastrin and gastrin/CCK_B receptors

Gastrin was mainly secreted from gastrin cells (G cells). G cells dispersed in the antral part of the gastric mucosa and in the proximal part of the duodenal mucosa. Gastrin was also released from cells other than the G cells. These other cells contributed only a little to circulating gastrin. In comparison with adult pancreatic tissue the fetal or neonatal pancreas processed a higher fraction to bioactive, C-terminally amidated gastrin in cats, dogs, humans, pigs and rats [8]. Gastrin also presented in the brain. Rehfeld and Johnsen reported that non-sulfated large gastrins, component I, and gastrin-34 constituted the main forms in the anterior lobe; however, gastrin-17, sulfated as well as non-sulfated, predominated in the intermediate and neural lobes of the pituitary [130].

Gastrin/CCK_B receptors were located in the brain and in the gastrointestinal tract, including the gallbladder [118]. In the stomach, gastrin/CCK_B receptors were found mainly on enterochromaffin-like (ECL) cells. Parietal cells also express gastrin/CCK_B receptors, but at a low level [127]. Gastrin/CCK_B receptors in the periphery are in physiological terms the receptor only for gastrin, since the 10-20 fold lower CCK concentrations in

plasma cannot compete; whereas, effects of CCK in the periphery are elicited essentially only via the CCK_A receptor, which do not bind gastrin. Gastrin/CCK_B receptors were also at a lower level in the exocrine and endocrine pancreas.

3.3. Roles of gastrin and its receptors in gastric acid and hormone secretion

Gastrin was defined by its effect on gastric acid secretion in monogastric animals as well as in ruminants [104]. The synthesis of gastrin was regulated by negative feedback from gastric acid via antral somatostatin cells. It is now generally thought that gastric acid secretion is regulated by gastrin through gastrin/CCK_B receptors on ECL cells, which then release histamine to stimulate parietal cells in the fundic mucosa. Gastrin stimulated the proliferation of gastric epithelial cells and increased the number of parietal and ECL cells.

In addition to regulation of gastric acid secretion, gastrin peptides are neurotransmitters and thought to function as an endocrine or autocrine growth factor [177]. .The role of gastrin in ghrelin release is a matter for discussion. Gastric microdialysis and abdominal infusion studies showed that human gastrin-17 had no measurable effect on the secretion of ghrelin in rats [23,27]. In a study of isolated rat stomach, gastrin-17 significantly inhibited vagally prestimulated ghrelin release [94]. However, IV administration of gastrin induced transient increases of ghrelin levels in a dose-dependent manner in vagotomized rats. Double immunostaining for ghrelin and gastrin receptor revealed that a proportion of ghrelin cells possess gastrin receptors [35]. Murakami et al. [113] showed that IP administration of gastrin increased ghrelin levels in fasted rats. An intravenous injection of gastrin increased the level of GHS-R mRNA after 2 h, but after 4 h, the level decreased in the rat nodose ganglion [143].

Pentagastrin infusion induced a rise in GH levels maximal at 60 min (9.1 ± 0.6 ng/ml), but this rise was abolished by pretreatment with GHRH in seven fasted healthy male subjects. However, gastrin did not modify basal GH release or GH responses to GHRH by rat anterior pituitary cells in monolayer culture. Therefore, the authors suggested that gastrin regulates GH secretion by acting at hypothalamic level [38]. Third ventricular injection of 5 μg dose of synthetic Leu^{15}-human gastrin induced a progressive elevation of plasma GH levels in conscious ovariectomized rats; however, IV injection of same dose gastrin had no effect on plasma GH level [168]. When gastrin was infused at 1.5 μg/kg.h for 100 min, both portal and systemic blood concentrations of somatostatin and pancreatic polypeptide rose significantly in six anesthetized dogs [46]. The interaction

of GH and somatostatin increased by gastrin is remained to be answered.

Intravenous injection of gastrin increased the concentration of insulin in the blood to a maximum within 2 min followed by a decline to basal levels after a further 10 min in normal young subjects [131]. Another reported also showed that a peak in insulin secretion occurred 1 min after injection the crude gastrin-containing extract of porcine antrum of stamach, and a rapid decline was observed immediately thereafter in dogs [165]. In isolated perfused porcine pancreas, gastrin increased insulin and glucagon secretion in a dose-dependent manner [67]. Duodenal luminal gastrin was reported to stimulate exocrine pancreatic secretion mediated by gastrin/CCK_B receptors in calves [179].

4. Bombesin-like peptides

4.1 The discovery of bombesin-like peptides and their receptors

During systematic screening for active polypeptides in the amphibian skin, it was found that methanol extracts of two European amphibians, *Bombina bombina* and *Bombina variegate variegate*, contain bombesin (BBS) [3]. Bombesin is a tetradecapeptide and displayed a number of pharmacological actions, including hypertension and stimulating the gastric secretion. [32]. Rantensin, an endecapeptide possessing a structure obviously different from BBS, was isolated from methanol extracts of the skin of the American frog, *Rana pipiens* [32]. Bombesin-like peptides (BLP), the generic name, was given to peptides that have homology with the C-terminal sequence of BBS. The first mammalian BLP was isolated from porcine non-antral gastric and intestinal tissue and named gastrin-releasing peptide (GRP) because of its potent gastrin releasing activity [101,102]. Gastrin-releasing peptide was shown to be a 27-amino acid peptide and shared the same seven C-terminal amino acid with BBS (Fig. 1-10) [101]. A decapeptide neuromedin B (NMB) was firstly purified from porcine spinal cord and named from suggesting to be involved in the neural communication system [107]. Neuromedin B is thought to be the mammalian homologue of ranatensin, as the C-terminal seven amino acid sequence is identical (Fig. 1-10) [101]. From a side fraction obtained in the isolation of NMB from porcine spinal cord, another decapeptide was found to be identical with the C-terminal sequence (18-27) of GRP and named neuromedin C (NMC) since it was closely related to NMB [108]. Gastrin-releasing peptide is expressed more widely and strongly compared to NMB in rat brain, and in most of the regions, distributions of the

GRP signals do not overlap with those of NMB[120], indicating that the functions of these two peptides were partly distinct and different receptor subtypes were present.

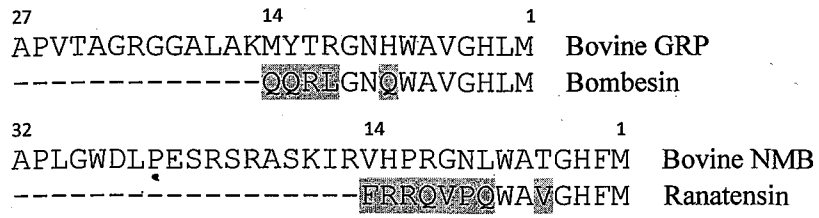

Fig. 1-10. Comparison of the amino acid sequences between bovine GRP (Uniprot accession number: Q863C3) and bombesin (P84213), between NMB (Q2T9U8) and ranatensin (P08950).

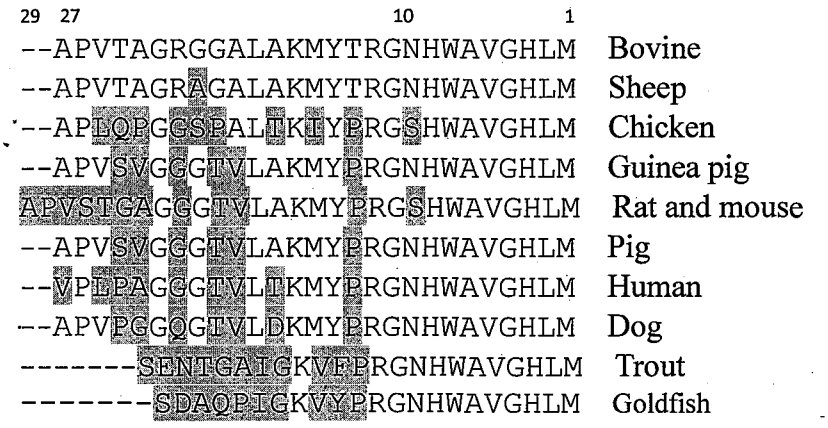

Fig. 1-11. Comparison of gastrin-releasing peptide (GRP) amino acid sequence among species: bovine(Uniprot accession number: Q863C3), sheep (P47851), chicken (P01295), guinea pig (P63152), rat (P24393), mouse(Q8R1I2), pig(P63153), dog(P08989), human (P07492), rainbow trout (Q9PS30), goldfish (Q9I8Z9). The amino acids shaded the background show differences compared to that of bovine GRP.

In 1988, the presence of receptors for BLPs was established using ^{125}I-GRP binding to murine Swiss 3T3 fibroblasts that were known to express high levels of receptors [140]. cDNAs encoding the bombesin/GRP receptor expressed by Swiss 3T3 cells were isolated using electrophysiological and luminometric *Xenopus* oocyte expression assays [147]. Sequence analysis showed that the receptor had 7 TM domains. These receptors had a high affinity for GRP and a lower affinity for NMB, termed GRP receptor, GRP-R, GRP-preferring receptor, or BB_2 receptor [140]. Subsequently, it was found that rat esophagus muscle possessed another specific receptor subtypes for BLPs, and the binding and causing contraction potencies of this receptor subtype as follow: NMB > BBS >

GRP=NMC [169]. The structure and expression of the cloned receptor with properties distinct from a GRP-preferring receptor in brain was reported [170]. This receptor subtype had a higher affinity for NMB than for GRP, termed NMB receptor, NMB-R, NMB-preferring receptor, or BB_1 receptor [169]. Then, the other cloning of a guinea-pig uterine cDNA encoded a new receptor subtype, which was designated as BLP receptor subtype-3 (BRS-3). The similarity between BRS-3 and two other cloned rodent bombesin receptors, the GRP-R and the NMB-R was 52% and 47%, respectively [39]. The affinity of BRS-3 for BBS was lower than that of GRP-R or NMB-R; moreover, GRP and NMB only had a poor potency for BRS-3[120]. No specific endogenous ligand has yet been found for the BRS-3. In amphibian, one clone encode for a bombesin receptor subtype distinct from the GRP-R was named BB4 [114]. The fifth receptor subtype of BLP was cloned from chick brain and named BRS-3.5 due to having sequence similarities to both mammalian BRS-3 and amphibian BRS-4 [65]. The BRS-3.5 showed moderate affinity for BBS, but low affinity for both GRP and NMB [65]. Binding studies suggested that GRP and NMB exerted their physiological effects through, at least, GRP-R and NMB-R in mammalian. To date, all the identified receptors of BLPs are coupled to guanine nucleotide binding proteins (G-protein) and have 7 TM domains [7].

4.2. Localization of BLPs and their receptors

Generally, bombesin-like immunoreactivities were detected in brain, spinal cord and gastrointestinal tissues [120]. The density of BLPs was great in hypothalamic area of rats [112]. Moreover, large quantities of GRP-like immunoreactivity were present in ovine pregnancy fluids [173]. Bombesin-like peptides （GRP, NMC and NMB） were purified from acid extracts of bovine adrenal medulla using HPLC [90]. Strong GRP-R immunoreactivities were observed in the hypothalamic regions of mouse [74]. Using in vitro receptor autoradiography, it was found that the bombesin receptor subtype in the rat stomach was GRP-R and that this subtype was responsible for the effects of BLPs on fundal smooth muscle contraction [88].

4.3. Roles of BLPs and their receptors

Bombesin-like peptieds have a wide range of physiological functions. Gastrin-releasing peptide was named for its first identified function of gastrin release. There were many studies supported this finding in monogastric animals. In conscious pigs, plasma gastrin levels were increased by BBS infusion with the doses ranged 50-100

pmol/kg/h [93]. In conscious dogs, IV infusions of BBS, GRP and NMC stimulated gastrin release and gastric acid secretion in a dose-dependent manner [11, 18]. Also in conscious dogs, synthetic porcine GRP (IV, 0.01, 0.1 and 0.5 μg/kg/h) stimulated the release of gastrin and CCK in a dose-related manner [61]. However, intracisternal injection of synthetic GRP induced a marked reduction in gastric secretory volume, acid concentration, and acid output in rats [151]. Intravenous infusions of either BBS or porcine GRP increased plasma glucagon, glucose and insulin concentration but did not change plasma either gastrin or CCK concentration in conscious 3-6 weeks calves [13]. In conscious sheep, GRP also did not elevated plasma gastrin levels [100]. These studies indicate that BLPs may play different roles in gastrin release between non-ruminant species and ruminants.

Bombesin-like peptides were involved in stimulation of other gastrointestinal hormones and pancreatic enzymes secretion. Intravenous injection of GRP was found to rapidly increase basal plasma levels of both insulin and glucagon in mice [124]. Neuromedin B was some 10-fold less potent than NMC, BBS and GRP in eliciting insulin secretion in isolated pancreatic acini of rats [121]. Intravenous infusions of synthetic porcine GRP stimulated the release of PP in a dose-related manner in conscious dogs [61]. Human GRP lowered microdialysate ghrelin level in rat stomach [23].

The function of gastrin on GH secretion was investigated by several groups of investigators resulting in opposite results. But results mainly supported the inhibitory effect of gastrin on GH release. Taché et al (1979) reported that plasma GH levels were depressed by bombesin injected intracerebroventricularly (IVT) under basal and cold stress conditions in fasted conscious rats [150]. Kentroti et al (1985) showed that IVT injection of porcine GRP_{1-27} decreased basal GH release and blocked the GH releasing action of IV human pancreatic GH-releasing hormone (GHRH) in ovariectomized female rats [79]. To determine if GRP had any direct action on the pituitary, they incubated pituitary cells with GRP and observed an increase in GH release [79]. Therefore, they suggested that GRP might act on periventricular structure to release somatostatin, which reduced GH release and blocked the response to GHRH. In fact earlier than Kentroti et al, Kabayama et al (1984) had done the similar studies and made the similar hypothesis [72]. Bicknell et al (1983) also supported that BBS stimulated GH secretion from bovine pituitary cultures during 90-min incubations [12]. Further studies by Kentroti et al. showed that GRP significantly stimulated somatostatin release in incubated rat median

eminence, and microinjection (IVT) of somatostatin antiserum prevented GRP from inhibiting the GH surge induced by IV GHRH [76]. Schaffer et al. reported that GRP receptors presented in rat fundic mucosal D cells, which were positive for somatostatin, and BBS, GRP_{1-27} and NMC were potent stimuli of somatostatin in D cells in primary culture [144]. Those results proved that somatostatin was a component of the mechanism whereby GRP inhibited GH release. Kentroti et al (1988) also showed evidence for a physiological role of hypothalamic GRP to suppress GH release through IVT injection a high specific antiserum against GRP to immunoneutrallize hypothalamic GRP resulted in an increase in plasma GH [77]. After near 10 years (1999), they interpreted data to indicate that GRP exerted its inhibitory action on the release of GH through a dopaminergic mechanism by subcutaneously pretreated with a dopamine receptor blocker in ovariectomized female rats.

By contrast, Rivier et al (1978) reported that IV and IVT injections of BBS resulted in a significantly increased GH secretion in anesthetized rats, but IVT injection seemed to be a less efficient method [137]. Intraperitoneal injection of 100 ng/g BW of BBS increased GH secretion and decreased forebrain pre-pro-somatostain gene expression in anesthetized goldfish [19]. Güllner (1982) found that synthetic porcine GRP (IV) had no significant effect on GH concentrations at any dose (0.1, 1, 10 ug) after 10, 20 and 30 min after injection of the peptide in anesthetized male rats. In contrast, it stimulated LH and suppressed TSH secretion in a dose-related fashion [44]

Studies have demonstrated that exogenous BLPs elicit a potent satiety effect when administered centrally or systematically [33,105]. After food ingestion, significant increased in the levels of BLPs in the hypothalamus, as well as in the antrum of the stomach in rats [75]. Intracerebroventricular (ICV) injection of GRP, NMB and NMC decreased feeding behavior in chicks while drinking behavior was not affected [152]. These results indicate that BLPs may play a physiological role in the control of food intake.

5. Objectives

Growth hormone (or somatotropin) is essential for somatic growth in humans and animals. Currently, recombinant bovine GH is marketed to enhance milk production in dairy cows. It has been used in the United States since it was approved by the Food and Drug Administration (FDA) in 1993, but its use is not permitted in the European Union,

Canada, and some other countries. It is putatively accepted that GH secretion from anterior pituitary is stimulated by hypothalamic GHRH and inhibited by somatostatin. Since ghrelin was first isolated from rat stomach, it has been demonstrated as a GH-releasing peptide [84]. Ghrelin also stimulates GH secretion in ruminants [48, 64, 154, 159, 160]. As ghrelin was more than endogenous GH secretagogue and defined as an important appetite regulator, energy conservator, and sympathetic nerve suppressor [115], the mechanisms by which regulate ghrelin secretion are interested. In literature review sections, we have made the research progress of ET, gastrin and BLPs on regulation of ghrelin, GH as well as insulin. However, most of the studies were carried out in monogastric species. Little is known about the endocrine system how to regulate ghrelin secretion in ruminants. Although some studies were done in ruminants, the administered peptides mentioned above may be not specific for species, for instant, using the peptides of rodent or human for ruminants. Furthermore, the established assay systems for measuring those hormones levels were mainly designed for rodents and/or humans.

This thesis studies were aimed to investigate whether bovine ET, gastrin and BLPs regulate ghrelin secretion in cattle. Moreover, as ghrelin can stimulate GH and insulin secretion, the regulatory effects of ET, gastrin and BLPs on GH, insulin as well as glucose were surveyed. After we obtained the results, the involvement of receptor subtypes of the administered peptide in ghrelin, GH and/or insulin secretion was determined. However, bovine peptide hormones and related first antibodies using in radioimmunoassay for the peptides are not always available commercially. Although some of the peptides can be specially ordered, the cost will be very high due to the special order and using in large animals. Therefore, we also designed to synthesize bovine related peptides which will be used in this studies. In addition, to understand the kinetics of administered peptides in plasma also will help us to illustrate what we observed. So, we planned to establish several RIA systems to measure the plasma levels of administered peptides. Accordingly, the objects of the thesis studies as follows:

To synthesize the peptides which will be used in the studies, especially the bovine peptides (Chapter 2).

To raise specific antibodies as the first antibodies in guinea pigs and/or hens for RIA (Chapter 2).

To establish the RIA systems for specific measurement of sulfated gastrin, total gastrin, GRP and other injected peptides (Chapter 2).

To investigate the dose-related effect of ET-3 on the secretion of ghrelin, GH, insulin as well as glucose and the involvement of GHS-R1a and ET receptor subtypes (Chapter 3).

To investigate the effects of several forms of gastrin on the secretion of ghrelin, GH, insulin as well as glucose and the involvement of GHS-R1a on the gastrin-induced GH change (Chapter 4 and 5).

To investigate the effects of BLPs (GRP, NMC and NMB) on the secretion of ghrelin, GH, insulin as well as glucose and the involvement of GHS-R1a and BLP receptor subtypes (Chapter 6).

To investigate whether the effects of gastrin and NMC on ghrelin and GH secretion are different between pre-weaned calves and post-weaned steers (Chapter 5 and 6).

The results obtained from this thesis studies were expected to contribute to illustrate, in part, the regulatory mechanisms of ghrelin, GH and insulin secretion in ruminants; to provide the basic data for further improvement of milk and meat production in ruminants.

Chapter 2

Peptide Synthesis and Establishment of Radioimmunoassay

1. Introduction

Most of the amino acid sequences of peptide hormone are different among animals even though part of the bio-activate sequences are identical for most species. Moreover, the different non-bioactive sequences may affect the binding affinity of peptide hormone for the receptor and the half-life of the peptide hormone in the body. Therefore, if we plan to investigate the regulatory effect of peptide hormones in cattle, the use of the species-specific peptide hormones seems to be of importance due to the large inter-species variations in amino acid sequences (see Table 1-7 and -11). On the other hand, if we want to measure plasma concentrations of peptide hormone produced by animals or injected, we need the related specific first antibody for radioimmunoassay (RIA). In this case, the peptide containing at least a cysteine residue, which can link the peptide to Mariculture Keyhole Limpet Hemocyanin (mcKLH), is required as a hapter for producing the first antibody. Moreover, the peptide containing at least one tyrosine or histidine is required for iodination of the peptide for RIA. However, bovine peptide hormones and related first antibodies are not commercially available. It is possible to directly order for them from manufactorers, but, high cost would be a major constraint to such option. In addition, large animals such as cattle require injection of large amount of peptide hormones. This will obviously increase the potential cost of the study.

In this chapter study, we aimed (1) to synthesis 23 peptides, such as bovine gastrins and bombesin-like peptides (shown in Table 2-1 and -2); (2) to raise the specific first antibody for peptide RIA in guinea pigs and chickens; (3) to establish the RIA system for gastrins, gastrin-releasing peptide and [D-Lys^3]-GHRP-6.

Table 2-1. List of synthesized gastrins

No.	Peptides	Amino acid sequences*	Uses in this study
1	[Pyr^{1}]-Gastrin II-34 (1-34, amide)	BLGLQDPPHMVADLSKKQGPWVEEEEAAY(SO$_3$H)GWMDF-NH$_2$	Injections, standard
2	[Cys^{1}]-Gastrin II-34 (1-34, amide)	CLGLQDPPHMVADLSKKQGPWVEEEEAAY(SO$_3$H)GWMDF-NH$_2$	As a hapten
3	[Cys^{17}]-Gastrin II-17 (17-34, amide)	CQGPWVEEEEAAY(SO$_3$H)GWMDF-NH$_2$	As a hapten
4	[Pyr^{18}]-Gastrin II-17 (18-34, amide)	BGPWVEEEEAAY(SO$_3$H)GWMDF-NH$_2$	Injections, standard
5	[Tyr^{18}]-Gastrin II-17 (18-34, amide)	YGPWVEEEEAAY(SO$_3$H)GWMDF-NH$_2$	For iodination
6	Gastrin II-9 (26-34, amide)	EAAY(SO$_3$H)GWMDF-NH$_2$	Injections, standard
7	[Tyr^{25}]-Gastrin II-9 (26-34, amide)	YEAAY(SO$_3$H)GWMDF-NH$_2$	For iodination
8	[Cys^{25}]-Gastrin II-9 (26-34, non-amide)	CEAAY(SO$_3$H)GWMDF-OH	As a hapten
9	[Pyr^{18}]-Gastrin I-17 (18-34, amide)	BGPWVEEEEAAYGWMDF-NH$_2$	Injections
10	[Cys^{25}]-Gastrin -9 (26-34, non-amide)	CEAAYGWMDF-OH	As a hapten
11	Gastrin II-9 (26-34, amide)	EAAYGWMDF-NH$_2$	As a standard

* Letters of amino acid shaded mean different from the natural peptides.

Table 2-2. List of synthesized bombesin-like peptides and other peptides

No.	Peptides	Amino acid sequences*	Uses in this study
12	GRP (1-27) amide	APVTAGRGGALAKMYTRGNHWAVGHLM-NH_2	Injections, standard
13	[Cys^1]-GRP (2-27) amide	CPVTAGRGGALAKMYTRGNHWAVGHLM-NH_2	Hapten
14	[Cys^{14}]-GRP (15-27) amide	CGNHWAVGHLM-NH_2	Hapten
15	[Cys^{28}]-GRP (18-27) amide	GNHWAVGHLMC-NH_2	Hapten
16	NMC or GRP (18-27) amide	GNHWAVGHLM-NH_2	Injections, standard
17	N-GRP-EE#	N-acetyl-HWAVGHL-OCH_2CH_3	GRP-R antagonist
18	NMB-10 (23-32) amide	GNLWATGHFM-NH_2	Injections
19	[Cys^{22}]-NMB-10 (23-32) amide	CGNLWATGHFM-NH_2	Hapten
20	[D-Lys^3]-GHRP-6	HwkWfK-NH_2	GHS-R1a antagonist
21	[Cys^0]- [D-Lys^3]-GHRP-6	CHwkWfK-NH_2	Hapten
22	GHRP-6 #	HwAWfK-NH_2	Standard
23	IRL 1620 #	succinyl-DEEAVYFAHLDIIW-NH_2	ET_B agonist

* Letters of amino acid shaded mean different from the natural peptides.

#: N-GRP-EE is N-acetyl-GRP(20-26)-OCH_2CH_3; GHRP-6 is (D-Trp^7,Ala^8,D-Phe^{10})-α-MSH (6-11) amide; IRL 1620 is suc-[Glu^9, $Ala^{11, 15}$]-ET-1 (8–21) amide.

2. Materials and method

2.1. Solid phase peptide synthesis

Solid phase peptide synthesis (SPPS) is based on sequential addition of α-amino and side-chain protected amino acid residues to an insoluble polymeric support.

2.1.1. Protected amino acids

Fmoc-Ala·H_2O, Fmoc-Arg(Pbf), Fmoc-Asn(Trt), Fmoc-Asp, Fmoc-Cys(Trt), Fmoc-Gln(Trt), Fmoc-Glu(OBu^t) ·H_2O, Fmoc-Gly, Fmoc-His(Trt), Fmoc-Ile, Fmoc-Leu, Fmoc-Lys(Boc), Fmoc-Met, Fmoc-Phe, Fmoc-Pro, Fmoc-Ser(Bu^t), Fmoc-Thr(Bu^t), Fmoc-Trp(Boc), Fmoc-Tyr(Bu^t) and Fmoc-Val were used as L-amino acids for synthesis of the peptides. Fmoc-D-Lys(Boc), Fmoc-D-Phe and Fmoc-D-Trp were used for synthesis of peptide No. 21-22, which was shown in Table 2-2. These Fmoc-amino acids were purchased from Peptide Institute, Inc, Japan.

2.1.2. Kaiser test

The presence or absence of free amino groups (deprotection / coupling) was tested using Kaiser test [73] with slight modifications. Approximate 0.5 mL resin beads were transferred from the reaction tube to a 10 mL glass centrifuge tube and washed with 0.7 mL of acetic acid/methanol (1/50, v/v) and dichloromethane (DCM)/methanol (3/2, v/v) sequentially. The washed beads were added 33 μL of phenol/ethanol (4 g/mL), 67 μL of 95% pyridine in water and 25 μL of ninhydrin/ethanol (0.05 g/mL). The mixture was heated by boiling water for 5 min. The presence of deprotected amino acids was indicated by blue resin beads or solution. However, some deprotected amino acids, such as histidine, proline, serine and glutamate, did not yield the expected dark blue. For example, deprotected histidine and asparagines showed red resin beads using this test. In the same way, if N-α-protected amino acid coupled with all the deprotected aimo acid, Kaiser test will show no color.

2.1.3. General procedures of SPPS

In this study, SPPS was conducted manually. Due to the synthesized peptide characteristics of N-terminus and the chain length of amino acid sequence, the resin was chosen for each peptide as shown in Table 2-3. Base-labile Fmoc-group was used for N-α-protection. One day before the synthesis day, each Fmoc-amino acid residue and

1-Hydroxybenzotriazole (HOBt, M.W. 135.12, 2.5 eq. relative to resin loading; Peptide Institute, Inc, Japan) were weighed into a 5 mL glass tube. In case of side-chain protecting with trityl (Trt) residue, weighed 1-[Bis (dimethylamino) methylene]-1 H-benzotriazolium (HBTU, M.W. 379.24, 2.45 eq. relative to resin loading; Peptide Institute, Inc., Japan) into the same tube. The tubes were sealed by Parafilm and stored in desiccators until use.

On the day of synthesis, 1 mL or more N,N-dimethylformamide (DMF, Wake Pure Chemical industries, Ltd., Japan) was added into the tube to dissolve the compounds followed by mixing. After the compounds were homogenized, the activation of the Fmoc-amimo acid was done by adding N,N'-Diisopropyl-carbodiimide (DIPCDI, M.W. 126.2, ρ=0.81g/ml, 2.5 eq. relative to resin loading; Wako Pure Chemical Industries, Ltd., Japan) into each tube, except the tubes containing side-chain protecting with trityl (Trt) residue, mixed well, and the mixture was left more than 1 h before being couple to the deprotected peptide-resin. For the side-chain protecting with trityl (Trt) residue, N-Ethyldiisopropylamine (EDIPA, M.W. 129.25, ρ=0.755g/ml, 5 eq. relative to resin loading; Wako Pure Chemical Industries, Ltd., Japan) was added into the tube just before coupling to the deprotected peptide-resin.

Table 2-3. Resins used for the peptide synthesis

Resin	Loading (mmol/g resin)	Mesh	Peptide No.*
2-Chlorotrityl chloride resin	1.00-1.60	200-400	6, 7, 10,11
H-Phe-2- Chlorotrityl resin	0.96		8
Rink Amide MBHA resin	0.70	100-200	18-22
Rink Amide MBHA resin	0.37	200-400	9
Rink Amide AM resin	0.40-0.80	200-400	12-14
Rink Amide AM resin	0.7	100-200	16
Fmoc-Leu-Wang resin	0.542		17
Fmoc-Trp(Boc)-Wang resin		100-200	23
Fmoc-Cys(Trt) -Wang resin		100-200	15
Sieber Amide resin	0.25-0.65	100-200	1-5

* Peptide No. means the peptide No. listed in Table 2-1 and -2

N-α-protection was cleaved by washing with approximate 1.5-fold resin volume of 10% piperidine (Wake Pure Chemical industries, Ltd., Japan) in DMF followed by the second treatment with the same reagent for 5 min. The deprotection was confirmed using Kaiser test which showed blue resin beads. After the Fmoc cleavage, the peptide-resin was washed with approximate 1.5-fold resin volume of DMF (×4). The next activated Fmoc-amino acid was added into the peptide-resin system for coupling followed by gentle agitation for 15 min. Then the complete coupling was checked using Kaiser test resulting in no color. The cleavage of Fmoc-group was done after the Fmoc-peptide-resin was washed with approximate 1.5-fold resin volume of DMF (×4). After deprotecion of the N-terminal Fmoc group of the last amino acid, the peptide-resin was washed with 2-fold volume of the peptide-resin of DMF(1,5,11), dichloromethane (2,4,6,10), 2-propanol(3,7,9) and methanol(8) as the sequence shown in the parentheses and then lyophilized.

Chemical synthesis of tyrosin O-sulfated peptides (peptide No. 1-8 in Table 2-1) is very difficult because of the acid-lability of the sulfate residue. The synthesis of these peptides was done using the method described by Kitagawa et al [82]. The ethyl ester of N-acetyl-GRP(20-26)-OCH_2CH_3 were synthesized using solution of N-acetyl-GRP(20-26) in ethanol with HCl gas at 0 °C [50].

2.1.4. Resin cleavage and deprotection of side-chain

Side-chain protecting groups are often chosen so as to be able to cleave simultaneously with detachment of the peptide from the resin. General protocols of resin cleavage and deprotection of side-chain describle in Novabiochem® Catalog were followed. Thorough washing and drying must be effective before cleavage. Optimum cleavage conditions are dependent on the individual amino acid residues present, their number and sequence, the side-chain protecting groups, and the type of linker attached to the resin. Final cleavage of the peptide-resin and side-chain deprotection requires strong acid, such as trifluoroacetic acid (TFA). The dry peptide-resin was placed in a flask and added TFA solution containing 10-25 mL/g resin of scavengers (Fig. 2-1). The flask was stoppered and the cocktail was swirled. Reaction time depends on the amino acid sequence and the choice of scavengers used. When the color of the cocktail changed from yellow to white, the reaction finished. The cocktail was thoroughly transferred to a 50 mL of plastic centrifuged tube. Cooled dry ether was added to reach 50 mL of total volume for precipitating the peptide. The precipitate was collected by centrifugation at 1870 × *g* at

4 °C for 15 min and washed twice with cooled dry ether as before. The collected precipitate containing cure peptide and resin was lyophilized and stored at -20 °C until purification.

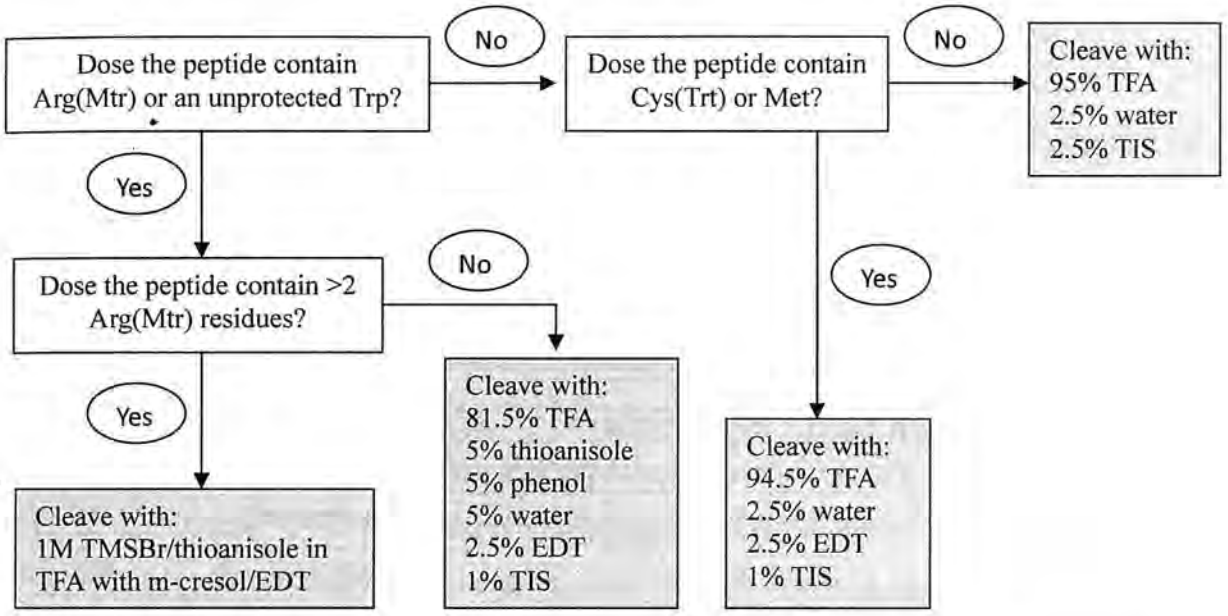

Fig. 2-1. Flow-chart for selecting cleavage cocktail for Fmoc SPPS (Modified from Novabiochem® 2002/3 catalog:3.15). EDT: 1,2-ethanedithiol; TIS: triisopropylsilane; TMSBr:trimethylsilyl bromide.

2.1.5 Purification of the synthesized peptide

The cure peptide containing resin was added to approximate 5-fold volume of 60% CH_3CN in 0.1% TFA and swirled for several hours. The supernatant was collected by centrifugation and then lyophilized. The cure peptide was purified by reverse-phase HPLC using TSKgel ODS-120A column. A solvent system, linear gradient and absorbance were depended on the peptide. The fractions containing the peptide were lyophilized to remove the solvent. The pure peptide was stored at -30 °C until use.

2.2. Raising the first antibodies

In order to measure plasma concentrations of peptide hormones or injected peptides, guinea pigs and chickens were used as host animals for producing specific antibodies.

2.2.1 Preparation of antigen

Peptides in Table 2-4 as hapters were conjugated to mcKLH using sulfo-SMCC (Pierce Chemical Company, U.S.A.) and purified by PD-10 column (GE Healthcare, UK).

Table 2-4. The list of haptens and hosts for producing antibodies

No.*	Haptens	Dose (μg/injection)	Days#	Hosts	Antibody No.&
8	[Cys^{25}]-Gastrin II-9-OH	250	42	guinea pig 1	09G01
				guinea pig 2	09G02
		500	64	Hen 17	10H17, 10Y17
				Hen 18	10H18, 10Y18
10	[Cys^{25}]-Gastrin I-9-OH	500	64	Hen 19	10H19, 10Y19
				Hen 20	10H20, 10Y20
2	[Cys^{1}]-Gastrin II-34	250	42	guinea pig 3	09G03
				guinea pig 4	09G04
13	[Cys^{1}]-GRP (2-27)	250	43	guinea pig 5	09G05
				guinea pig 6	09G06
		500	65	Hen 13	10H13
			64	Hen 14	10H14
14	[Cys^{14}]-GRP (15-27)	250	43	guinea pig 7	09G07
				guinea pig 8	09G08
		500	82	Hen 9	09H09
				Hen 10	09H10
15	[Cys^{28}]-GRP (18-27)	500	65	Hen 11	10H11
			64	Hen 12	10H12
19	[Cys^{22}]-NMB-10 (23-32)	250	78	guinea pig 9	10G11
				guinea pig 10	10G10
21	[Cys^{0}]- [$_{D}$-Lys^{3}]-GHRP-6	250	46	guinea pig 13	11G13
				guinea pig 14	11G14

* means the peptide No. shown in Table 2-1 and -2.

\# Days when the animal suffered to be sampled all blood after the first immunization.

& The first two numbers of antibody's name was the immunization starting year; the middle capital letters "G", "H" or "Y" indicate guinea pig serum, hen serum or IgY, respectively; the last two number are the host animal No..

The conjugate was divided into several tubes, depending on the number of booster injections and dose injected per booster for each animal, and stored at -20 °C until use. On the immunization day, one tube of the frozen conjugate was dissolved and emulsified in Freund's complete adjuvant (Wake Pure Chemical industries, Ltd., Japan) at a 1:1 ratio (v/v). In this study, we chosen guinea pigs and/or hens as host animals, and two same species animals per antigen were used.

2.2.2 Guinea pigs as hosts

The emulsified conjugate, which contained 250 μg peptide, was subcutaneously injected into the back part of a guinea pig. The interval for subsequent booster was one week. After several booster injections, the animal was suffered ether inhalation anesthesia and immediate heart puncture for collecting all blood. Blood samples were stored at 4 °C for 24 h, then serum was isolated by centrifugation at 8000 rpm for 20 min. The serum was stored at -20 °C until assay. Titer and specific check of antiserum were done by RIA. After immunization 42-43 days, average body weight (BW) of guinea pig 1-8 was 756 ± 21 g, the volume of sampled whole blood was 4% BW followed by 2% BW of serum obtained. Each antiserum was named in Table 2-3, such as No. 09G01, the first two numbers mean the immunization starting year, 2009; the middle capital letters "G", "H" or "Y" indicate the host species, guinea pig serum, hen serum or IgY, respectively; the last two number are the host animal number, guinea pig 1.

2.2.3. Chickens as hosts

In this study, hens of laying breeds were used after start to produced eggs for one month. The emulsified conjugate, which contained 500 μg peptide, was subcutaneously injected into the neck of chicken. The interval for subsequent booster was one week. Approximate 1 mL blood was sampled from the wing vein for monitor the titer of antibodies before and after immunization. All eggs were collected for extraction of immunoglobulin in yolk (IgY) before and after immunization since chickens can transfer high quantities of IgY into the egg yolk. After more than 7 booster injections, hens were suffered collection of all blood from wing vein followed heart puncture. Treatment of blood samples was identical to that of guinea pigs.

2.2.4. Extraction of IgY

Extraction of IgY by the water dilution method was done as described by Akita and

Nakai [1] with a slight modification. An Egg was weighed and its yolk was separated from the white. Whole yolk with membrane was thoroughly washed with distilled water followed by removing the membrane. The near 2 or 3 days' yolk was pooled, weighed, mixed and marked as the earliest day's yolk. The mixed yolk was processed as shown in Fig. 2-2. From hen 17-20, 27 randomly weighed eggs were collected. The average weight of eggs was 65.5 ± 0.7 (mean ± SME) g/egg, and the wet weight of yolk and extract IgY obtained were approximately 22% and 0.6% of egg weight.

2.2.4. Detection of the titer, best final dilution and specificity of antiserum and IgY

The detection of the titer, final dilution and specificity of antiserum and IgY was performed using RIA. Titer of antiserum or IgY was tested by a 1:5000 final dilution in 400 μL cocktail. The best final dilution was chosen based on the dilution of antiserum or IgY made of 20-30% ^{125}I-antigen binding in total binding (B/T). Specificity of antibodies was the property which enables them to react with some determinants and not with others. Specificities of antiserum and IgY were determined by constructing binding curves with serial dilution standards.

2.3. Establishment of RIA systems for peptides

In this study, the general method of RIA was described as follows. The peptide containing tyrosine or histidine residues was radio-iodinated using the method of McConahey and Dixon [99]. Assay buffer (pH 7.4) contained 0.05M NaH_2PO_4, 0.9% NaCl, 0.025M EDTA, 0.08% NaN_3 and 1% BSA. A serial of 2-fold dilution of cold standard was prepared with assay buffer. The first antibodies were diluted with assay buffer based on the final dilution required. Assay cocktail per assay tube was 100 μL assay buffer, 100 μL plasma or cold standard, 100 μL final dilution antiserum, and 100 μL tracer (8,000-10,000 cpm, containing 1% normal serum of the fist antibody's host animal). The reactants were incubated for 24 hours at 4 °C. Then, 1 mL/tube of precipitating reagent (2-3% second antibody, 0.9% NaCl, 0.025M Na_2EDTA, 0.08% NaN_3, 0.05% Triton X-100, 3% PEG-6000 and 0.05M NaH_2PO_4, pH 7.4) was added and incubated for 30 min at 4 °C. The bound and free ligands were separated by centrifugation at 1,870 × *g* for 30 min at 4 °C. The radioactivity of the precipitate was counted with an auto well gamma system (ARC-1000M, Aloka, Tokyo, Japan). Sensitivity, intra- and inter-assay CVs and recovery rate were calculated.

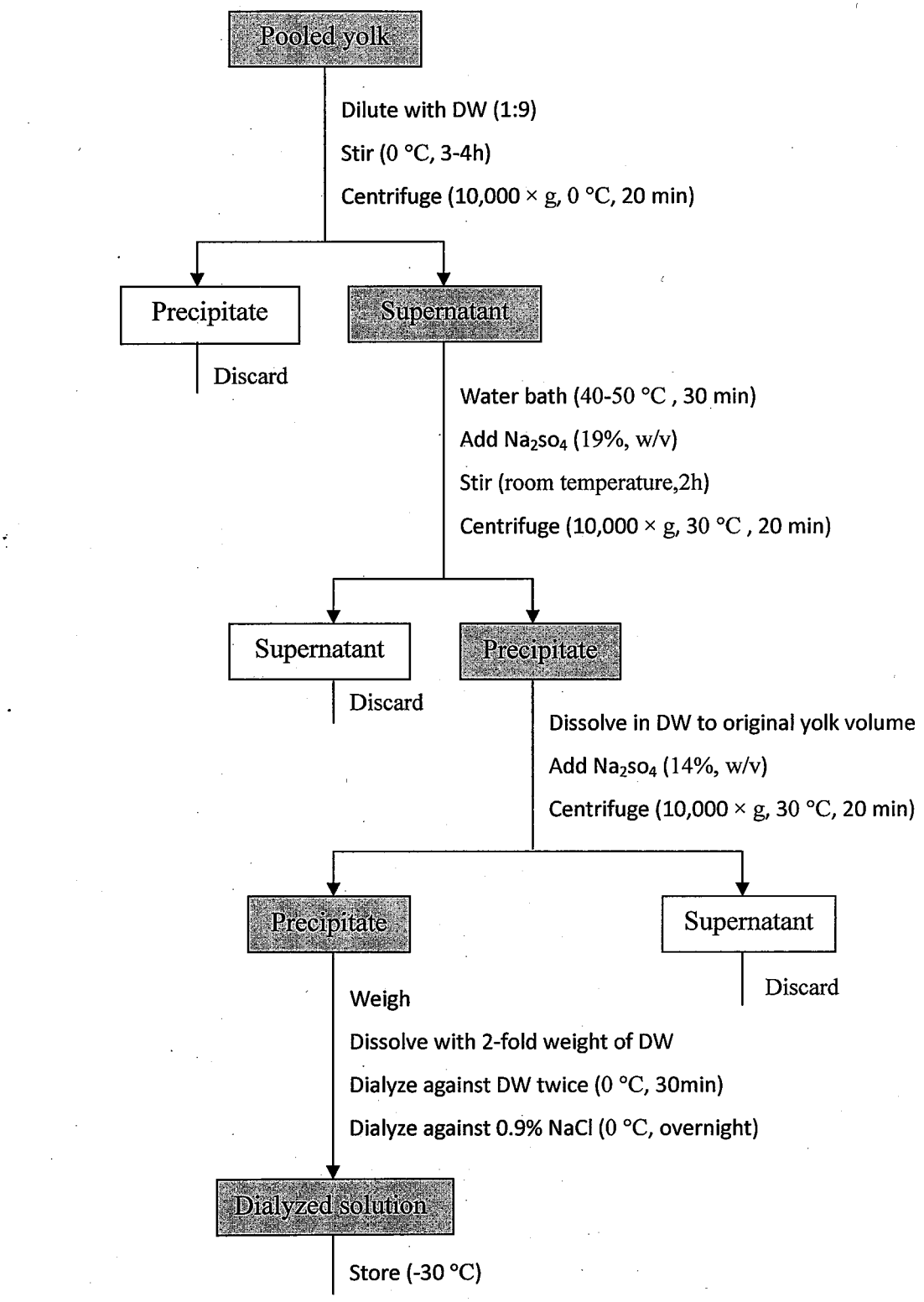

Fig. 2-2. A general outline of IgY purification from egg yolk by the water dilution method. DW: distilled water.

3. Results

3.1. Characters of antibodies

3.1.1. Guinea pig anti-gastrin antisera

On 42-day after the first immunization, guinea pig 1-4 suffered to be sampled all blood. The binding abilities of the antisera 09G01, 09G02, 09G03 and 09G04 were compared using ^{125}I-sulfated gastrin-9 and ^{125}I-non-sulfated gastrin-9 as tracer ligands. Figure 2-3 shows that binding of 09G01 and 09G02 were much stronger with sulfated gastrin-9 than non-sulfated gastrin-9 based on the results of B/T; however, 09G03 and 09G04 equally bound with both sulfated and non-sulfated gastrin-9. Inhibition by various peptides on the 4 antisera binding of ^{125}I-sulfated gastrin-9 is presented in Fig. 2-4. Figure 2-4A and B further proved that inhibition of sulfated form gastrins on binding of ^{125}I-sulfated gastrin-9 with antisera 09G01 and 09G02 was stronger than that of non-sulfated gastrins, and any form of CCK did not inhibit the binding. Fig.2-4 C presents that all forms of gastrin and CCK investigated inhibited the bind of ^{125}I-sulfated gastrin-9 with 09G03.

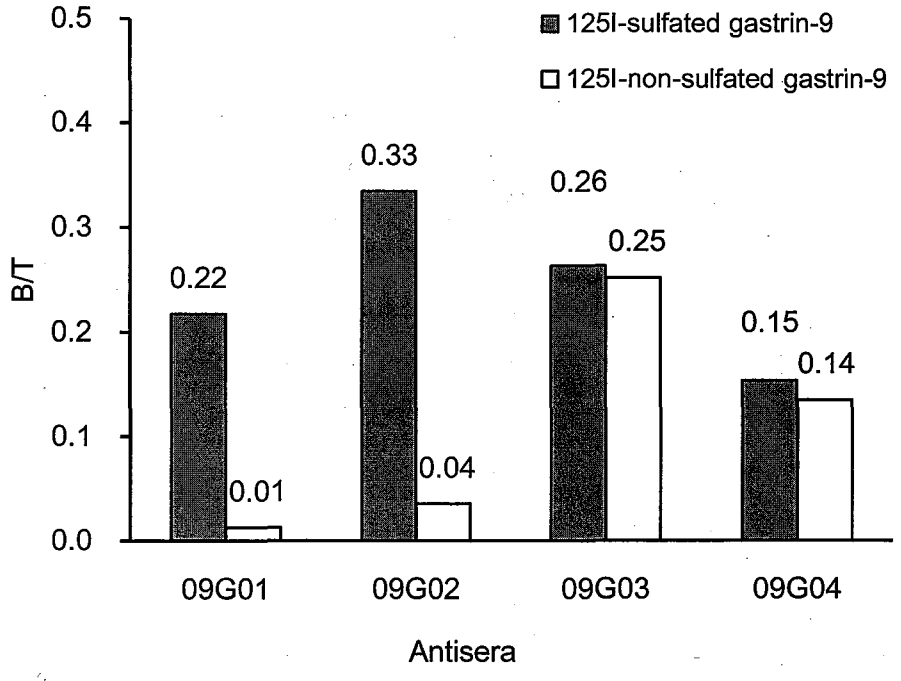

Fig. 2-3. Comparison of the binding ability of guinea pig anti-gastrin sera with sulfated and non-sulfated gastrin. The tracers are ^{125}I-sulfated gastrin -9 and ^{125}I-sulfated gastrin-9.The antiserum 09G01, 09G03, 09G01 and 09G01 are guinea pig 1 and 2 anti- [Cys^{25}-sulfated gastrin 9(26-34)-OH] sera and guinea pig 3 and 4 anti- [Cys^{1}-sulfated gastrin 34(2-34) amide] sera, respectively, at a 1:5000 final dilution. B/T represents binding/binding in total.

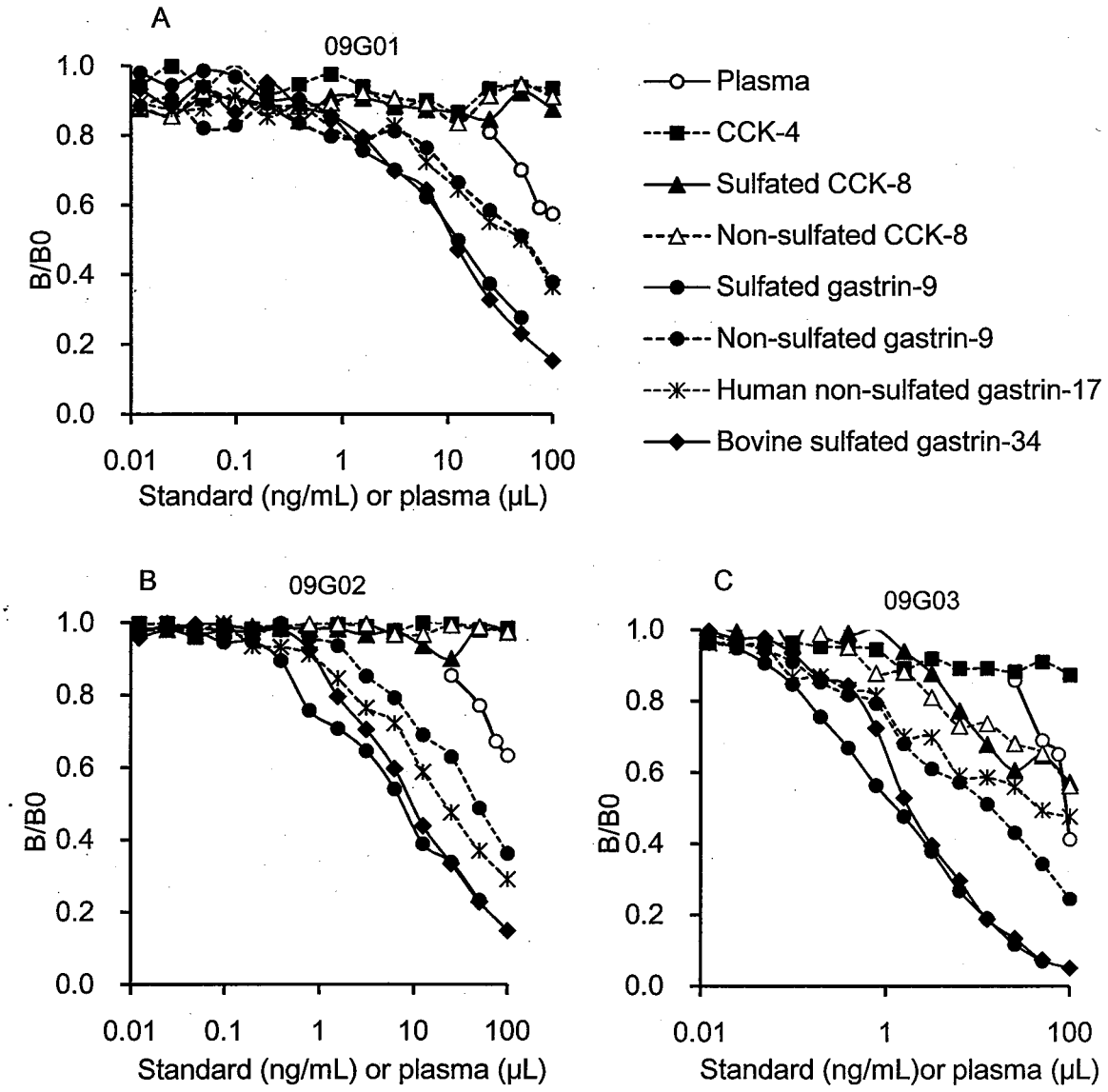

Fig. 2-4. Inhibition by various peptides on the anti-gastrin sera binding of ^{125}I-labeled bovine sulfated gastrin-9. Antiserum 09G01 (A), 09G02 (B) and 09G03 (C) were investigated with a serial dilution of CCK-4, sulfated CCK-8, bovine sulfated gastrin-9, bovine non-sulfated gastrin-9, human non-sulfated gastrin-17, bovine sulfated gastrin-34 and pooled bovine plasma. Each point is the mean of triplicate determinations. B/B0 represents bound/bound in zero standard.

3.1.2 Chicken anti-gastrin antibodies

All blood was collected after the first day of immunization at 55 days for hen 18, and 64 days for hen 17, 19 and 20. Moreover, for investigation the titer changes of antibodies during the first month of the immunization, 1 mL of blood samples was obtained weekly, and subsequently changed to two-day interval. Figure 2-5 shows that the binding of each

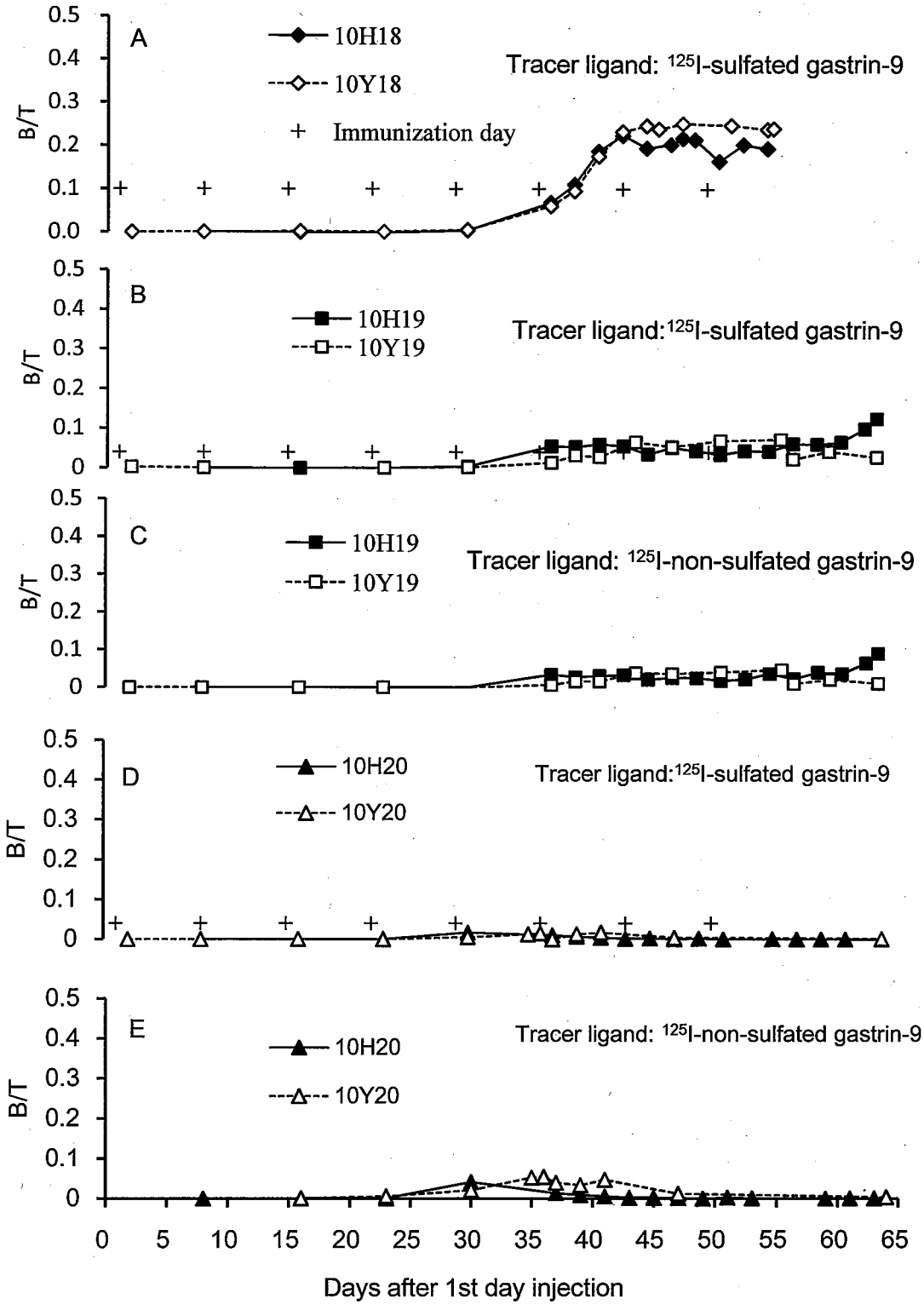

Fig. 2-5. Comparison of the binding ability of chicken anti-gastrin antibodies with ^{125}I-sulfated gastrin -9 (A,B and D) and ^{125}I-non-sulfated gastrin-9 (C and E). The antibody No. 10H18 and 10Y18 are hen 18 anti- [Cys^{25}-sulfated gastrin 9(26-34)-OH] serum and IgY, respectively; and the antibody No. 10H19 and 10Y19 are hen 19 anti- [Cys^{25}-non-sulfated gastrin 9(26-34)-OH] serum and IgY, respectively; the antibody No.10H20 and 10Y20 are hen 20 anti- [Cys^{25}-non-sulfated gastrin 9 (26-34)-OH] serum and IgY, respectively. The antibody was used at a 1:5000 final dilution. B/T represents binding/binding in total.

radio-ligand, ^{125}I-sulfated gastrin-9 or ^{125}I-non-sulfated gastrin-9 to each antiserum or IgY at a 1:5000 of the final dilution. Both radioligands could not bind with hen 17's serum and IgY (10H17 and 10Y17, data not shown).

Figure 2-5A shows that on 37-day after the first immunization, the serum and yolk of hen 18 (10H18 and 10Y18) had presented the antibody which could bind with sulfated gastrin (B/T = 0.068 and 0.061 for 10H18 and 10Y18, respectively), and on 43-day arrived the peak (B/T = 0.225 for 10H18) and nearly continued to the last sampling day, 55-day. However, ^{125}I-non-sulfated gastrin-9 could not bind with 10H18 and 10Y18 (data not shown). The inhibition of sulfated or non-sulfated gastrin and CCK (concentration ranged from 100,000-12 pg/mL) on the binding of ^{125}I-sulfated gastrin-9 to 10H18 on 48-day further confirmed that 10H18 selectively bound with sulfated form gastrins, and with over 25 ng/mL of non-sulfated gasrtin-9; however, human non-sulfated gastrin-17 and any forms of CCK investigated could not inhibit the binding of ^{125}I-sulfated gastrin-9 to 10H18 (Fig. 2-6A).

Figure 2-5B and C show that on 37-day after the first immunization, the antibody which could bind with both sulfated and non-sulfated gastrin-9 have presented with low titer (B/T = 0.055 for 10H19 to ^{125}I-sulfated gastrin-9). The titer was still low till the 59-day, even though the last sampling day, 64-day, it was slightly increased (B/T = 0.124 for 10H19 to ^{125}I-sulfated gastrin-9) on 60 day. Results (Fig.2-6B) from the inhibition test were observed that 10H19 on 64-day bound with all forms of gastrin and CCK detected, except CCK-4.

Figure 2-5D and E show that on 30-day after the first immunization, hen 20 was observed to produce the antibody which binding with non-sulfated gastrin-9 (B/T= 0.042 for 10H20) was stronger than with sulfated gastrin-9 (B/T= 0.017 for 10H20), however, the titer was too low and did not increase till the last sampling day, 64-day.

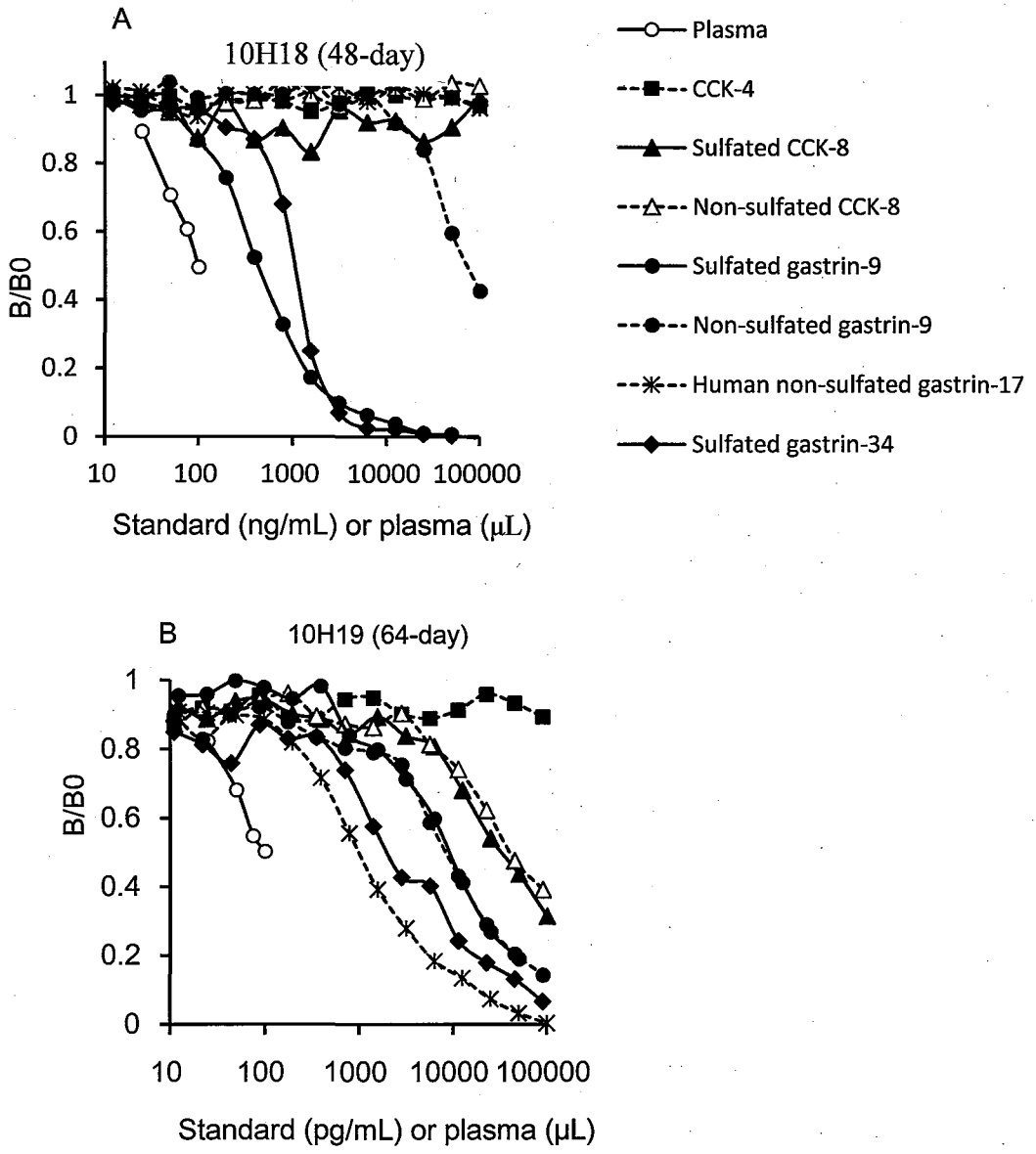

Fig. 2-6. Inhibition by various peptides on antiserum 10H18 and 10H19 binding of ^{125}I-labeled bovine sulfated gastrin-9. Hen 18 anti- [Cys^{25}-sulfated gastrin 9(26-34)-OH] serum 10H18 and hen 19 anti-[Cys^{25}-Gastrin I-9-OH] serum 10H19 were investigated with serials dilution of CCK-4, sulfated CCK-8, bovine sulfated gastrin-9, bovine non-sulfated gastrin-9, human non-sulfated gastrin-17, bovine sulfated gastrin-34 and pooled bovine plasma. Each point is the mean of triplicate determinations. B/B0 represents bound/bound in zero standard.

3.1.3. Guinea pig and hen anti-GRP antibodies

Guinea pig 5-8 were suffered sampling all blood on 43-day after the first immunization. The antisera from the four guinea pigs bound with both ^{125}I-NMC and ^{125}I-GRP, but the bindings with ^{125}I-GRP were stronger than with ^{125}I-NMC (Fig. 2-7). These results also were proved by the inhibition test of antiserum 09G05 (Fig.2-9A) and 09G06 (Fig.2-9B). In another test, antiserum 09G05 was found also can react with N-GRP-EE, the antagonist of GRP-R (Fig. 6-1).

The antiserum 10H11 from hen 11 on 37-day after the first immunization have presented the antibodies against NMC (B/T= 0.284, Fig. 2-8B). On-53 day, B/T of 10H11 was much higher for ^{125}I-NMC than for ^{125}I-GRP (Fig. 2-7). The available final dilution in 400 μL of cocktail for 10H11 on-65 day was 1:30,000 (B/T = 0.293 with ^{125}I-NMC as tracer). The antiserum 10H13 from hen 13 on 37-day after the first immunization have presented the antibodies against GRP (B/T= 0.139, Fig. 2-8A). On-53 day, B/T of 10H13 was much higher for ^{125}I-GRP than for ^{125}I-NMC (Fig. 2-7). The available final dilution in 400 μL of cocktail for 10H13 on-64 day was 1:8000 (B/T = 0.307 with ^{125}I-GRP as tracer). Hen 9, 10, 12 and 14 did not produce the detectable antibodies against NMC and/or GRP (Fig. 2-7 and Fig. 2-8A)

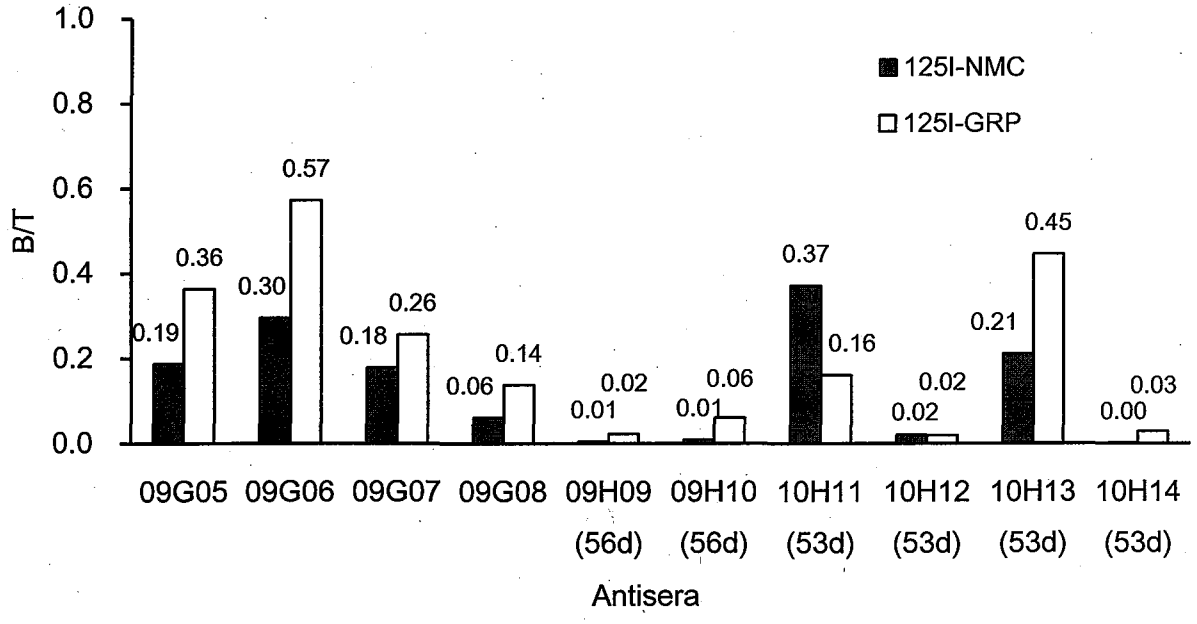

Fig. 2-7. Comparison of the binding ability of guinea pig and chicken anti-GRP sera with radioligands. The radioligands were ^{125}I-NMC and ^{125}I-GRP.The antisera No. 09G05, 09G06, 09H13 and 09H14 were guinea pig 5 , 6, and hen 13, 14 anti- [Cys^{1}]-GRP (2-27) sera, respectively; 09G07, 09G08, 09H09 and 09H10 were guinea pig 7 , 8, and hen 9, 10 anti- [Cys^{14}]-GRP (15-27) sera, respectively; 09H11 and 09H12 were hen 11 and 12 anti- GRP (18-27)-[Cys^{28}] sera, respectively. The sera were at a 1:5000 final dilution. B/T represents total binding counts/total counts.

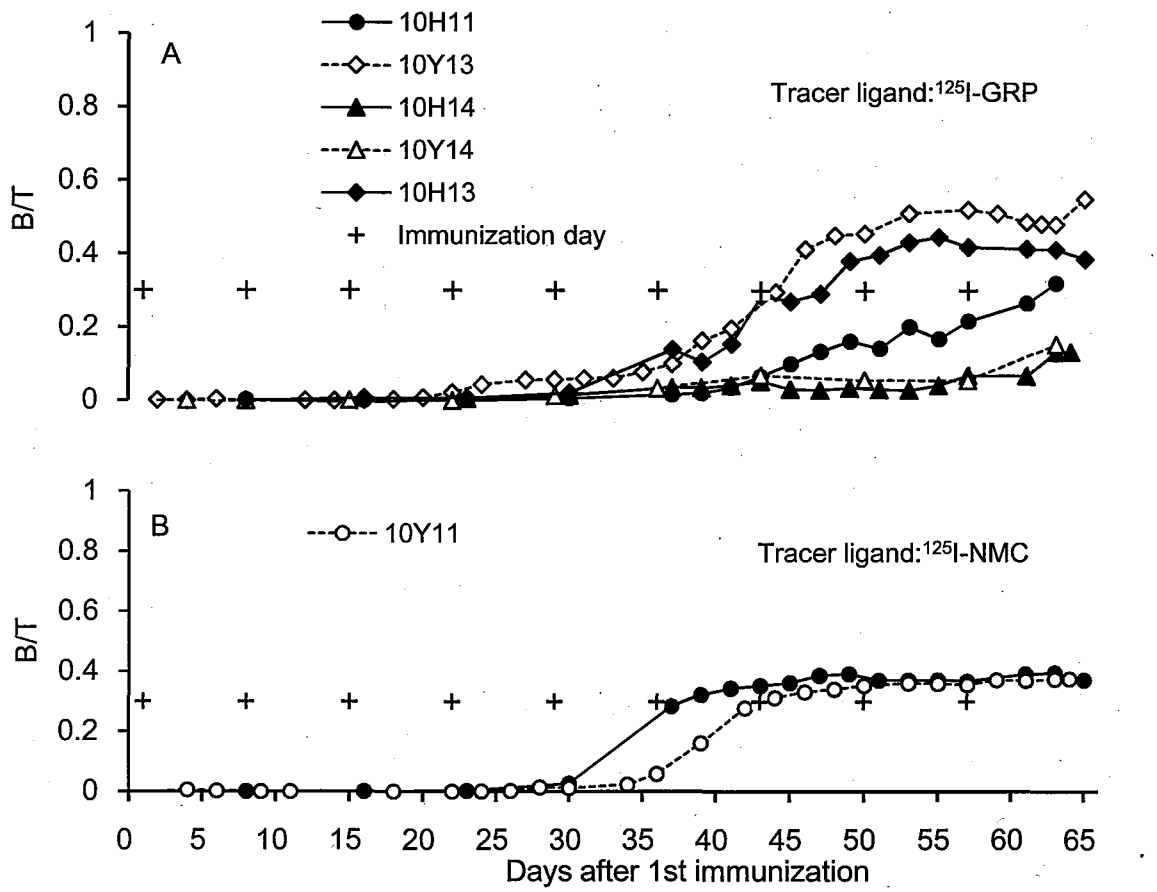

Fig. 2-8 The binding chances of chicken anti-GRP antibodies with ^{125}I-GRP (A) and ^{125}I-NMC (B) after immunization. H means hen serum and Y means egg IgY. The antibodies were used at a 1:5000 final dilution. B/T represents total binding counts/total counts.

3.1.4 Guinea pig anti-[D-Lys^3]-GHRP-6 sera

Guinea pig 13 and 14 were suffered sampling a serial blood after the first immunization and all blood was sampled on 46-day after the first immunization. The antisera 11G13 and 11G14 on 37-day after the first immunization have presented the antibodies against [D-Lys^3]-GHRP-6 (B/T= 0.082 for 10G13, B/T = 0.259 for 10G14, Fig. 2-10). The available final dilution in 400 μL of cocktail for 11G13 and 11G14 on-46 day was 1:3000 and 1:5000, respectively. When the radioligand was ^{125}I-[D-Lys^3]-GHRP-6, the half maximal inhibitory concentration (IC_{50}) of 11G13 was 20.50 ng/mL for [D-Lys^3]-GHRP-6, and more than 320 ng/mL for GHRP-6 and GHRP-2 (Fig.2-11A); IC_{50} of 11G14 was 13.70, 62.29, 85.12 ng/mL for [D-Lys^3]-GHRP-6, GHRP-6 and GHRP-2, respectively (Fig.2-11B). Both 11G13 and 11G14 did not bind with bovine ghrelin and α-MSH (Fig.2-11).

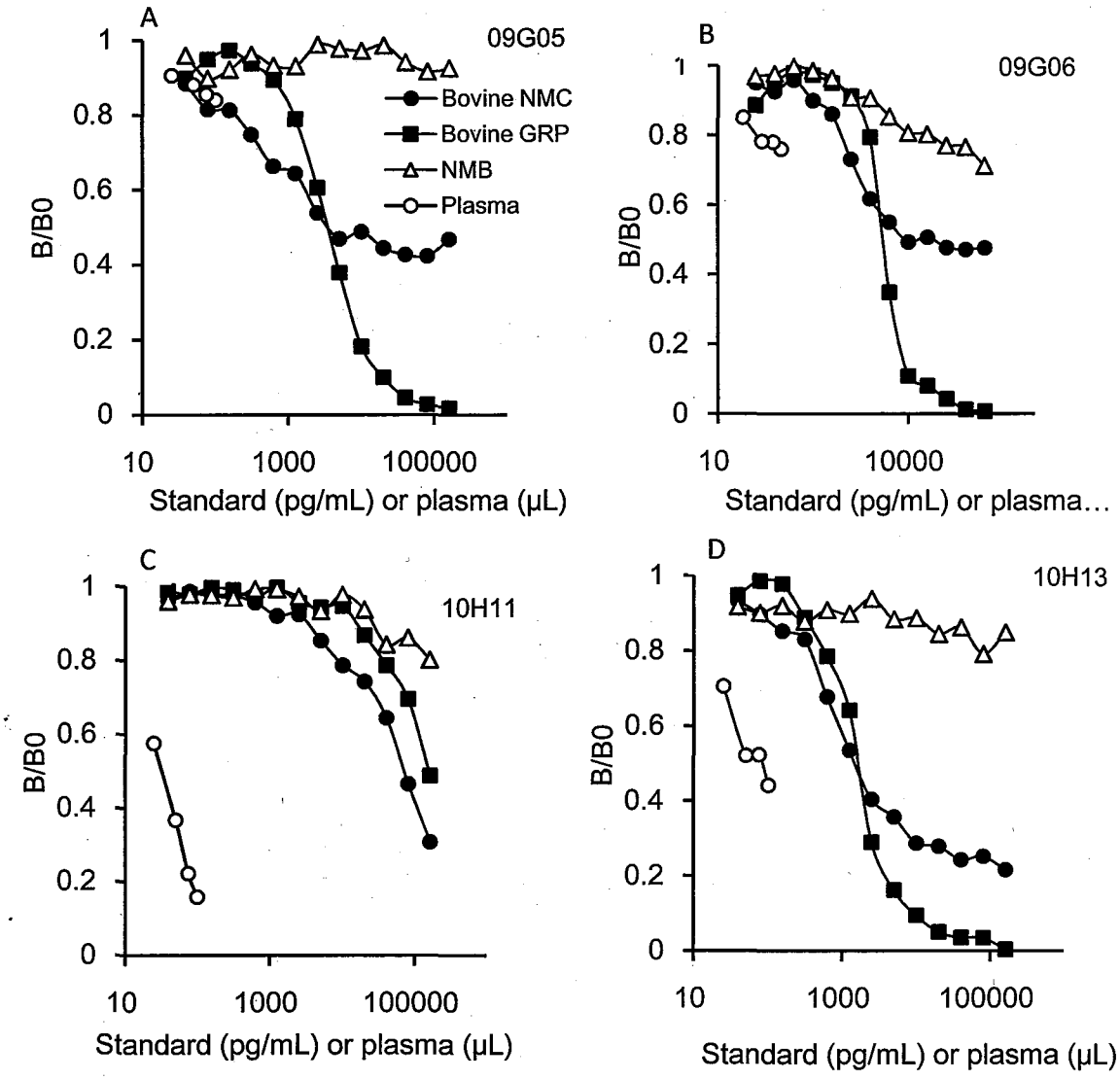

Fig. 2-9. Inhibition by various peptides on guinea pig and chicken antisera binding of ^{125}I-bovine GRP. Antisera 09G05 (A), 09G06(B), 10H11(C) and 10H13 were investigated with serials dilution of bovine NMC, GRP, NMB and pooled bovine plasma. Each point is the mean of triplicate determinations. B/B0 represents bound/bound in zero standard.

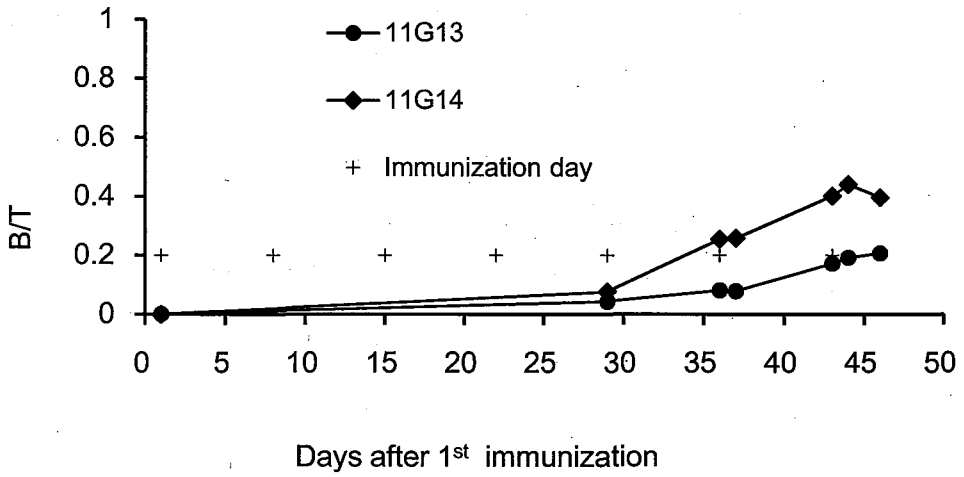

Fig. 2-10. The binding chances of guinea pig anti-[D-Lys^3]-GHRP-6 with ^{125}I-[D-Lys^3]- GHRP-6 after immunization. The antisera were used at a 1:5000 final dilution. B/T represents total binding counts/total counts.

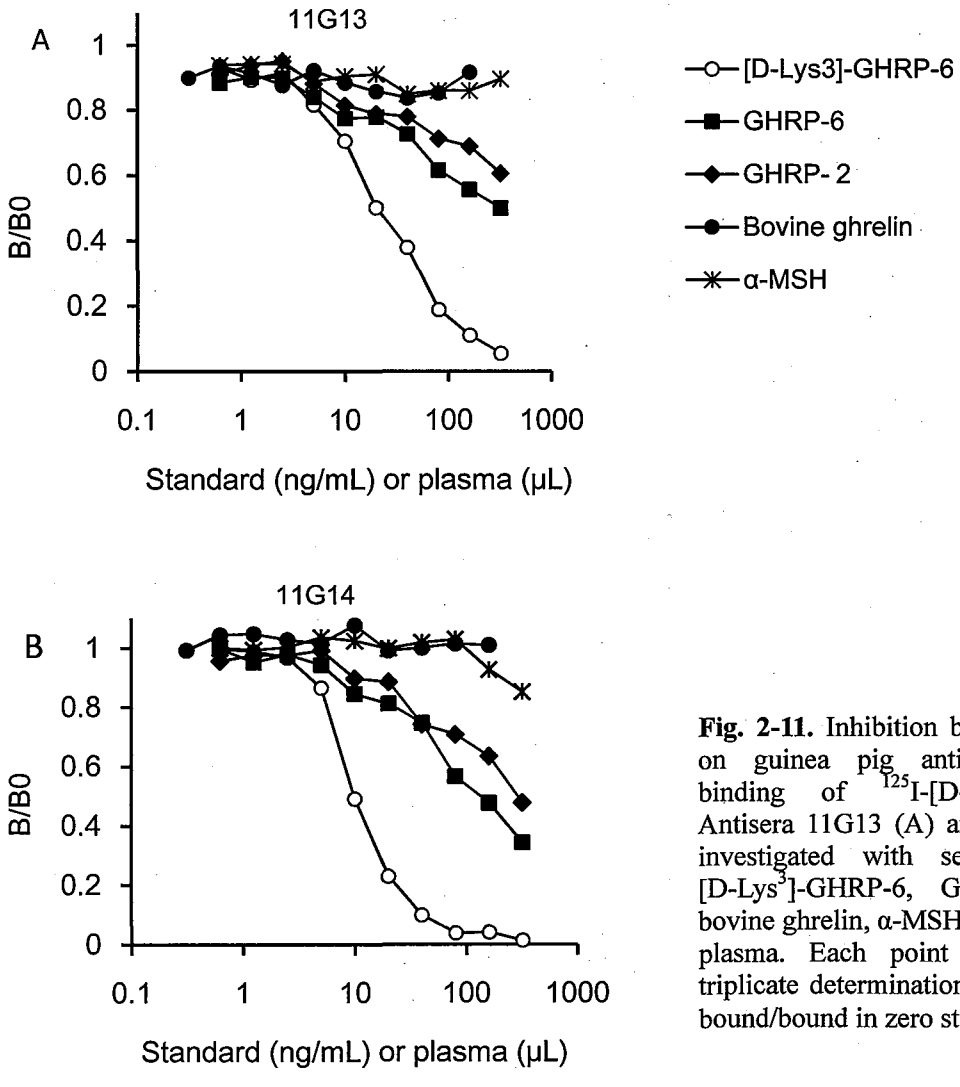

Fig. 2-11. Inhibition by various peptides on guinea pig anti-[D-Lys^3]-GHRP-6 binding of ^{125}I-[D-Lys^3] -GHRP-6. Antisera 11G13 (A) and 11G14(B) were investigated with serials dilution of [D-Lys^3]-GHRP-6, GHRP-6, GHRP-2, bovine ghrelin, α-MSH and pooled bovine plasma. Each point is the mean of triplicate determinations. B/B0 represents bound/bound in zero standard.

3.1.5 Guinea pig anti-NMB sera

Fig. 2-12 shows that B/T of 10G09 and 10G10 until 78-day after the first immunization was less than 0.05 at 1:5000 final dilution binding with ^{125}I-NMB-10. Therefore, those two sera were not available for RIA for NMB.

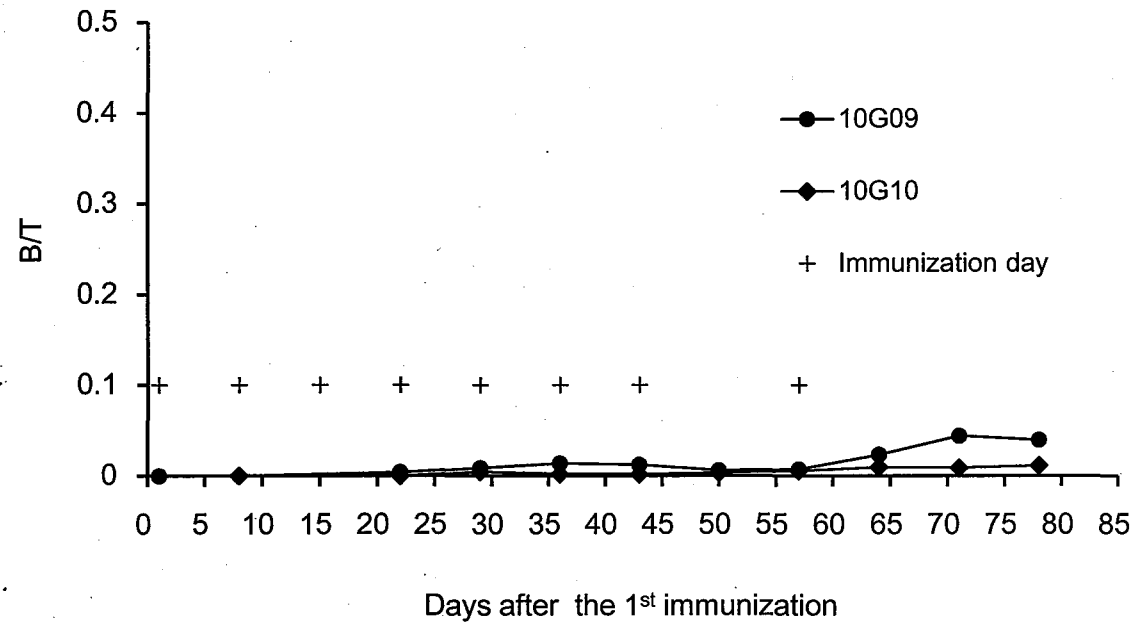

Fig. 2-12. The binding chances of guinea pig anti-[Cys^{22}]-NMB-10 (23-32) with ^{125}I- NMB-10 after the first immunization. The sera were used at a 1:5000 final dilution. B/T represents total binding counts/total counts.

3.2. RIA systems for peptides

In this study, we have established the RIA for sulfated gastrin, total gastrin (including sulfated and non-sulfated gastrin), GRP, N-GRP-EE (an antagonist for GRP-R) and [D-Lys^3]-GHRP-6 (an antagonist for GHS-R1a) using the antibodies from guinea pigs and/ or chickens. The tracer, the first antibody and cold standard of each RIA system were shown in Table 2-5.

Table 2-5. RIAs for gastrin, GRP, N-GRP-EE and [D-Lys^3]-GHRP-6

RIA for	Tracers	1st Ab		2nd Ab	Cold standards		
		No.	Final dilution		Peptide	Ranged (ng/mL)	IC_{50}(ng/mL)
Sulfated gastrin	Gastrin II-9	10H18	1:5000	Y*	Gastrin II-9	25-0.01	0.6
					bGastrin II-34	12.5-0.1	0.7
Total gastrin	Gastrin II-9	09G01	1:5000	G*	Gastrin II-9	200-0.1	10.6
					hGastrin I-17	200-0.1	48.5
					bGastrin II-34	200-0.2	7.6
		09G02	1:5000	G	Gastrin II-9	200-0.1	7.3
					hGastrin I-17	200-0.1	11.8
					bGastrin II-34	200-0.4	8.4
GRP	bGRP(1-27)	09G05	1:5000	G	bNMC	10-0.08	12.1
					bGRP(1-27)	160-0.2	2.6
		10H13	1:10000	Y	bNMC	20-0.2	3.7
					bGRP(1-27)	40-0.2	1.8
N-GRP-EE	bNMC	09G05	1:3000	G	N-GRP-EE	160>-<0.02	0.4
[D-Lys^3]-GHRP-6	[D-Lys^3]-GHRP-6	11G13	1:1000	G	[D-Lys^3]-GHRP-6	320-2.5	20.5
		11G14	1:3000	G	[D-Lys^3]-GHRP-6	80-2.5	12.7

* Y: 3% goat anti-IgY serum; G: 3% goat anti-IgY serum.

5. Discussion

After the first immunization, we collected a serial of blood samples from all hens and some guinea pigs, and extracted IgY from each egg, so we could investigate when the animals produced high titer antibodies. Generally, results from hen 11, 13 and 18 and guinea pig 13 and 14 (Fig. 2-5, -8 and -10) showed that the specific antibodies had been produced by at least one month after the first immunization and achieved the relative high titer on 40-day. The name IgY was proposed in 1969 by Leslie and Clem based on physical-chemical and antigenic differences between the immunoglobulin in chicken serum as well as yolk, and immunoglobulin G (IgG) in mammalian serum [92]. We observed that the B/T curves of IgY were usually shifted to the right compared with those of antisera from the same hen, indicating produce of the specific IgY in yolk was later than that in serum. This phenomenon confirmed the theory that yolk IgY was transferred from serum [71]. For raising specific antibodies, we recommended that at least two animals of the same species per antigen should be used. This principle can reduce potential total failure resulting from non-responsiveness to antigens of individual animals. For example, in this study, Hen 11 and 12 were immunized with [Cys ^{28}]-GRP(15-27), but only hen 11 produced anti-GRP antibodies (Fig. 2-8).

In mammalian, amino acid sequences of gastrin and CCK are identical in the C-terminal five amino acids (-Gly-Trp-Met-Asp-Phe-NH_2). Therefore, it is difficult to raise antibodies which are only specific for gastrin or CCK due to the close homology of gastrin and CCK. On the other hand, gastrin has two forms, tyrosyl sulfate or non-sulfate in position 6 as counted from the C-terminus. In order to yield antibodies that can specifically bind with bovine sulfated gastrin or total gastrin (including sulfated and non-sulfated form), three haptens (Peptide No. 2, 8 and 10 in Table 2-3) were designed based on the amino sequences of bovine gastrin and CCK. Peptide No. 8, [Cys^{25}]-Gastrin II-9-OH, as a hapten was immunized to two guinea pigs (NO. 1 and 2) and two hens (NO. 17 and 18). The reason for using the C-terminal non-amidated form of gastrin was the hope of raising an antibody without cross-reaction to the amidated C-terminus, which is common in gastrin and CCK. Surprisingly, the antisera (09G01 and 09G02) from both guinea pigs bound with both sulfated and non-sulfated gastrin and displayed no reactivity with any forms of CCK (Fig. 2-4A and B); however, the antiserum (10H18) from hen 18 specifically bound with sulfated gastrin and showed no reactivity with non-sulfated gastrin

or any forms of CCK investigated (Fig. 2-6A). Therefore, antisera 09G01 and 09G02 as the first antibodies were used for total gastrin RIA; while 10H18 was used for sulfated gastrin RIA (Table. 2-4). These results indicated that the specificity of antibodies from different species host animals might be distinct even using a same antigen. The different specificity of anti-gastrin antibodies was mainly due to the quite difference of amino acid sequence of gastrin between guinea pigs and hens (Fig. 1-7). Especially, aside from the C-terminal tetrapeptide and the tyrosyl residue, the molecule of chicken did not resemble other known forms of gastrin or CCK [26]. In a way, chickens may not have real gastrin. The antisera from guinea pig 3 and 4 (09G03 and 09G04) and hen 19 (10H19) were found to bind with all forms of gastrin and CCK investigated, except CCK-4. The results indicated that these three antibodies may, at least, specially recognize the glycine residue in position 5, which CCK-4 has no, as counted from the C-terminus. So, 09G03 and 09G04 can use as the first antibodies for assay the total peptide including gastrin and CCK by RIA, but 10H19 is not available since the titers of specific antiserum and IgY for gastrin and CCK were too low (at a 1:500 final dilution).

In order to yield antibodies that can specifically bind with bovine GRP_{1-27} or NMC, three haptens (Peptide No. 13, 14 and 15 in Table 2-3) were designed based on the amino sequences of bovine GRP. Among the three haptens, Peptide No.13, [Cys^1]-GRP(2-27) made guinea pig 5 and hen 13 produced the satisfactory antibodies, 09G05 and 10H13, respectively, for GRP RIA (Table. 2-4). Although the titer of antiserum 10H11 was relatively high (at a 1:30,000 final dilution) compared with other antibodies raised in this study, but the basal level of ir-GRP in bovine plasma measured by RIA using this antiserum was too high , more than 200 ng/mL, compared with less than 5 ng/mL reported in humans [43, 119]. This result indicated that the specificity of 10H11 was low for plasma samples and 10H11 could not be used without further purification.

For real-time monitoring the plasma concentration of [D-Lys^3]-GHRP-6 after administration, guinea pig 13 and 14 were immunized with [Cys^0]-[D-Lys^3]-GHRP-6 and produced useful antisera 11G13 and 11G14 though the titers of the two antisera were not so high (Table. 2-4). The specificity of 11G13 was higher than that of 11G14 since 11G14 slightly bound with GHRP-2 and -6, which were not the natural peptides in the body (Fig. 2-11). However, if [D-Lys^3]-GHRP-6 and GHRP-2 and/or -6 were not administered together, the different specificity of the two antisera may not affect the assay for [D-Lys^3]-GHRP-6.

5. Summary

In the Chapter 2 study, 23 peptides were synthesized, including [Pyr^{1}]-Gastrin II-34, [Cys^{1}]-Gastrin II-34, [Cys^{17}]-Gastrin II-17, [Pyr^{18}]-Gastrin II-17, [Tyr^{18}]-Gastrin II-17, Gastrin II-9, [Tyr^{25}]-Gastrin II-9, [Cys^{25}]-Gastrin II-9-OH, [Pyr^{18}]-Gastrin I-17, Gastrin II-9, [Cys^{25}]-Gastrin-9, GRP_{1-27}, [Cys^{1}]-GRP_{2-27}, [Cys^{14}]-GRP_{15-27}, [Cys^{28}]-GRP_{18-27}, NMB-10, NMC, N-GRP-EE, [Cys^{22}]-NMB-10, [D-Lys^{3}]-GHRP-6, [Cys^{0}]- [D-Lys^{3}]-GHRP-6, GHRP-6, IRL 1620. These peptides were used as haptens for generating the first antibodies, cold standards and iodine-labeled as tracers for RIA or administered to animals. Guinea pigs and/or hens have produced specific antibodies which include antibodies 10H18 and 10Y18 for sulfated gastrin, antisera 09G01 and 09G02 for total gastrin, antisera 09G03 and 09G04 for both gastrin and CCK, antibodies 09G05 and 10H13 for both GRP and N-GRP-EE (an antagonist for GRP-R), antisera 11G13 and 11G14 for [D-Lys^{3}]-GHRP-6 (an antagonist for GHS-R1a). Therefore, RIA systems using the raised first antibodies were established for measurement of sulfated gastrin, total gastrin, GRP, N-GRP-EE and [D-Lys^{3}]-GHRP-6 in plasma.

Chapter 3

Involvement of Receptors in Endothelin-induced Increase of Ghrelin and Growth Hormone

1. Introduction

Ghrelin, an endogenous ligand for growth hormone (GH) secretagogue receptors, was firstly purified from stomach and reported as a GH-releasing peptide in rat by Kojima et al. [55, 84]. Exogenous ghrelin also stimulates GH secretion in ruminants [48, 64, 154, 159, 160]. Growth hormone secretagogue receptor type 1a (GHS-R1a) has been shown to mediate the GH-releasing action of ghrelin in vitro and in vivo [57, 103]. Acyl modification on Ser^3 is essential for the binding of ghrelin to GHS-R1a. However, des-acyl ghrelin does not interact with this receptor. Both acyl and des-acyl ghrelin are circulating in bloodstream in significant amounts [56, 160].

In addition to the simulation of GH secretion, ghrelin has other multifaceted roles, including the regulation of food intake and body weight (BW) [164]. It is of interest to examine the regulation of ghrelin secretion by hormones. Gastrin, cholecystokinin, adrenaline and noradrenaline were found to increase ghrelin levels [23, 35, 113]. Our previous study has demonstrated that endothelins (ETs) stimulate ghrelin secretion in cattle [157]. Endothelins are a family of three peptides known as endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3) in mammalian species [175]. Endothelins, besides their vasopressor action, exert some influence on various endocrine organs such as the adrenals, gonads, thyroid, parathyroid and pituitary [166, 167]. The effects of ETs are mediated through two G protein-coupled receptor subtypes termed endothelin A (ET_A) and endothelin B (ET_B).

In our previous study, we also found that ET-1 or ET-3 increased the levels of GH in plasma, and that ET-3 was more effective than ET-1 [157]. Therefore, we suggest that the effects of ET-1 or ET-3 on the release of ghrelin and GH may be mediated by ET_B receptors, as ET_A receptors have lower affinity for ET-3 than ET-1, while ET_B receptors

associate equally with these two peptides [6, 141]. The present study was designed to determine the dose-dependent effects of ET-3 on the release of ghrelin, GH, glucose and nonesterified fatty acid (NEFA) in Holstein steers. This study was also attempted to unravel the pharmacological roles of IRL 1620, one of the most widely used selective synthetic agonists [22, 156], in the secretion of ghrelin and GH as well as characterize the involvement of ET_B receptors in this process. In addition, [D-Lys^3]-GHRP-6, a putative GHS-R1a antagonist, was used to examine whether ET-3-induced endogenous ghrelin stimulates GH secretion through GHS-R1a.

2. Materials and Methods

All experimental procedures involving animals were approved (animal protocol numbers: 21-9) by the Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine, Japan.

2.1. Animals

Eight Holstein steers (7-month-old, 209 ± 7 kg BW) were fed with 2 kg/head/day of concentrate (crude protein 16.0%, crude fat 2.5%, crude fiber 9.0% and crude ash 10.0%, Nishin Marubeni, Japan) twice daily (at 09:00 and 16:00). Timothy hay, salt block and water were supplied ad libitum. One day before the experiment, a sterilized polyethylene catheter was inserted non-surgically into the external jugular vein of animals for both peptide injection and blood sampling. The patency of catheter was maintained with heparin saline. During administration and sampling periods, animals were loosely chained to the stanchion and allowed hay and water ad libitum.

2.2. Peptides

Bovine ET-3 (CTCFTYKDRECVYYCHLDIIW), IRL1620 ([succinyl-(Glu^9, Ala^{11,15})-ET-1(8–21)], suc-DEEAVYFAHLDIIW) and [D-Lys^3]-GHRP-6 (HwkWfK-NH_2) were synthesized by Fmoc (9-fluorenylmethoxycarbonyl) solid-phase peptide synthesis procedures and purified by reverse-phase HPLC (TSKgel ODS-120A column; linear gradient of 0%-60% CH_3CN). Purified peptides were lyophilized and stored at -30 °C. These peptides were used for administration or as the cold standard, or they were labeled for radioimmunoassay (RIA). All ^{125}I-labeled peptides used for RIA were radioiodinated by the chloramine T method [153] and purified by HPLC.

2.3. Administration of peptides and blood sampling procedures

Endothelin-3 and [D-Lys^3]-GHRP-6 were dissolved in sterilized double distilled water at a concentration of 1 mg/mL and 10 mg/mL, respectively. IRL 1620 was dissolved in sterilized 10 mM NaOH at a concentration of 1 mg/mL and neutralized to pH 8 with 1 M HCl. The dissolved peptides were stored at -30 °C. On the day of administration, frozen peptides were thawed and diluted into 5 mL of 0.1% bovine serum albumin (BSA) in saline.

Steers were randomly assigned to a complete blocked Latin Square Design (eight animals × eight treatments × eight days of treatment with one or two days recovery between each treatment) to receive intravenous bolus injections of 0.1% BSA-saline as vehicle, ET-3 (0.1, 0.4, 0.7, 1.0 μg/kg BW), IRL1620 (2.0 μg/kg BW), [D-Lys^3]-GHRP-6 (20.0 μg/kg BW), and ET-3(1.0 μg/kg BW) combined with [D-Lys^3]-GHRP-6 (20.0 μg/kg BW), respectively, at 12:00. The highest dose of ET-3, 1.0 μg/kg, was decided based on the results of a previous study [157] and 3.3 μg/kg bolus of ET-1 causing death in sheep [146]. The dose of IRL1620, 2.0 μg/kg, was expected to have the same effects as 1.0 μg/kg of ET-3, as some effects of IRL1620 were approximately one third of ET at the same molar concentration [36, 116]. The dose of [D-Lys^3]-GH RP-6, 20 μg/kg, was determined according to a study in sheep [154].

Blood samples were withdrawn from the catheter at -30, -15, 0, 5, 10, 15, 20, 25, 30, 35, 40, 50, and 60 min relative to injection time. Samples were put into pre-ice-chilled tubes containing heparin (8 IU/mL blood). Plasma was isolated after centrifugation (1870×g, 30min, 4 °C) and stored at -30 °C. For ghrelin assay, 50 μL of 1 M HCl was added to 1 mL of plasma and stored at -30 °C until analyzed.

2.4. Measurement of plasma hormones, IRL 1620 and metabolites

2.4.1. Measurement of plasma hormones and IRL 1620

Concentrations of acyl and total ghrelin in plasma were measured by double antibody RIA as previously described [160]. Sensitivities of ghrelin assay were 33.0 pg/mL and 0.32 ng/mL, intra-assay coefficients of variation (CVs) were 11% and 12%, inter-assay CVs were 13% and 15% for acyl ghrelin and total ghrelin RIA, respectively.

The concentration of GH in plasma was assayed by double antibody RIA procedures [138] with a slight modification. NIDDK-oGH-I-5 (lot AFP12855B) was used as standard and iodinated ligand. Samples were incubated at 4 °C throughout the assay. Sensitivity,

intra- and inter-assay CVs were 0.11 ng/mL, 10% and 14%, respectively.

Radioiodinated bovine ET-3 was used for assays of ETs and IRL 1620 in plasma as reported by ThanThan et al. [157]. Anti-endothelin-1 antiserum (catalog number E1645, Lot: 126K4848, Sigma Chemical Company) was used at a final dilution of 1:5000 for the ETs RIA system. The antiserum had almost equal affinity for bovine ET-1, human ET-2 and bovine ET-3 [157]. In this study, we found that the antiserum equally recognized bovine ET-3 and IRL 1620 (Fig. 3-1). Therefore, the concentrations measured by the ETs RIA system, irrespective of containing IRL 1620, were termed ETs concentrations. Sensitivity, intra- and inter-assay CVs were 0.5 ng/mL, 11% and 13%, respectively.

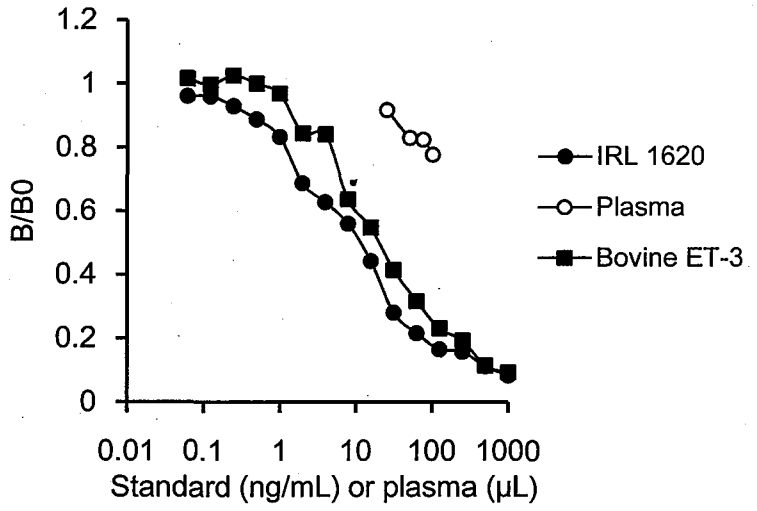

Fig. 3-1. Standard radioimmunoassay curves for bovine endothelin-3 (ET-3) and IRL 1620. Inhibition of ^{125}I-labeled bovine ET-3 binding to anti-ET-1 antiserum by a serial dilution of bovine ET-3, IRL1620 and pooled bovine plasma was obtained. Each point was the mean of triplicate determinations. B/B0, bound/ bound in zero standard.

2.4.2. Measurement of plasma [D-Lys^3]-GH RP-6

In order to measure concentration of [D-Lys^3]-GHRP-6 in plasma, a polyclonal antiserum (11G14) against [Cys^0]-[D-Lys^3]-GHRP-6 raised from a guinea pig was used at a final dilution of 1: 8000 in this study. The specificity of the antiserum was determined by constructing a number of standard curves (Fig. 2-11B). [D-Lys^3]-GHRP-6 was radio-iodinated using the method of McConahey and Dixon [99]. [D-Lys^3]-GHRP-6 was used as cold standard ranged from 320 to 0.6 ng/mL. Plasma sample from 5 min to 90 min after [D-Lys^3]-GHRP-6 administration was 2 times diluted using assay buffer. Sensitivity,

intra-assay CVs and recovery rate were 2.14 ng/mL, 11% and 98%, respectively. The standard curve used in this chapter study is shown in Fig. 3-2.

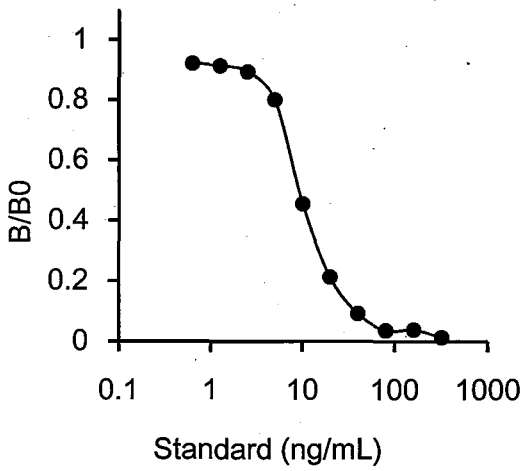

Fig. 3-2. Standard radioimmunoassay curves for [D-Lys^3]-GHRP-6. Inhibition of ^{125}I-labeled [D-Lys^3]- GHRP-6 binding to antiserum 11G14 by a serial dilution of [D-Lys^3]-GHRP-6 was obtained. Each point was the mean of triplicate determinations. B/B0, bound/ bound in zero standard.

2.4.3. Measurement of plasma glucose and NEFA

Plasma glucose and NEFA concentrations were measured by commercially available kits (Code No. 439-90901 and 279-75401, respectively, Wako Chemicals, Osaka, Japan).

2.5. Statistical analysis

The results are presented as means ± SEM. Differences in plasma hormone concentrations were analyzed by repeated measure ANOVA. All data were analyzed using SPSS for Windows version 15, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Dose-dependent effects of ET-3 on the levels of ETs, ghrelin and GH in plasma

Endothelin-3 dose-dependently increased concentrations of ETs in plasma. The peak concentrations were 1.6-, 3.1-, 5.0- and 8.7-fold higher than those of pre-injection at 5 min after administration of 0.1, 0.4, 0.7 and 1.0 μg/kg of ET-3, respectively. Concentrations of ETs returned to the basal level within 10 or 15 min after each dose of ET-3 administration (Fig. 3-3A). Dose-dependent effects of ET-3 on plasma concentrations of acyl and total ghrelin were shown in Fig. 3-3B and 3-3C, respectively. Concentrations of acyl and total

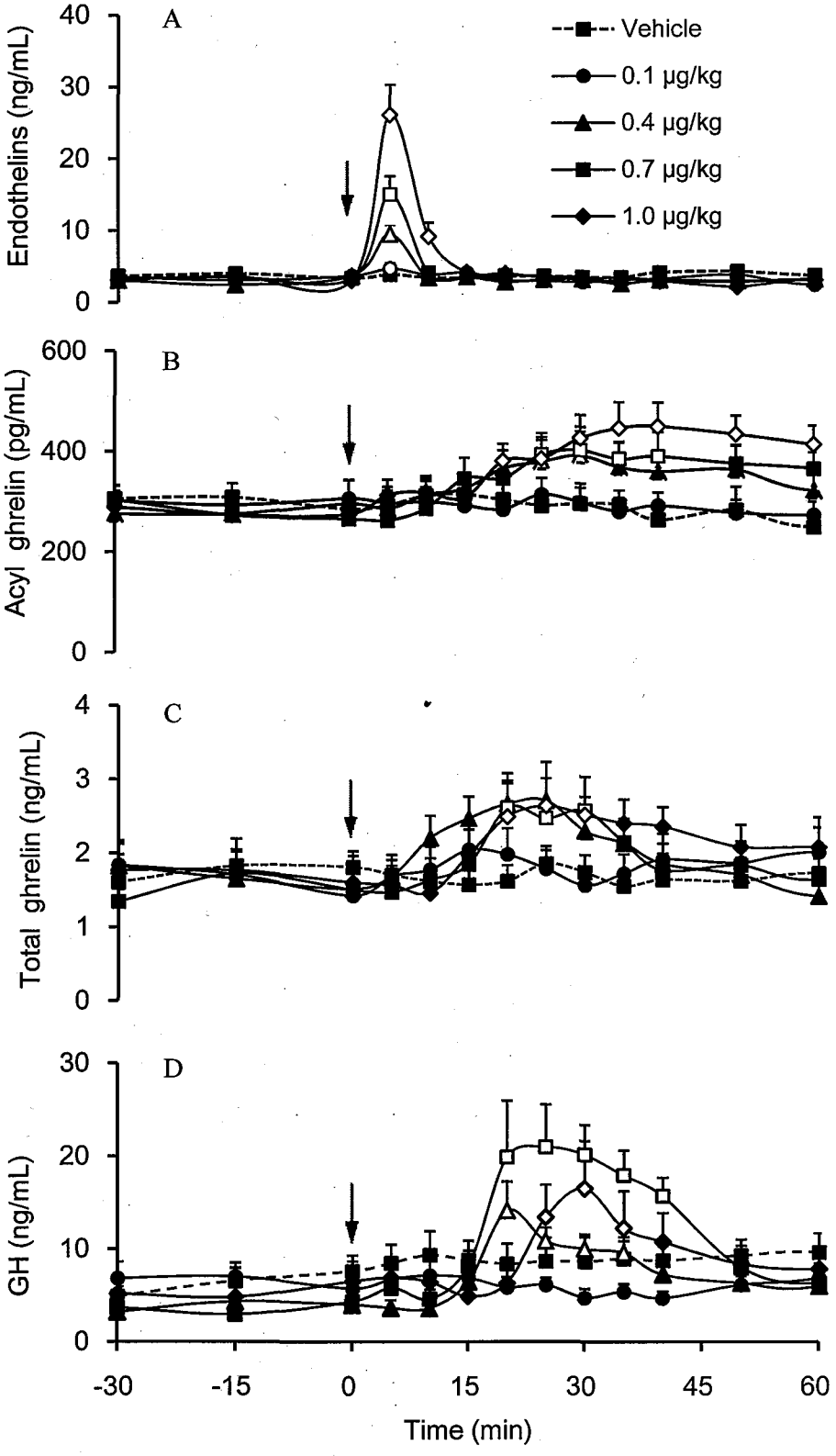

Fig. 3-3. Changes in plasma endothelins (A), acyl ghrelin (B), total ghrelin(C) and growth hormone (D) in response to synthetic endothelin-3 administration in Holstein steers. Animals were intravenously injected with vehicle, 0.1, 0.4, 0.7 and 1.0 µg/kg BW of endothelin-3 at 0 min as indicated by the arrow. Values are presented as means ± SEM (n=8). Open symbols indicate a significant difference ($P < 0.05$) compared with the mean of pre-injected values (-30, - 15 and 0 min).

ghrelin were not significantly altered by administration of vehicle and 0.1 μg/kg of ET-3 throughout the sampling periods. The ratio of basal level of acyl ghrelin to total ghrelin was 1: 5.7. Concentrations of acyl ghrelin were significantly enhanced after 25 min, and returned to basal level 35 and 50 min after administration of 0.4 and 0.7 μg/kg of ET-3, respectively. Concentration of acyl ghrelin significantly increased 30 min after administration of 1.0 μg/kg of ET-3, and maintained the same level until the last sampling time. The peak amplitudes and durations of acyl ghrelin increment by ET-3 were found in a dose-dependent manner. Concentrations of total ghrelin after administration of 0.4, 0.7 and 1.0 μg/kg of ET-3 were markedly elevated after 20, 20 and 25 min. The minimum effective dose of ET-3 to increase plasma ghrelin levels was 0.4 μg/kg.

Growth hormone levels were increased by different doses of ET-3 treatments (Fig. 3-3D). Concentrations of GH were not significantly changed throughout the experiment in vehicle and 0.1 μg/kg of ET-3 administrated groups. Growth hormone levels after administration of 0.4, 0.7 and 1.0 μg/kg of ET-3 were markedly elevated after 20, 20 and 25 min, and returned to the basal level after 40, 50 and 40 min, respectively. The peak amplitude and duration of GH increment by 0.7 μg/kg of ET-3 were greater than by 1.0 μg/kg of ET-3.

3.2. Effects of ET_B receptor agonist on the levels of ETs, ghrelin and GH in plasma

Plasma concentration of ETs, including IRL 1620, surged after IRL1620 administration (Fig. 3-4A). The peak concentration (38.8 ± 5.9 ng/mL) was observed after 5 min, and after 40 min the concentration returned to the basal level. Concentrations of acyl ghrelin, total ghrelin and GH response to IRL 1620 injection were significantly enhanced compared to their basal levels (Fig. 3-4B, C and D). Changes of plasma GH after administration of IRL 1620 were similar to those after administration of 0.7 μg/kg of ET-3.

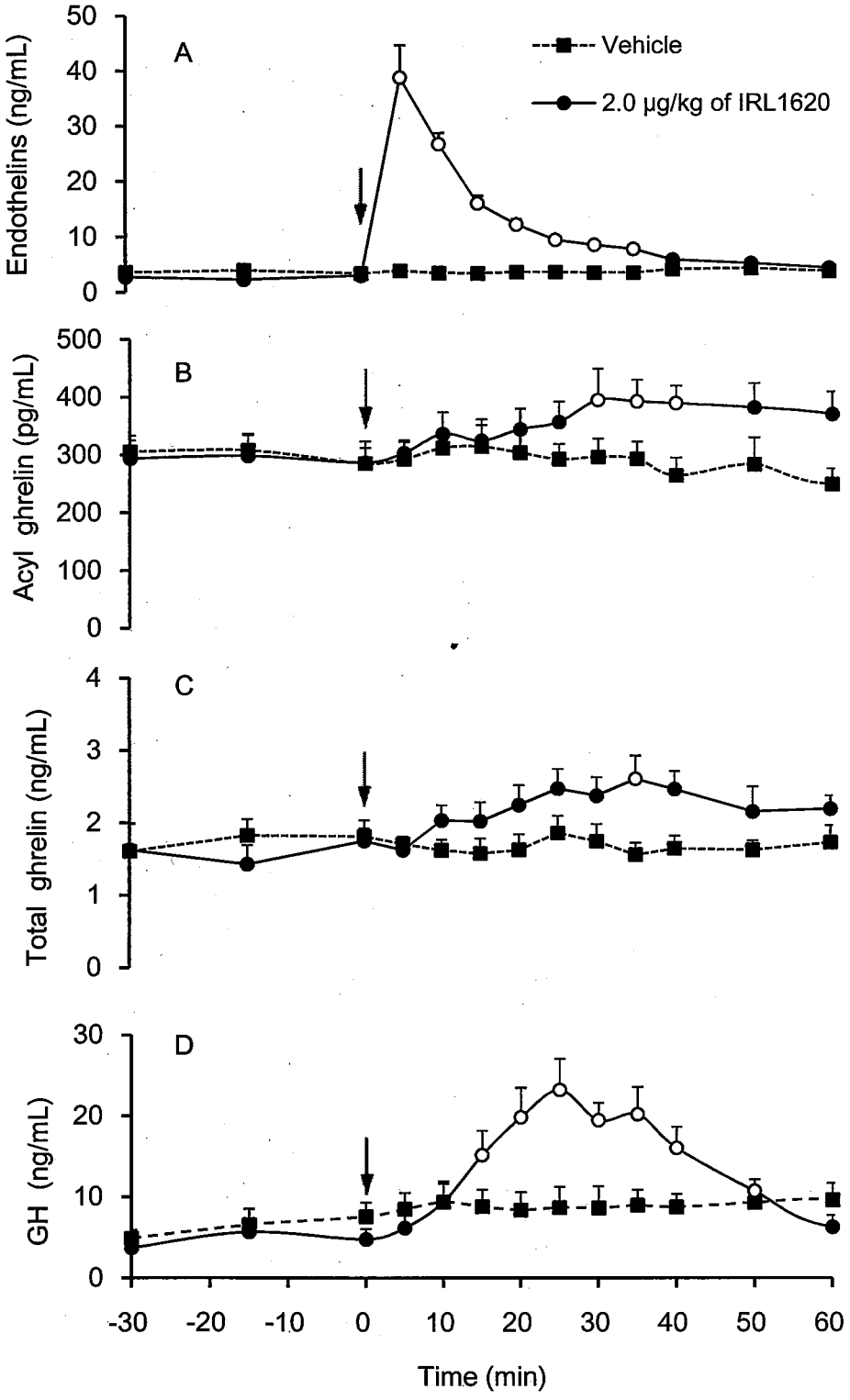

Fig.3-4. Changes in plasma endothelins (A), acyl ghrelin (B), total ghrelin (C) and growth hormone (GH, D) in response to administration of IRL 1620, a selective ET_B receptor agonist, in Holstein steers. Animals were intravenously injected with 0.1% BSA-saline as vehicle and 2.0 µg/kg of IRL 1620 at 0 min as indicated by the arrow. Values are presented as means ± SEM (n=8). Open symbols indicate a significant difference ($P < 0.05$) compared with the mean of pre-injected values (-30, -15 and 0 min).

3.3. Effects of GHS-R antagonist on the levels of GH in plasma

Figure 3-5 shows that concentrations of [D-Lys^3]-GHRP-6 in plasma were 235.1 ± 30.2 or 240.7 ± 23.4 ng/mL at 5 min after injections of 20.0 μg /kg BW of [D-Lys^3]-GHRP-6 alone or combined with ET-3, respectively. Moreover, concentration of [D-Lys^3]-GHRP-6 in plasma was more than 30 ng/mL at 90 min after injection. Changes of acyl and total ghrelin after administration of [D-Lys^3]-GHRP-6 combined with 1.0 μg/kg of ET-3 were similar with those after administration of 1.0 μg /kg of ET-3 alone (Fig. 3-6B and C). However, the ET-3-induced increase of GH level was blocked by [D-Lys^3]-GHRP-6 (Fig. 3-6D). Administration of [D-Lys^3]-GHRP-6 alone did not significantly affect the concentrations of ETs, acyl ghrelin, total ghrelin and GH throughout the experiment (Fig. 3-6).

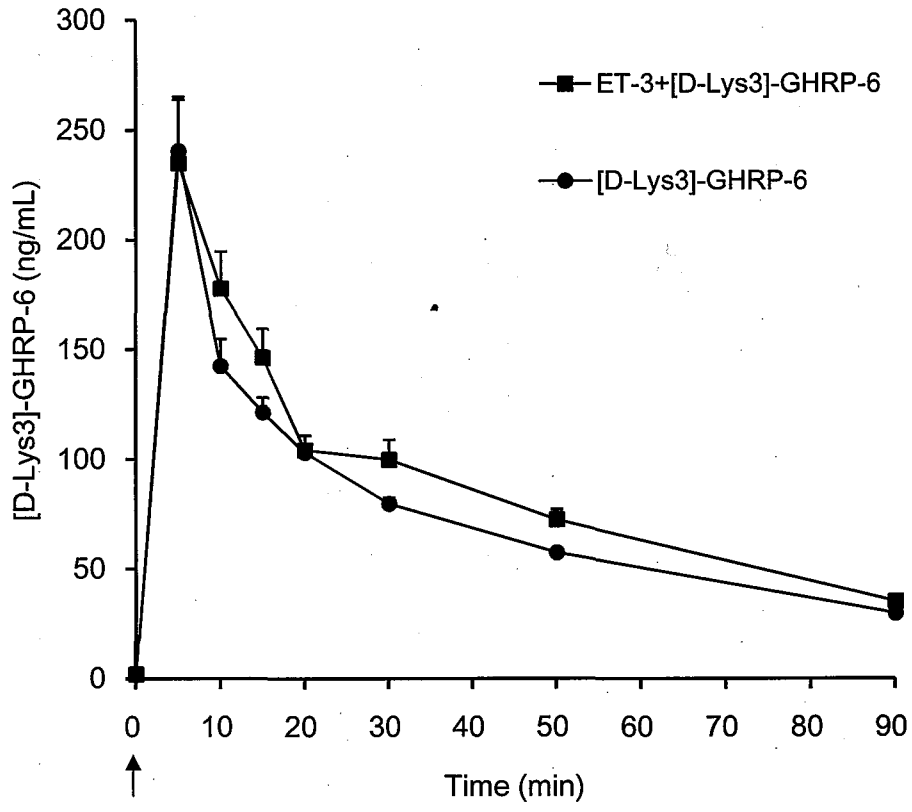

Fig. 3-5. Changes in plasma [D-Lys^3]-GHRP-6 level in response to administration of [D-Lys^3]-GHRP-6 in Holstein steers. Animals were intravenously injected with 20.0 μg/kg [D-Lys^3]-GHRP-6, a GHS-R1a antagonist, with 1.0 μg/kg endothelin-3 or without at 0 min as indicated by the arrow. Values are presented as means ± SEM (n=8).

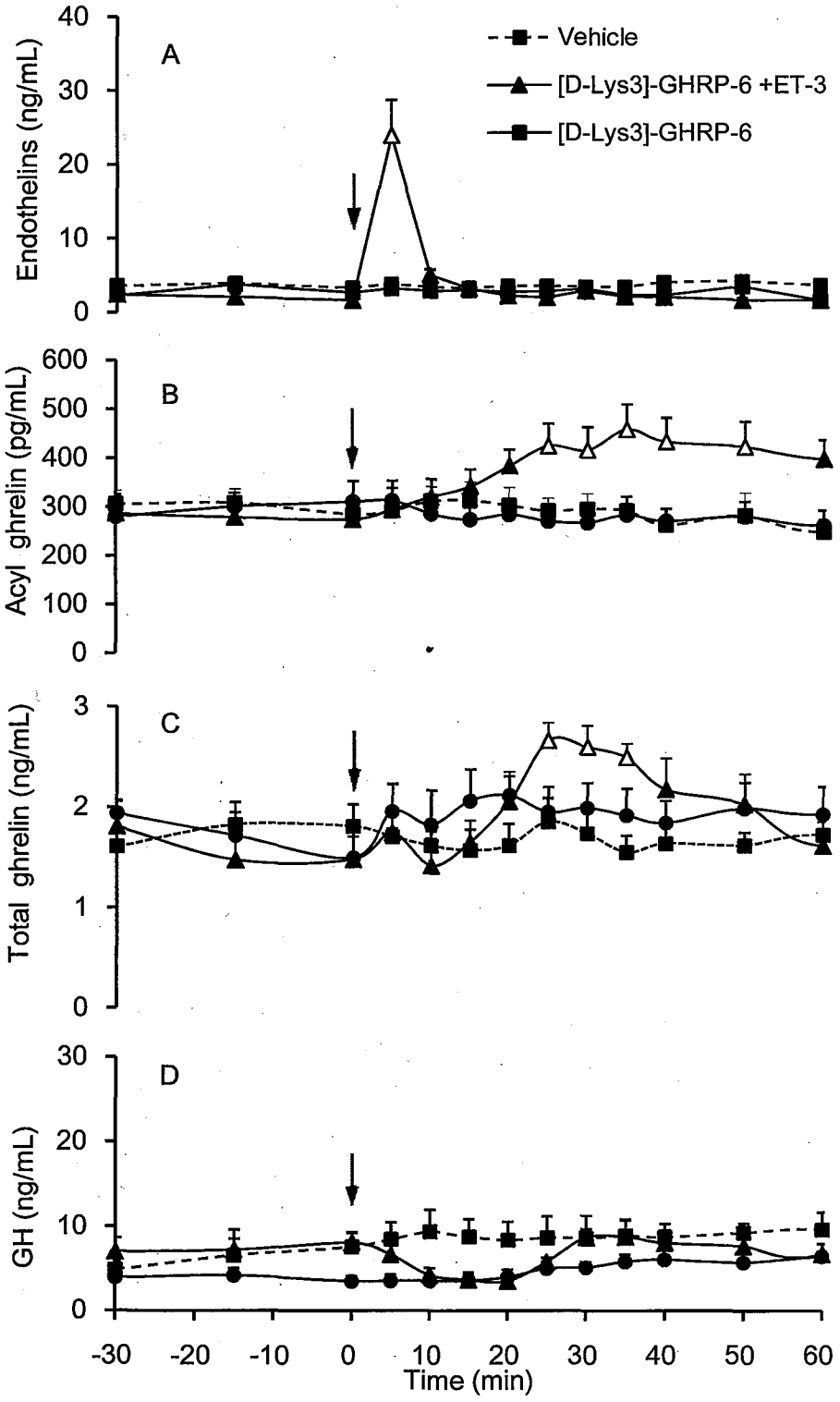

Fig. 3-6. Changes in plasma endothelins (A), acyl ghrelin (B), total ghrelin (C) and growth hormone (GH, D) in response to administration of [D-Lys^3]-GHRP-6, a GHS-R1a antagonist, in Holstein steers. Animals were intravenously injected with 0.1% BSA-saline as vehicle, 20.0 μg/kg [D-Lys^3]-GHRP-6 with 1.0 μg/kg endothelin-3 or without at 0 min as indicated by the arrow. Values are presented as means ± SEM (n=8). Open symbols indicate a significant difference ($P < 0.05$) compared with the mean of pre-injected values (-30, -15 and 0 min).

3.4. Effects of different dose of ET-3 on the levels of glucose and NEFA in plasma

Plasma glucose levels were increased from 10 min to 60 min, the last sampling time point, after 0.4, 0.7 and 1.0 μg/kg BW of ET-3 injection (Fig. 3-7A). Injections of vehicle and 0.1 μg/kg BW of ET-3 did not change plasma glucose levels during the sampling period. However, plasma NEFA levels were increased from 10 min after each dose of ET-3 injection (Fig. 3-7B).

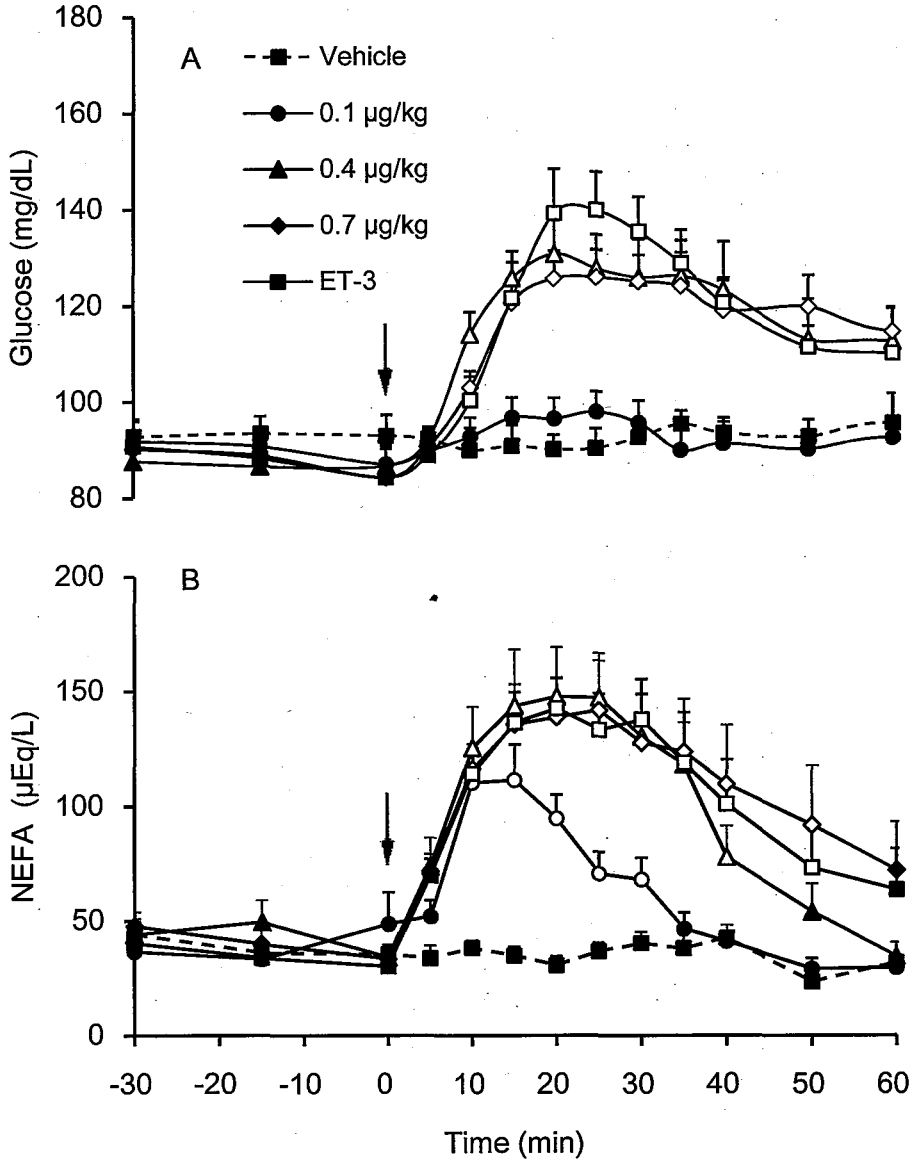

Fig. 3-7. Changes in plasma glucose (A) and nonesterified fatty acid (NEFA, B) in response to synthetic endothelin-3 administration in steers. Animals were intravenously injected with vehicle, 0.1, 0.4, 0.7 and 1.0 μg/kg BW of endothelin-3 at 0 min as indicated by the arrow. Values are presented as means ± SEM (n=8). Open symbols indicate a significant difference ($P < 0.05$) compared with the mean of pre-injected values (-30, - 15 and 0 min).

3.5. Effects of ET_B receptor agonist and GHS-R antagonist on the levels of glucose and NEFA in plasma

IRL 1620 mimicked the effects of ET-3 increase plasma glucose and NEFA levels from 10 min after administration (Fig. 3-8). Moreover, [D-Lys^3]-GHRP-6 combined with ET-3 did not affect the ET-3-induced plasma glucose and NEFA elevation. Injection of [D-Lys^3]-GHRP-6 alone did not change the basal levels of glucose and NEFA during all the sampling time points.

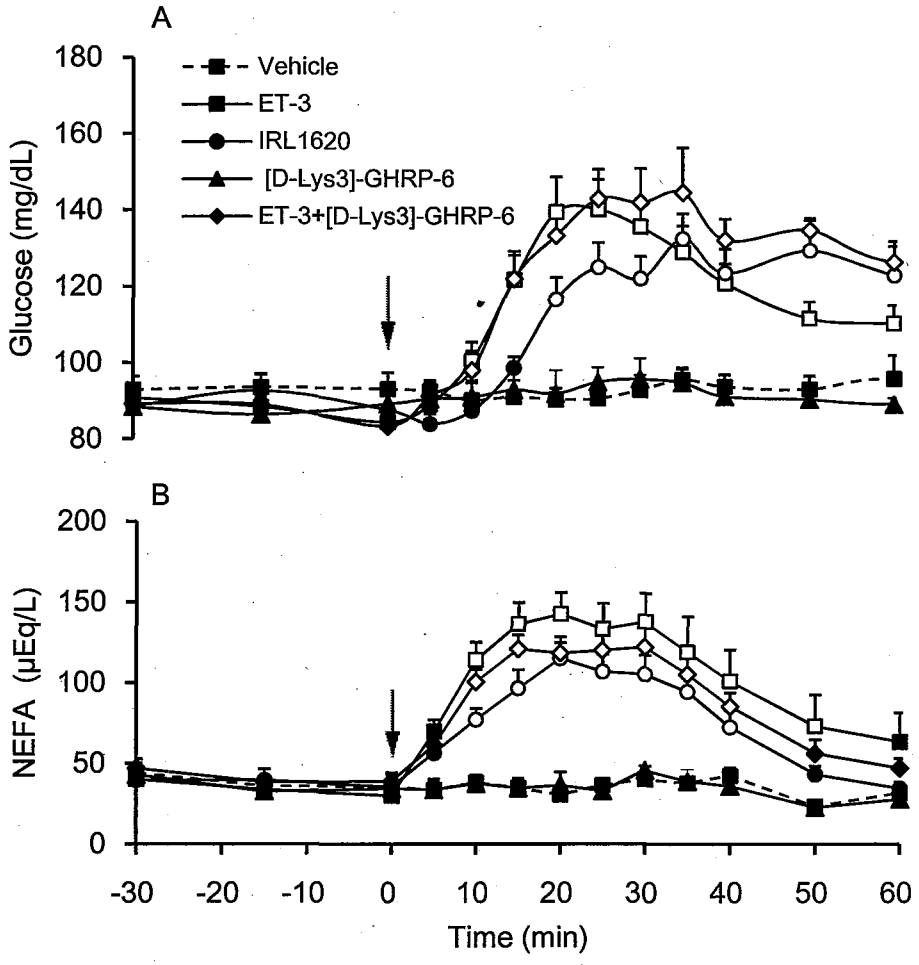

Fig. 3-8. Changes in plasma glucose (A) and nonesterified fatty acid (NEFA, B) in response to administration of IRL 1620 and [D-Lys^3]-GHRP-6 in steers. Animals were intravenously injected with 0.1% BSA-saline as vehicle, 20.0 μg/kg BW of IRL 1620, 20.0 μg/kg [D-Lys^3]-GHRP-6 with 1.0 μg/kg endothelin-3 or without at 0 min as indicated by the arrow. Values are presented as means ± SEM (n=8). Open symbols indicate a significant difference ($P < 0.05$) compared with the mean of pre-injected values (-30, -15 and 0 min).

4. Discussion

This chapter study showed that administration of ET-3 increased the levels of both acyl and total ghrelin in plasma in a dose-dependent manner. Total ghrelin includes acyl ghrelin and des-acyl ghrelin. Des-acyl ghrelin exists in considerably larger amounts than acyl ghrelin does in plasma [56, 160]. To date, it has not yet been clearly demonstrated whether des-acyl ghrelin is produced by deacylation of acyl ghrelin, or directly synthesized from its prepro-ghrelin. Two recent studies reported that pro-ghrelin was acylated by ghrelin O-acyltransferase (GOAT) before being transported to the Golgi apparatus [45, 175]. Several esterases, including butyrylcholinesterase, contribute to ghrelin des-octanoylation in human serum in vitro [24]. These studies indicated that acyl ghrelin was deacylated into des-acyl ghrelin. Therefore, in the present study, we suggest that the increment in total ghrelin in plasma originates from ET-3-stimulated acyl ghrelin and its decomposed forms.

IRL 1620, a synthetic analogue of ET-1, is a highly selective ET_B receptor agonist, and 120, 000 times more selective to ET_B receptors than to ET_A receptors. Moreover, IRL 1620 is 60 times more selective than ET-3 for the ET_B receptors in porcine lung membranes [156]. IRL 1620 is presently in a phase I clinical trial (NCT00613691) in the United States for patients with recurrent or progressive carcinoma [91]. The pharmacokinetics of IRL 1620 has not been reported extensively. In the present study, changes of IRL 1620 in plasma after an intravenous bolus injection was determined by RIA using anti-endothelin-1 antiserum. However, we could only determine the relative concentrations of IRL 1620, since the antiserum has equal affinity for IRL 1620 and endogenous ETs. The duration of high level IRL 1620 in plasma was significantly longer than that of ET-3 (35 vs. 10 min) after administration of each peptide, indicating that IRL 1620 had a longer half life than ET-3 in the circulatory system. In this study, IRL 1620 was used to confirm the involvement of ET_B receptors in the release of ghrelin and GH. The results showed that IRL 1620 mimicked the effects of ET-3 on ghrelin secretion, suggesting that ET_B receptors were involved in the ET-3-induced increase of ghrelin. However, the effects by ET_A receptors could not be excluded since the selective ET_B receptor antagonist was not used in this study. IRL 1620 did not significantly affect heartbeat and systemic blood pressure as compared to ETs [91]. We supposed that IRL 1620 might be one of the candidates which could pharmacologically up-regulate ghrelin

and GH in the subjects with related growth and /or anorexia diseases.

The secretion of ET-3 is proved in pituitary cells [98] and primary astrocytes [29] of rat in vitro. In ruminants, ghrelin-immunoreactive cells are widely distributed in the digestive tract [49], the hypothalamus and the pituitary [106]. Moreover, ET_B receptors are also known to exist in these organs [54]. The foregoing and our results indicate the possibility that ET_B receptors mediate the effects of ET-3 on the secretion of ghrelin in peripheral organs and the central nervous system. The levels of ET-3 are higher than that of ET-1 in rat pituitary [97] and in human cerebrospinal fluid [87]. Therefore, we suggest that ET-3 may play a role as neuropeptide in the central nervous system.

It is commonly accepted that GH release is stimulated by hypothalamic GH-releasing hormone (GHRH) and inhibited by somatostatin. Ghrelin also has been well known to stimulate the secretion of GH. Several reports demonstrated that the secretion of GH was increased by peripherally-injected ghrelin [48, 64, 154, 158, 159, 160]. However, little information is known about the relationship of endogenous ghrelin and GH. In order to investigate whether the secretion of GH was stimulated by endogenous ghrelin after administration of ET-3, the GHS-R1a antagonist combined with ET-3 was administrated. ET-3-induced increase of GH was blocked by [D-Lys^3]-GHRP-6, indicating the increase in the concentration of GH was stimulated by endogenous ghrelin through GHS-R1a. In dose-response studies, it was found that 0.7 μg/kg of ET-3 exhibited a stronger effect than 1.0 μg/kg of ET-3 on the levels of GH in plasma. The reduction of GH response to ET-3 administration at the higher dose may be due to desensitization of endothelin receptors. The similar reduction of response at higher dose had been observed using one of the ET_B receptor agonists, sarafotoxin S6c, in venous smooth muscle contraction [149]. On the other hand, ET receptor, GHS-R1a and GHRH receptor (GHRH-R) belong to G protein-coupled receptors. It is well accepted that ligands of G protein-coupled receptors can regulate the expression level of their own receptors. Ghrelin significantly reduced the expression of its own receptor and GHRH-R in porcine pituitary cell in vitro [95]. Accordingly, the reduction of GH response to a higher dose of ET-3 administration also may be due to ET-3 and/or ET-3-induced endogenous ghrelin decrease of their receptors expression.

Although plasma acyl ghrelin was maintained at a high level from 40 to 60 min after administration of 1.0 μg/kg of ET-3, plasma GH level did not increase in this period. Likewise, it was reported that an increase in ghrelin concentrations and a related GH surge

was not detected in Holstein cows [110]. In addition, ghrelin was not involved in the postprandial increase in GH concentrations in milk-fed goats [83]. These results indicate that ghrelin increase is not always related to GH secretion.

Endothelin-3 was observed to increase both glucose and NEFA levels in plasma. Those results were similar to that previously found by our laboratory [157]. Endothelin B receptors may involve in ET-3-induced elevation of glucose and NEFA since IRL 1620, the selective agonist for ET_B receptors, mimicked the effects of ET-3 on the elevation of glucose and NEFA (Fig. 3-8). Single intravenous injection of GH was noted a significant rise in circulating glucose and NEFA in sheep [47]. Although, in the present study, ET-3-induced elevation of plasma GH was blocked by [D-Lys^3]-GHRP-6, [D-Lys^3]-GHRP-6 was failed to block ET-3-induced elevation of glucose and NEFA. These results indicated that ET-3 stimulated production of glucose and NEFA with a GH-independent manner. Infusion of ET-1 or ET-3 into the portal vein increased glucose, and both ET_A and ET_B receptors were suggested to be involved in the metabolic effects of circulating ET in rat liver [20]. Therefore, the ET-3-induced glucose elevation may be caused by stimulation of glycogenolysis in cattle.

In conclusion, this chapter studies demonstrated that: (1) intravenous bolus injections of ET-3 dose-dependently stimulated ghrelin secretion, and the minimum effective dose was 0.4 μg/kg in cattle; (2) ET_B receptors involved in the ET-3-induced secretion of ghrelin and GH; (3) endogenous ghrelin response to ET-3 regulated GH levels through GHS-R1a; (4) ET-3 stimulated production of glucose and NEFA with a GH-independent manner in ruminants.

5. Summary

This chapter study was designed to determine the dose-dependent effects of endothelin-3 (ET-3) on the secretion of ghrelin, growth hormone (GH), glucose and nonesterified fatty acid (NEFA) and to characterize the receptors involved in these effects. Eight Holstein steers were randomly assigned to receive intravenous bolus injections of vehicle (0.1% bovine serum albumin in saline), bovine ET-3 (0.1, 0.4, 0.7 and 1.0 μg/kg), IRL1620 (a selective agonist for ET_B receptors, 2.0 μg/kg), [D-Lys^3]-GHRP-6 (an antagonist for GH secretagogue receptor type 1a [GHS-R1a], 20.0 μg/kg) and bovine ET-3 (1.0 μg /kg) combined with [D-Lys^3]-GHRP-6 (20.0 μg/kg), respectively. Blood samples were collected at -30, -15, 0, 5, 10, 15, 20, 25, 30, 35, 40, 50 and 60 min relative to

injection time. Concentrations of acyl ghrelin, total ghrelin, glucose and NEFA were significantly increased by ET-3 in a dose-dependent manner. Concentrations of GH were markedly elevated by administration of 0.4, 0.7 and 1.0 μg/kg of ET-3, and the effect of 0.7 μg /kg was greater than that of 1.0 μg/kg. The minimum effective dose of ET-3 in the elevation of ghrelin, GH and glucose was 0.4 μg/kg. IRL 1620 mimicked the effects of ET-3 on the elevation of ghrelin, GH, glucose and NEFA in plasma. Endothelin-3-induced increase of plasma GH was blocked by [D-Lys^3]-GHRP-6, but the increase of glucose and NEFA was not affected. These results indicated that endogenous ghrelin response to ET-3 injection stimulated GH secretion through GHS-R1a; regulatory effects of ET on the levels of ghrelin, glucose and NEFA may be mediated by ET_B receptors with a GH-independent manner in ruminants.

Chapter 4

Effects of Sulfated Gastrin-34 on the Circulating Levels of Ghrelin, Growth Hormone and Insulin

1. Introduction

Gastrin was discovered in 1905 by Edkins, and the name was defined by its effect on gastric acid secretion [28]. Gastrin is predominantly secreted by antrum G-cells in monogastrics [28,42] as well as in ruminants [14] There are various molecular forms of gastrin based on the number of amino acids in the peptide chain; and gastrin-17 and gastrin-34 are the main forms. Gastrin has a tyrosyl residue in position six, as counted from the C-terminus, that can be sulfated [42,133]. The degree of sulfation of antral extracts was reported to be more than 50% of total gastrin [5,15,133]. Two receptor subtypes, CCK_A and gastrin/CCK_B, mediate the bioactivity of the gastrin and CCK family. Gastrin/CCK_B receptors are less selective than CCK_A receptors, which bind only sulfated CCK. However, gastrin/CCK_B receptors have equally high affinity to bind with both sulfated and non-sulfated gastrin and CCK [135].

Ghrelin, an endogenous ligand for growth hormone (GH) secretagogue receptor (GHS-R), is secreted from X/A-like cells in the fundic glands of the stomach [21]. In addition to stimulating GH [159], ghrelin increases gastric acid secretion [96]. Moreover, ghrelin and gastrin have a synergistic action on gastric acid secretion [35]. It is of interest to examine whether gastrin regulates the secretion of ghrelin, since both peptides are mainly secreted from the stomach and both stimulate gastric acid secretion. The role of gastrin in ghrelin release is a matter for discussion. Gastric microdialysis and abdominal infusion studies showed that human gastrin-17 had no measurable effect on the secretion of ghrelin in rats [23,27]. In a study of isolated rat stomach, gastrin-17 significantly inhibited vagally prestimulated ghrelin release [94]. However, Fukumoto et al. [35] and Murakami et al. [113] showed that intravenous (IV) and intraperitoneal (IP) administration of gastrin increased ghrelin levels in fasted rats. In these previous studies, administered

forms of gastrin were short and non-sulfated. Therefore, the sulfated and long forms of gastrin, such as sulfated gastrin-34, should be investigated in studies of the function of gastrin. In addition, when the regulatory effect of gastrin on ghrelin is examined, the regulatory effect of gastrin on GH is also of interest since ghrelin is a secretagogue of GH [160].

The stimulatory effect of gastrin on insulin has been reported in dogs [62,50] and humans [131]. Insulin is believed to act as a long-term regulator of feed intake in ruminants. In general, insulin causes cells to take up glucose from the blood, then glucose is stored as glycogen in the liver and muscle. On the contrary, glucagon causes the increase of blood glucose; therefore, when researching the action of gastrin on blood glucose level, the effect of gastrin on insulin and glucagon secretion should be studied.

These previous studies were carried out in monogastric species. However, the effect of gastrin on hormones mentioned above was not clear in ruminants. Feeding dependence of the plasma levels of gastrin, ghrelin and other gut regulatory peptides are very pronounced in monogastric species. Conversely, no or only very little variation in gastrin, ghrelin and acid secretion may be observed in ruminants, in which the influx of material into the abomasums is relatively continuous [162]. The existence of interspecies differences for hormonal regulation in ghrelin secretion and the paucity of studies on the effect of gastrin on ghrelin release in ruminants led us to investigate the effects of bovine sulfated gastrin-34 on plasma levels of ghrelin, GH, insulin, glucagon and glucose in cattle.

2. Materials and Methods

All experimental procedures involving animals were approved (animal protocol numbers: 21-101 and 22-88) by the Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine, Japan.

2.1. Animals

Eight Holstein steers aged 9 months and weighing 327 ± 8 kg were used. Animals were fed with 4 kg/head/day of concentrate (crude protein 16.0%, crude fat 2.5%, crude fiber 9.0% and crude ash 10.0%, Nishin Marubeni, Japan) twice daily, at 09:00 and 16:00. Timothy hay, salt block and water were supplied ad libitum. One day before the experiment, a sterilized polyethylene catheter was non-surgically implanted into the left or

right external jugular vein for the injections of peptides and blood sampling. The patency of the catheter was maintained with heparin saline. During the administration and sampling periods, animals were loosely chained to the stanchion and allowed hay and water ad libitum.

2.2. Peptides

Bovine sulfated gastrin-34 (Pyr-LGLQDPPHMVADLSKKQGPWVEEEEAAY [SO_3H] GWMDF-NH_2, UniProt ID: P01352), bovine sulfated gastrin-9 (EAAY [SO_3H] GWMDF- NH_2), bovine non-sulfated gastrin-9 (EAAY GWMDF-NH_2), bovine sulfated CCK-8 (DY[SO_3H] MGWMDF-NH_2, UniProt ID: P41520), bovine non-sulfated CCK-8 (DYMGWMDF-NH_2), Tyr^0-bovine sulfated gastrin-9 (YEAAY [SO_3H] GWMDF- NH_2) and Cys^0-bovine sulfated gastrin-9-OH (CEAAY [SO_3H] GWMDF-OH) were synthesized by Fmoc (9-fluorenylmethoxycarbonyl chloride) solid-phase peptide synthesis (SPPS) procedures. A modified SPPS method [82] was used for the synthesis of sulfated peptides. The crude sulfated gastrin was purified by semi-preparative (TSKgel ODS-120A column) HPLC using a linear gradient for 60 min from 5 mM sodium phosphate (pH 6.5) to 60% CH_3CN in double-distilled water, and the absorbance was detected at 305 nm. Then, the peak fraction was 2-fold diluted in double-distilled water and desalted by semi-preparative HPLC using a linear gradient for 5 min from double-distilled water to 60% CH_3CN. After lyophilization, the purity of gastrin, including sulfated and non-sulfated forms, was more than 98%, and that of sulfated gastrin was approximately 90% [82]. Other crude peptides were purified by reverse-phase HPLC (TSKgel ODS-120A column; linear gradient of 0%-60% CH_3CN containing 0.1% TFA), lyophilized and stored at -30 °C. Cholecystokinin-4 (Peptide Institute, Inc., Japan) and human non-sulfated gastrin-17 (Phoenix Pharmaceuticals, Inc., U.S.A.) were obtained commercially.

2.3. Administration of peptide and blood sampling procedures

On the day of administration, bovine sulfated gastrin-34 was dissolved in sterilized double-distilled water to a concentration of 10 mg/mL. Then, based on the desired dose of gastrin for each animal, this solution was diluted with 0.1% bovine serum albumin in saline (BSA-saline, as vehicle) to obtain 5 mL of total injection volume.

Animals were randomly assigned to an incomplete blocked Latin Square Design (eight animals × four treatments × four days of treatment with one day recovery between each treatment) to receive an intravenous bolus injection of vehicle, 0.8, 4.0 and 20.0

μg/kg body weight (BW) of sulfated gastrin-34, at 11:30. The highest dose of gastrin-34 was decided based on our preliminary studies in steers (data were not shown).

Blood samples were withdrawn at -10, 0 (just prior to injection), 5, 10, 15, 20, 30, 45, 60, 75, 90, 120 and 150 min relative to injection time. Samples were put into ice-chilled tubes containing heparin (8 IU/mL blood, Wako, Japan). Plasma was isolated by centrifugation at 1 870 × *g* at 4 °C for 30 min, and stored at -30 °C. For the ghrelin assay, 50 μL of 1M HCl was added to 1 mL of plasma and stored at -30 °C until analyzed.

2.4. Measurement of hormones and glucose in plasma

2.4.1. RIA for acyl ghrelin, total ghrelin, GH, insulin and glucagon

Concentrations of acyl ghrelin and total ghrelin [160], GH [180], insulin [160] and glucagon [157] were quantified as described previously. Sensitivity, intra-assay coefficients of variation (CVs) and recovery rate were 77.5 pg/mL, 10% and 110% for acyl ghrelin assay; 0.10 ng/mL, 14% and 114% for total ghrelin assay; 0.36 ng/mL, 13% and 106% for GH assay; 0.05 ng/mL, 4% and 100% for insulin assay; 0.49 ng/mL, 14% and 95% for glucagon assay, respectively.

2.4.2 RIA for total gastrin

In order to measure gastrin, a polyclonal antiserum (09G02) against Cys^0-bovine sulfated gastrin-9-OH was raised from a guinea pig. The reason for using the C-terminal non-amidated form of gastrin was the hope of raising an antibody without cross-reaction to the amidated C-terminus which is common in gastrin and CCK. The specificity of the antiserum raised from one of guinea pigs was determined by constructing a number of standard curves (Fig. 2-4B). It binds bovine gastrin-9, human gastrin-17 and bovine gastrin-34, irrespective of the degree of tyrosine-O-sulfation. However, it displays no cross-reactivity with CCK-4, bovine sulfated and non-sulfated CCK-8. Therefore, concentrations of gastrin measured by using this antiserum were expressed as total gastrin, including sulfated and non-sulfated gastrin. In this chapter for total gastrin RIA, Tyr^0-bovine sulfated gastrin-9 amide was radio-iodinated using the method of McConahey and Dixon [99]. Bovine sulfated gastrin-34 was used as cold standard ranged from 320 to 0.16 ng/mL, and 09G02 was at a final dilution of 1: 8000. Sensitivity, intra-assay CVs and recovery rate were 0.16 ng/mL, 10% and 104%, respectively. The standard curve used in this chapter study is shown in Fig. 4-1.

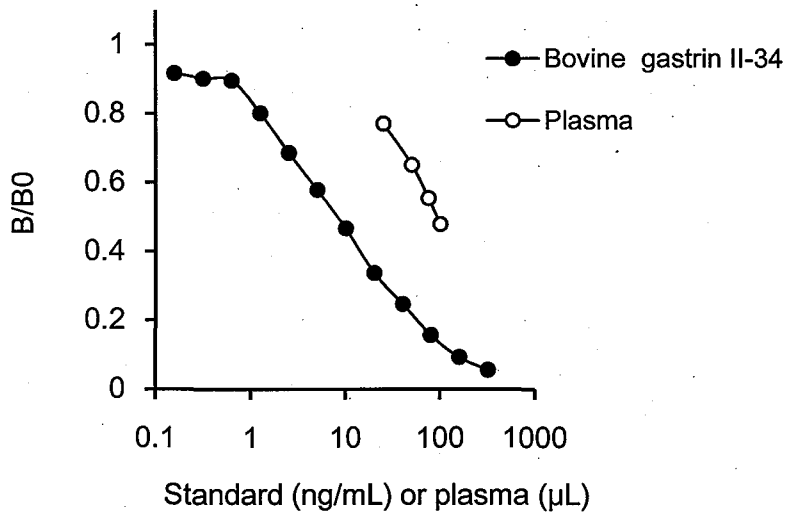

Fig. 4-1. Standard curve for total gastrin RIA. The tracer is ^{125}I-labeled bovine sulfated gastrin-9. The antibody was 09G02 at 1:8000 final dilution. The concentration of cold standard, bovine sulfated gastrin-34, ranged from 320 to 0.16 ng/mL. Each point is the mean of triplicate determinations. B/B0 represents bound/bound in zero standard.

2.4.3 Measurement of glucose

Concentrations of glucose in plasma were analyzed by an enzymatic method using commercially available kits (Wako Pure Chemical Industries, Ltd., Japan).

2.5. Statistical analysis

Interaction between the effect of treatment and time on the levels of assayed hormones and glucose were analyzed using a two-way ANOVA followed by post hoc multiple comparisons. The mean difference in the level of assayed hormone and glucose among treatments at each time point was compared using univariate analysis. The mean difference is significant at the 0.05 level. SPSS version 16 for Windows was used.

3. Results

3.1. Dose-response effects of gastrin-34 on the levels of total gastrin

Concentrations of total gastrin in response to gastrin-34 injection were dose-dependently increased (Fig. 4-2 and Table 4-1). Peaks of total gastrin at 5 min after the administration of 0.8, 4.0 and 20.0 μg/kg BW of gastrin-34 were 5-, 8- and 34-fold higher than that of vehicle injection (5.73 ± 0.06 ng/mL), respectively. Gastrin levels remained high until 150 min after the administration of 20 μg/kg BW of gastrin-34.

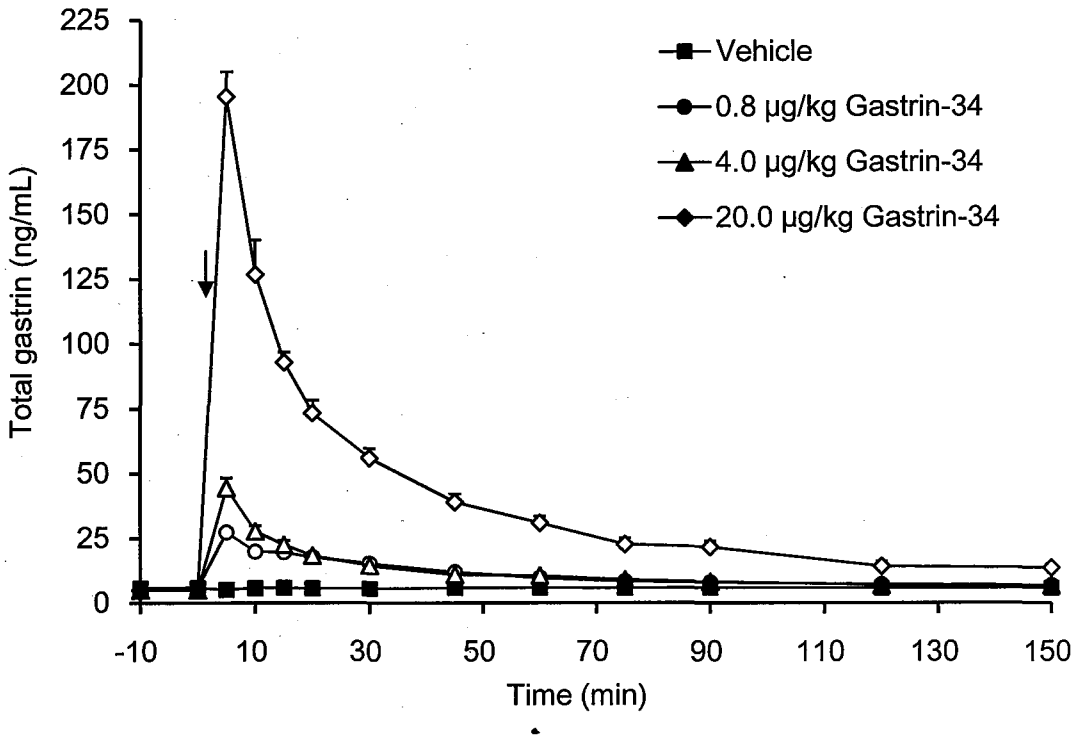

Fig. 4-2. Plasma concentrations of total gastrin in response to gastrin-34 injection in cattle. Animals were intravenously injected with 0.1% bovine serum albumin in saline (as vehicle), 0.8, 4.0 and 20.0 μg/kg body weight of bovine sulfated gastrin-34. The arrow shows the injection time at 0 min. Each value represents the mean ± SEM for eight animals. Open symbols indicate that the value is significantly different from the corresponding levels of vehicle injection ($P < 0.05$).

3.2. Dose-response effects of gastrin-34 on the levels of ghrelin and GH

There were significant interactions between the effect of treatment and time on acyl ghrelin, total ghrelin and GH (Table 4-1). Concentrations of acyl ghrelin were significantly increased from 192.4 ± 13.2 pg/mL to 241.0 ± 13.3 pg/mL within 10 min after 0.8 μg/kg BW of gastrin-34 injection (Fig. 4-3A). Higher doses of gastrin-34 increased acyl ghrelin levels over a longer period. Concentrations of total ghrelin were significantly elevated at 20 and 30 min after 0.8 μg/kg of BW gastrin-34 injection compared to that of vehicle injection (0.89 ± 0.03 ng/mL) (Fig. 4-3B). Moreover, 4.0 and 20.0 μg/kg BW of gastrin-34 injection enhanced total ghrelin levels for up to 150 min, the last sampling time. Concentrations of GH were increased by the administration of each doses of gastrin-34 (Fig. 4-3C). Peaks were seen for 20.4 ± 9.4 ng/mL at 20 min, for 28.0 ± 7.1 ng/mL at 30 min, and for 17.2 ± 4.5 ng/mL at 30 min after the administration of 0.8, 4.0 and 20.0 μg/kg BW of gastrin-34, respectively. However, the peak of GH was the lowest in the highest dose of gastrin-34 treated group.

Table 4-1. Effect of treatment and time on the mean of variables in steers injected with vehicle, 0.8, 4.0 and 20.0 μg /kg of BW gastrin-34.

Variables	Vehicle	Gastrin-34 (μg/kg of BW)			SEM	*P* values	
		0.8	4.0	20.0		Treatment	Treatment×Time
Total gastrin (ng/mL)	5.73^{a}	12.55^{b}	14.60^{b}	53.65^{c}	0.08	0.000	0.000
Acyl ghrelin (pg/mL)	160.6^{a}	220.2^{b}	247.9^{c}	278.0^{d}	0.8	0.000	0.000
Total ghrelin (ng/mL)	0.89^{a}	1.29^{b}	1.59^{c}	1.73^{c}	0.01	0.000	0.000
GH (ng/mL)	2.94^{a}	8.35^{b}	8.65^{b}	7.21^{b}	0.09	0.000	0.000
Insulin (ng/mL)	1.32^{a}	0.42^{b}	0.58^{c}	0.69^{d}	0.01	0.000	0.000
Glucagon (ng/mL)	1.04^{a}	1.16^{a}	1.04^{a}	0.97^{a}	0.01	0.214	1.000
Glucose (mg/dL)	92.8^{a}	95.9^{b}	95.3^{bc}	93.5^{ac}	0.1	0.022	0.522

^{a,b,c,d} Values within a row with different superscript letters were significantly different ($P < 0.05$).
Time: at -10, 0, 5, 10, 15, 20, 30, 45, 60, 75, 90, 120 and 150 min relative to injection time.
Data were analyzed using two-way ANOVA.

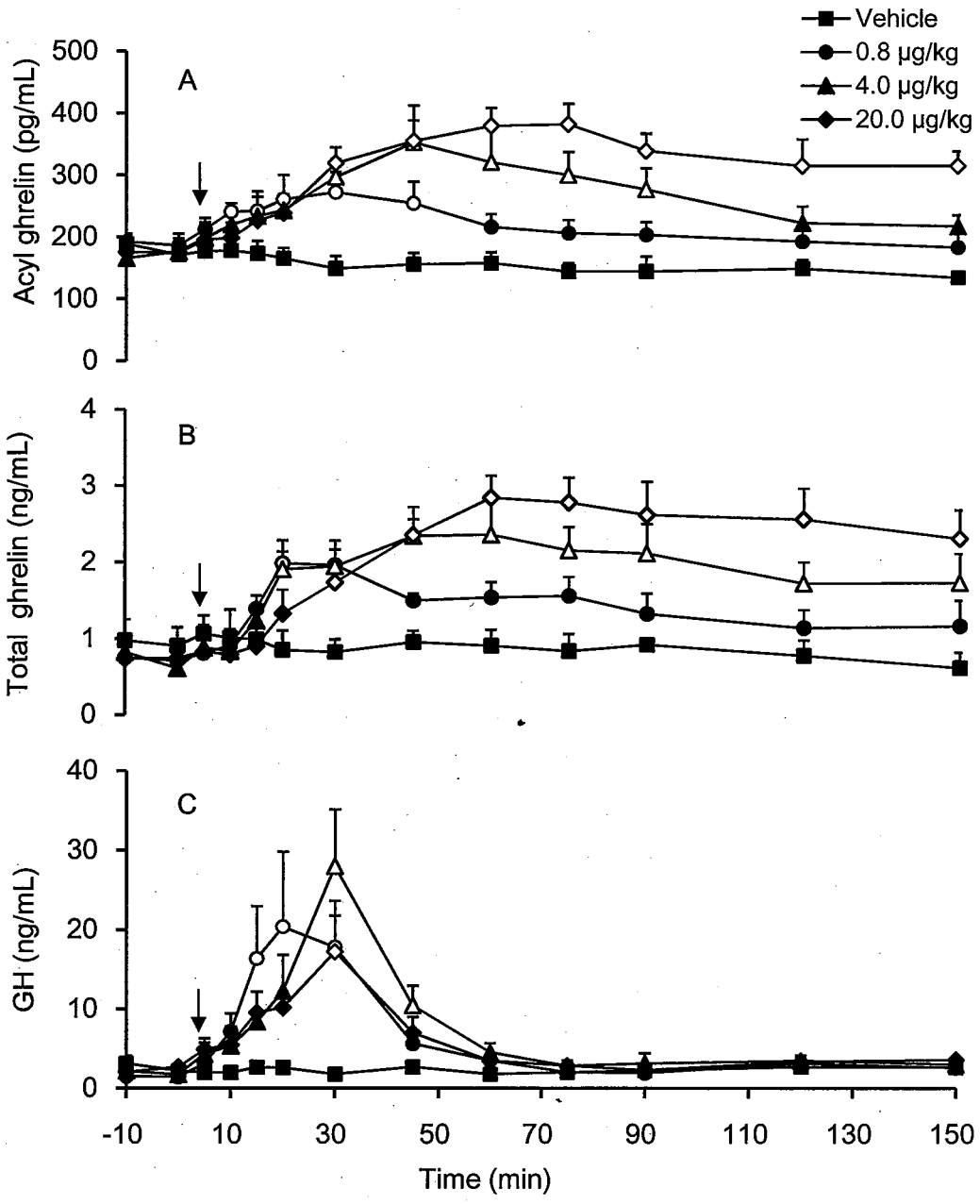

Fig. 4-3. Plasma concentrations of acyl ghrelin (A) , total ghrelin (B) and growth hormone (GH, C) in response to gastrin-34 injection in cattle. Animals were intravenously injected with 0.1% bovine serum albumin in saline (as vehicle), 0.8, 4.0 and 20.0 μg/kg body weight of bovine sulfated gastrin-34. The arrows show the injection time at 0 min. Each value represents the mean ± SEM for eight animals. Open symbols indicate that the value is significantly different from the corresponding levels of vehicle injection ($P < 0.05$).

3.3. Dose-response effects of gastrin-34 on the levels of insulin, glucagon and glucose

The injection of each dose of gastin-34 significantly reduced basal insulin levels (1.32 ± 0.04 ng/mL) within 15 min after the administration, and these levels remained low until the last sampling time, 150 min (Fig. 4-4A). Levels of insulin were significantly

different among the three doses of gastrin-34 treated groups (Table 4-1). However, 20.0 μg/kg BW of gastrin-34 at 5 min caused a slight increase ($P > 0.05$) in the concentration of insulin compared to that of vehicle injection. All treatments did not change concentrations of glucagon (1.05 ± 0.06 ng/mL) throughout the experiment (Fig.4-4B and Table 4-1). The highest dose of gastrin-34 did not affect the concentrations of glucose (92.7 ± 0.6 mg/dL) throughout the experiment (Table 1). However, concentrations of glucose were increased ($P < 0.05$) compared to that of vehicle injection at 120 and 150 min after both 0.8 and 4.0 μg/kg BW of gastrin-34 injection (Fig. 4-4C).

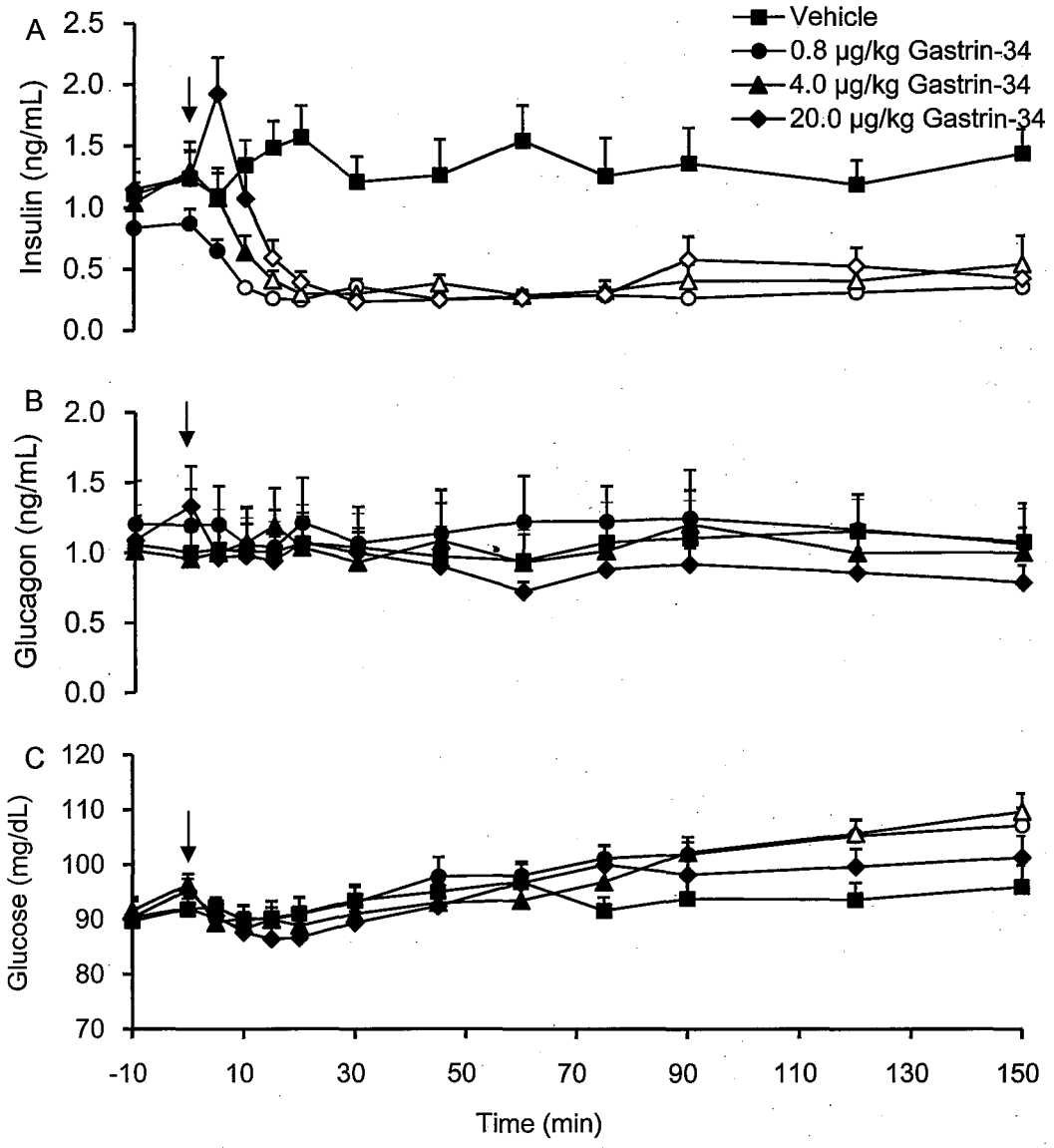

Fig. 4-4. Plasma concentrations of insulin (A), glucagon (B) and glucose (C) in response to gastrin-34 injection in cattle. Animals were intravenously injected with 0.1% bovine serum albumin in saline (as vehicle), 0.8, 4.0 and 20.0 μg/kg body weight of bovine sulfated gastrin-34. The arrows show the injection time at 0 min. Each value represents the mean ± SEM for eight animals. Open symbols indicate that the value is significantly different from the corresponding levels of vehicle injection ($P < 0.05$).

4 . Discussion

This chapter study showed that IV bolus injection of sulfated gastrin-34 increased the plasma levels of ghrelin in a dose-dependent manner in cattle. The results were, in general, in agreement with those obtained earlier in fasted rats [35,113]. However, gastric microdialysis and abdominal infusion studies showed that human Leu^{15}-gastrin-17 had no effect on the secretion of ghrelin in rats [23,27]. Lippl et al. showed that gastrin-17 significantly inhibited vagally prestimulated ghrelin release in the isolated rat stomach [94]. Some factors may account for the discrepancy between earlier results and the present data. One important factor may be that different forms of gastrin peptide were administered. In the present study, bovine sulfated gastrin-34 was effective in the stimulation of ghrelin in cattle. Other factors, such as routes of administration, doses of peptide, and feeding conditions, may have affected the results. The duration of increment of ghrelin was long in the high dose of gastrin injected group (Fig. 4-3A and B). We suggest that the long duration of ghrelin release may be related to the long duration of plasma high gastrin levels after the injection of gastrin-34. In the present study, elevations of plasma total gastrin levels lasted 45, 60 and 150 min after the administration of 0.8, 4.0 and 20.0 μg/kg BW of gastrin-34, respectively. Walsh et al. [171] reported a half-life for non-sulfated gastrin-34 after rapid intravenous injection that was 8-fold longer than that for non-sulfated gastrin-17. Hirst et al. [53] showed that the metabolic clearance rate of non-sulfated gastrin-6 was 3- and 6-fold higher than that of human non-sulfated gastrin-17 and sulfated gastrin-6, respectively. These reports imply that increasing N-terminal extensions of gastrin or sulfation of tyrosine residues in gastrin fragments increases the half-life of the peptides. Therefore, we suggest that the employment of long gastrin peptide with sulfation may result in the long life of gastrin that caused the long response of ghrelin secretion in the present study.

Gastrin is released to stimulate gastric acid secretion after food intake [131]. On the other hand, it was shown that ghrelin is increased by fasting and then decreased by re-feeding [148]. Accordingly, it can be assumed that gastrin decreases ghrelin release. However, in the present study, we found the opposite; gastrin increased plasma ghrelin levels. Plasma levels of ghrelin were reported to exhibit two diurnal peaks corresponding to the minimal and maximal gastric contents in physiological conditions in rats [113,143]. The peak observed during gastric emptying may stimulate food intake, whereas the other

one may stimulate gastric acid secretion [143]. This synergistic action of gastrin and ghrelin on gastrin acid secretion was reported in rats [35]. Therefore, the elevation of ghrelin levels in response to gastrin is reasonable, in physiological conditions, to stimulate gastric acid secretion. Gastrin/CCK_B receptors have been detected on the border of ghrelin cells [35], and gastrin binds only to gastrin/CCK_B receptors, not CCK_A receptors, so gastrin may stimulate ghrelin secretion mediated by gastrin/CCK_B receptors.

Gastrin was reported to increase the concentration of GH in plasma in humans and rats [38,168]. We assumed GH would be released after the administration of gastrin-34, since ghrelin was elevated by gastrin, and ghrelin is the secretagogue of GH [160]. As expected, the administration of gastrin-34 increased plasma GH levels in our study's cattle. However, in the present study, the duration of increment of plasma GH levels was shorter than that of ghrelin indicating that ghrelin may not involve in the gastrin-induced GH release. On the other hand, significant amounts of gastrin peptide and the expression of gastrin gene are present in the pituitary of pigs, cows and mice [125,128,134]. It is possible that gastrin directly regulates GH secretion, since under physiological conditions significant amounts of gastrin as well as gastrin/CCK_B receptors are localized in the pituitary [117] where GH is secreted.

It was found that the high dose of gastrin exhibited weak effects on the levels of GH compared with the low dose of gastrin (Fig. 4-4). This phenomenon was also found for the effect of endothelin-3 on the secretion of GH in our previous study [180], where it was explained by the desensitization of endothelin receptors and down-regulation of GHS-R1a and GHRH-R. This explanation is also possible in the present study. On the other hand, gastrin was reported to stimulate the secretion of somatostatin, which is known to inhibit the secretion of GH [46,62].

In the present study, plasma insulin levels decreased after the administration of each dose of gastrin-34, and the low levels of insulin lasted more than 2 hours (Fig. 4-5). However, gastrin has been reported to have a rapid and short-lived (1-2 min) insulin-release activity in monogastric species [62,128,165]. The effect of gastrin on significant and long-term decrease of insulin levels was not reported in those studies. We cannot exclude the possibility that plasma insulin levels was rapidly increased and returned to the baseline within 5 min after the administration of gastrin-34, since the first sampling time after the administration was 5 min in the present study. The reason for the

discrepancy in the long-term response of insulin remains unclear, but it may reflect the different effects of gastrin on the regulation of insulin in monogastrics and ruminants.

Plasma glucose levels in response to gastrin injection were not significantly changed, excluding the increases which occurred at 120 and 150 min after 0.8 and 4.0 μg/kg BW of gastrin-34 injection (Fig. 4-4). Levels of glucagon were not altered by any dose of gastrin-34 injection. When blood glucose levels rise too much, insulin increases the glucose disappearance rate and inhibits glucose production [16]. On the other hand, when blood glucose levels fall too low, glucagon causes the liver to convert stored glycogen into glucose, which is released into the bloodstream [16,17]. Therefore, it is reasonable that plasma glucose levels were not changed since insulin levels were decreased and simultaneously glucagon levels were not changed after gastrin injection. The increases of plasma glucose levels at 120 and 150 min may be due to the long-term lower insulin levels leading to a reduced glucose disappearance rate, and subsequent glucose accumulation in the blood.

Taking all these results together, we conclude that (1) intravenous bolus injections of sulfated gastrin-34 dose-dependently stimulates ghrelin and GH secretion; (2) endogenous ghrelin in response to gastrin may not contribute to the release of GH; and (3) gastrin may be involved in maintaining the homeostasis of blood glucose through down-regulation of insulin in ruminants.

5. Summary

This chapter studies were aimed to seek the effects of sulfated gastrin-34 on the circulating levels of ghrelin, growth hormone (GH), insulin, glucagon and glucose in ruminants. Animals were randomly assigned to receive intravenous bolus injections 0.1% bovine serum albumin in saline as vehicle, 0.8, 4.0 and 20.0 μg/kg body weight (BW) of bovine sulfated gastrin-34. Blood samples were collected from -10 to 150 min relative to injection time. Concentrations of acyl and total ghrelin in response to gastrin injection were significantly increased ($P< 0.05$) in a dose-dependent manner. Concentrations of GH were also markedly elevated ($P< 0.05$) by gastrin injection; however, the effect of 20.0 μg/kg was weaker than that of 4.0 μg/kg. The three doses of gastrin equally decreased ($P< 0.05$) insulin levels within 15 min and maintained the level until the time of last sampling. Gastrin had no effect ($P > 0.05$) on the levels of glucagon and glucose. The present results indicate that sulfated gastrin-34 stimulates both ghrelin and GH release, and sulfated gastrin seems to indirectly maintain the homeostasis of blood glucose through down-regulation of insulin in ruminants.

Chapter 5

Effects of Different Forms of Gastrin on the Circulating Levels of Ghrelin, Growth Hormone and Insulin

1. Introduction

There are various molecular forms of gastrin based on the number of amino acids in the peptide chain; and gastrin-17 and gastrin-34 are the main forms. In antral tissue of ruminants, gastrin-17 was reported to be the principal molecular form with gastrin-34 constituting 5% or less [5,40]. However, one-third of total gastrin in the blood was gastrin-34 [126,145]. Gastrin has a tyrosyl residue in position six, as counted from the C-terminus, that can be sulfated [42,133]. The degree of sulfation of antral extracts was reported to be more than 50% of total gastrin [5,15,133]. The reported effects of gastrin on ghrelin secretion were opposite in monogastric species [23,27, 35, 94, 113]. In chapter 4 study, we have found that bovine sulfated gastrin-34 increased plasma ghrelin and GH levels with a dose dependent manner in steers. However, the effect of sulfated and non-sulfated gastrin-17 on ghrelin and GH secretion was still not clear in ruminants, since the peptide chain length or sulfation of C-terminal gastrin fragments can affect the biological potency and the specificity of gastrin/CCK_B receptor binding [35, 53, 163, 171]. Moreover, results from chapter 4 study showed that the kinetics of the gastrin-induced increase in ghrelin and GH did not seem overlap in time (Fig. 4-3); therefore, whether ghrelin contributes to GH secretion should be confirmed.

This chapter study was aimed to investigate (1) whether sulfation was essential for gastrin action on the release of ghrelin and GH; (2) whether the gastrin chain length affected on the release of ghrelin and GH; (3) whether the effects of gastrin on ghrelin and GH release were different during pre-weaning and post-weaning; and (4) whether endogenous ghrelin in respond to gastrin stimulated GH secretion in cattle.

2. Materials and methods

All experimental procedures involving animals were approved (animal protocol numbers: 22-115 and 22-132) by the Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine, Japan.

2.1. Peptides

Bovine sulfated gastrin-17 (BGPWVEEEEAAY [SO_3H] GWMDF-NH_2, UniProt ID: P01352), bovine non-sulfated gastrin-17, bovine sulfated gastrin-9 (EAAYGWMDF-NH_2), Tyr^0-bovine sulfated gastrin-9 (YEAAY [SO_3H] GWMDF- NH_2) and [$D\text{-}Lys^3$]-GHRP-6 (HwkWfK-NH_2) were synthesized by Fmoc (9-fluorenylmethoxycarbonyl chloride) solid-phase peptide synthesis (SPPS) procedures. A modified SPPS method [82] was used for the synthesis of sulfated peptides. On the day of administration, peptides were dissolved in sterilized double-distilled water to a concentration of 1 mg/mL for gastrins and 10 mg/mL for [$D\text{-}Lys^3$]-GHRP-6. Then, based on the desired dose of gastrin for each animal, this solution was diluted with 0.1% bovine serum albumin in saline (BSA-saline, as vehicle) to obtain 5 mL of total injection volume.

2.2. Experimental design

2.2.1. Experiment 1: Effect of gastrin on ghrelin and GH levels in pre-weaned calves

The results obtained from chapter 4 showed that plasma ghrelin and GH levels were elevated by sulfated gastrin-34 in steers, whose digestive system is matured. However, the digestive system of pre-weaned claves does not develop well [133], we hypothesized that the different developing stage of digestive system might affect the function of gastrin on ghrelin and/or GH. Therefore, seven pre-weaned Holstein claves aged 27 ± 1 days and weighing 41.4 ± 0.4 kg were used. Each calf was raised in an individual pen under the natural light-dark conditions. Animals were bottle-fed with 300 g of milk replacer diet (crude protein 25%, crude fat 20%, crude fiber 1% and crude ash 8%; Snow Brand Seed, Japan) in 2 L of 39 °C water per meal, twice daily (at 9:00 and 16:00). Calf starter (crude protein 25%, crude fat 2%, crude fiber 7% and crude ash 10%; Manna Pro Corp., St. Louis, MO, USA), timothy hay and water were supplied *ad libitum*. One day before the experiment, a sterilized polyethylene catheter was non-surgically implanted into the left or right external jugular vein for injections and blood sampling. The patency of the catheter was maintained with heparin saline (10 IU heparin/mL saline, Wako, Japan). During the administration and sampling periods, animals were freely on individual pen allowed hay and water *ad libitum*. Each animal randomly received an intravenous bolus injection of 0.1%

BSA-saline as vehicle and 1.0 μg/kg (equal to 0.5 nmol/kg) BW of bovine sulfated gastrin-17 at 11:30. Blood samples were withdrawn at -10, 0 (just prior to injection), 5, 10, 15, 20, 30, 45, 60, 75, 90, 120 and 150 min relative to injection time. Samples were put into ice-chilled tubes containing heparin (8 IU/mL blood, Wako, Japan). Plasma was isolated by centrifugation at 1,870 × *g* at 4 °C for 30 min, and stored at -30 °C. For the ghrelin assay, 50 μL of 1M HCl was added to 1 mL of plasma and stored at -30 °C until analyzed.

2.2.2. Experiment 2: Effect of different forms of gastrin on ghrelin and GH levels in steers

Eight Holstein steers aged 4 months and weighing 119 ± 3 kg were used. Those animals had been used in Experiment 1 except one of them. Each animal was fed with 3 kg/day of concentrate (crude protein 16.0%, crude fat 2.5%, crude fiber 9.0% and crude ash 10.0%, Nishin Marubeni, Japan) twice daily, at 09:00 and 16:00. Timothy hay, salt blocks and water were supplied *ad libitum*. During the administration and sampling periods, animals were loosely chained to the stanchion and allowed hay and water *ad libitum*. Animals were randomly assigned to receive an intravenous bolus injection of vehicle, 1 nmol/kg BW of bovine sulfated gastrin-17, bovine non-sulfated gastrin and sulfated gastrin-9, at 11:30. Those doses of gastrins were decided based on the results from chapter 4 and this chapter Experiment 1. Other procedures and treatment of samples were identical to Experiment 1.

2.2.3. Experiment 3: Roles of GHS-R1a on the gastrin-induced GH elevation

The results from Experiment 1 and 2 showed that plasma ghrelin and GH levels were elevated by gastrin injection. Moreover, ghrelin has a putative GH-releasing function [160]. Therefore, in order to determine whether endogenous ghrelin in response to gastrin stimulates GH via GHS-R1a, we used [D-Lys^3]-GHRP-6, a selective antagonist for GHS-R1a. Eight steers which were same batch with Experiment 2, aged 9 months and weighing 268 ± 4 kg, were randomly assigned to an incomplete blocked Latin Square Design (eight animals × three treatments × three days of treatment with one day recovery between each treatment) to receive an intravenous bolus injection of vehicle, 0.53 μg/kg (equal to 0.25 nmol/kg) BW of bovine sulfated gastrin-17 alone or combined with 20.0 μg/kg BW of [D-Lys^3]-GHRP-6, at 11:30. The dose of [D-Lys^3]-GHRP-6 was decided based on our previous study [180]. In a preliminary experiment, the effect of 1 nmol/kg

BW of sulfated gastrin-17 on GH release was not blocked by 20.0 μg/kg BW of [D-Lys^3]-GHRP-6; therefore, in this experiment, 1/4 dose of gastrin-17 was used. Blood samples were withdrawn at -10, 0 (just prior to injection), 5, 10, 15, 20, 30, 45 and 60 min relative to injection time. The treatment of blood samples was identical to Experiment 1.

2.3. Measurement of hormone concentration in plasma

Concentrations of acyl ghrelin [160], GH [180], insulin [160] were quantified as described previously. RIA for total gastrin was identical to that descripted in chapter 5, except that sulfated gastrin-9 ranged from 320 to 0.039 ng/mL was used as cold standard (Fig. 5-1). Sensitivity, intra-assay coefficients of variation (CVs) and recovery rate were 77.5 pg/mL, 10% and 110% for acyl ghrelin assay; 0.36 ng/mL, 13% and 106% for GH assay; 0.05 ng/mL, 4% and 100% for insulin assay; 0.03 ng/mL, 10% and 95% for total gastrin assay, respectively.

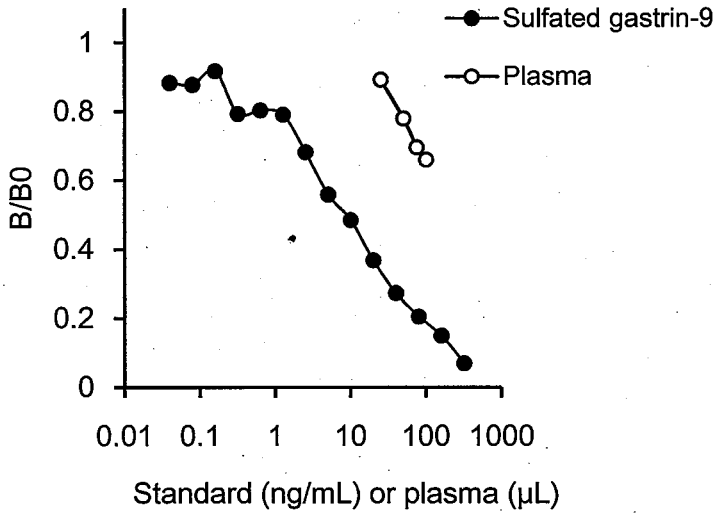

Fig. 5-1. Standard curve for total gastrin RIA. The tracer was ^{125}I-labeled bovine sulfated gastrin-9. The antibody was 09G02 at a 1:7000 final dilution. Sulfated gastrin-9 ranged from 320 to 0.039 ng/mL was used as cold standard. Each point is the mean of triplicate determinations. B/B0 represents bound/bound in zero standard.

2.4. Statistical analysis

The mean ± SEM difference in the level of assayed hormone among treatments at each time point was compared using univariate analysis. Interaction between the effect of treatment and time on the levels of assayed hormones was analyzed using a two-way

ANOVA followed by post hoc multiple comparisons. The mean difference is significant at the 0.05 level. SPSS version 16 for Windows was used.

3. Results

3.1. Effect of gastrin on plasma ghrelin and GH levels in pre-weaned calves

Concentrations of total gastrin were approximately 3-fold higher than basal level (2.05 ± 0.10 ng/mL) at 5 min after sulfated gastrin-17 injection in pre-weaned calves (Fig. 5-2A). The changes of acyl ghrelin could not be analyzed due to the great variation of acyl ghrelin levels among animals and time points in both vehicle and gastrin injection groups (Fig. 5-2B). Plasma GH level significantly increased at 15 and 20 min after sulfated gastrin-17 injection compared with vehicle injection (Fig. 5-2C).

3.2. Effect of different forms of gastrin on plasma ghrelin and GH levels in steers

Figure 5-3A shows that peak levels of total gastrin at 5 min were 3-fold different between sulfated (23.97 ± 2.68 ng/mL) and non-sulfated (7.90 ± 1.33 ng/mL) gastrin-17 injected groups though the molar dose of both gastrins injected was same, 1 nmol/kg BW. Moreover, the duration of total gastrin increase in response to sulfated gastrin-17 injection (10 min) was longer than that of non-sulfated gastrin-17 injection (5 min). However, the effects of sulfated and non-sulfated gastrin-17 on acyl ghrelin and GH release were not different (Fig. 5-3B and C). Surprisingly, we did not find the change of total gastrin level after 1 nmol/kg BW of sulfated gastrin-9 injection (Fig. 5-3A). And also, sulfated gastrinp-9 did not affect on plasma levels of both ghrelin and GH (Fig. 5-3B and C).

3.3. Role of GHS-R1a in the gastrin-induced GH release

Plasma levels of acyl ghrelin and GH were not significantly different between administration of sulfated gastrin-17 alone and combined with [D-Lys^3]-GHRP-6 (Table 5-1). Figure 5-4A confirms that levels of acyl ghrelin were increased at 10 and 20 min after administration of sulfated gastrin-17 alone or combined with [D-Lys^3]-GHRP-6. Figure 5-4B shows that levels of GH were elevated by gastrin-17; however, combined administration of [D-Lys^3]-GHRP-6 did not block the elevation of GH.

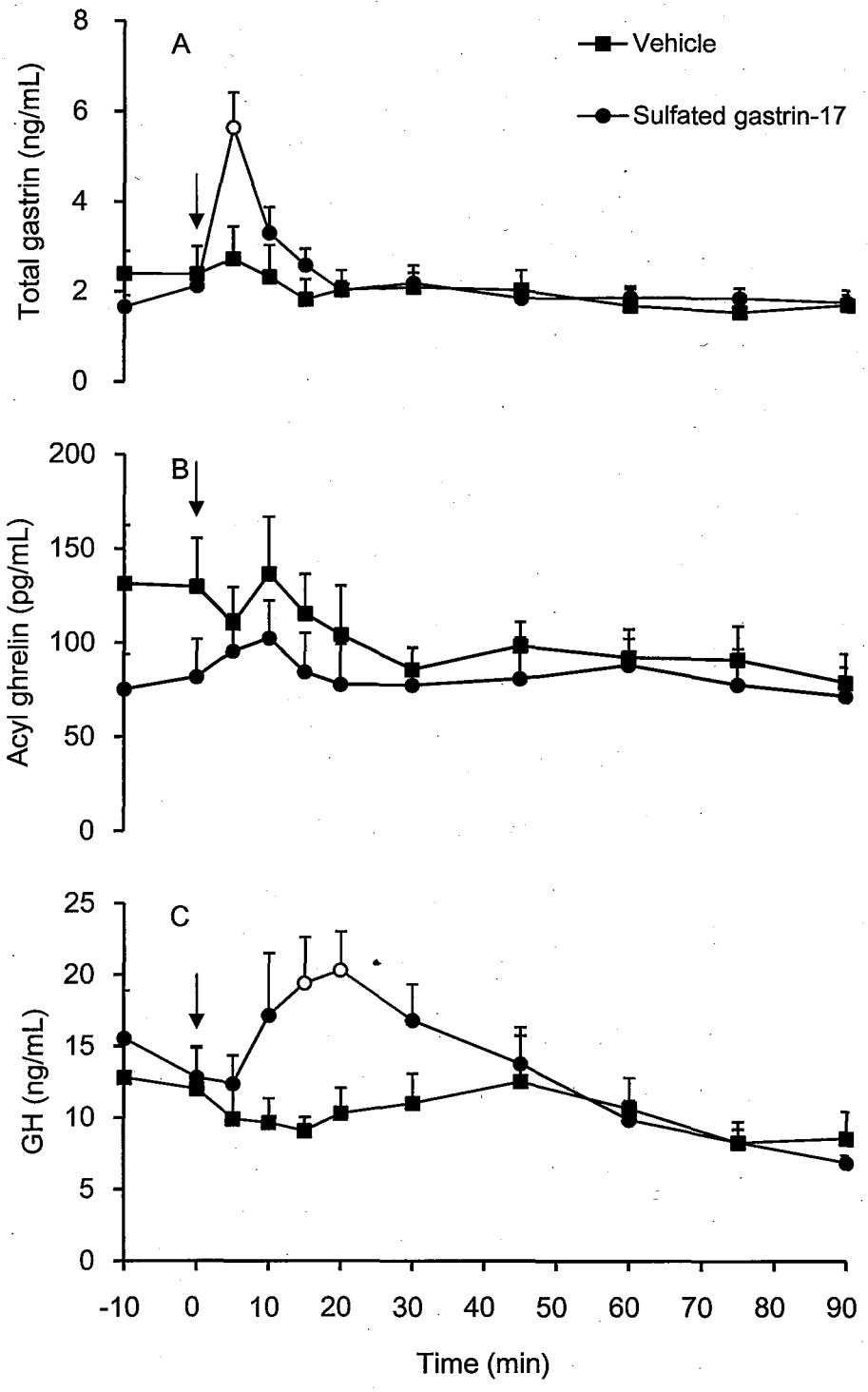

Fig. 5-2. Plasma concentrations of total gastrin (A), acyl ghrelin(B) and growth hormone (GH, B) in response to sulfated gastrin-17 injection in cattle. Animals were randomly received a bolus intravenous injection of 0.1% bovine serum albumin in saline (as vehicle) and 1 nmol/kg body weight of sulfated gastrin-17 during pre-weaning (27 days old) and post-weaning (4 months old). The arrow shows the injection time at 0 min. Each value represents the mean±SEM for seven animals. Open symbols indicate that the value is significant different from the corresponding levels of vehicle injection ($P<0.05$).

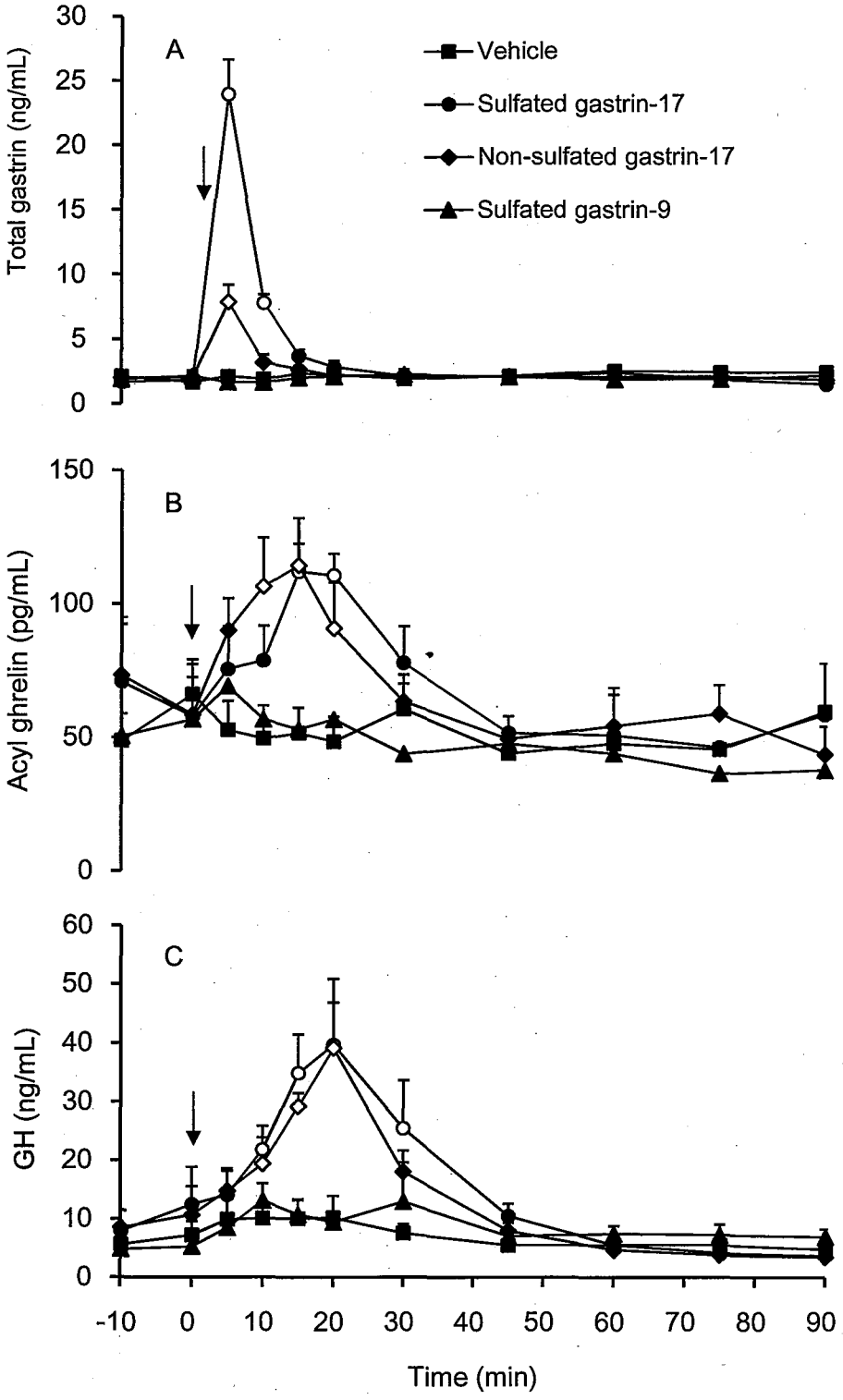

Fig. 5-3. Plasma concentrations of total gastrin (A), acyl ghrelin (B) and growth hormone (GH, C) in response to injections of three form of gastrins in steers. Animals were randomly received a bolus intravenous injection of 0.1% bovine serum albumin in saline (as vehicle), 1 nmol/kg body weight of sulfated gastrin-17, non-sulfated gastrin and sulfated gastrin-9. The arrow shows the injection time at 0 min. Each value represents the mean±SEM for six animals. Open symbols indicate that the value is significant different from the corresponding levels of vehicle injection ($P<0.05$).

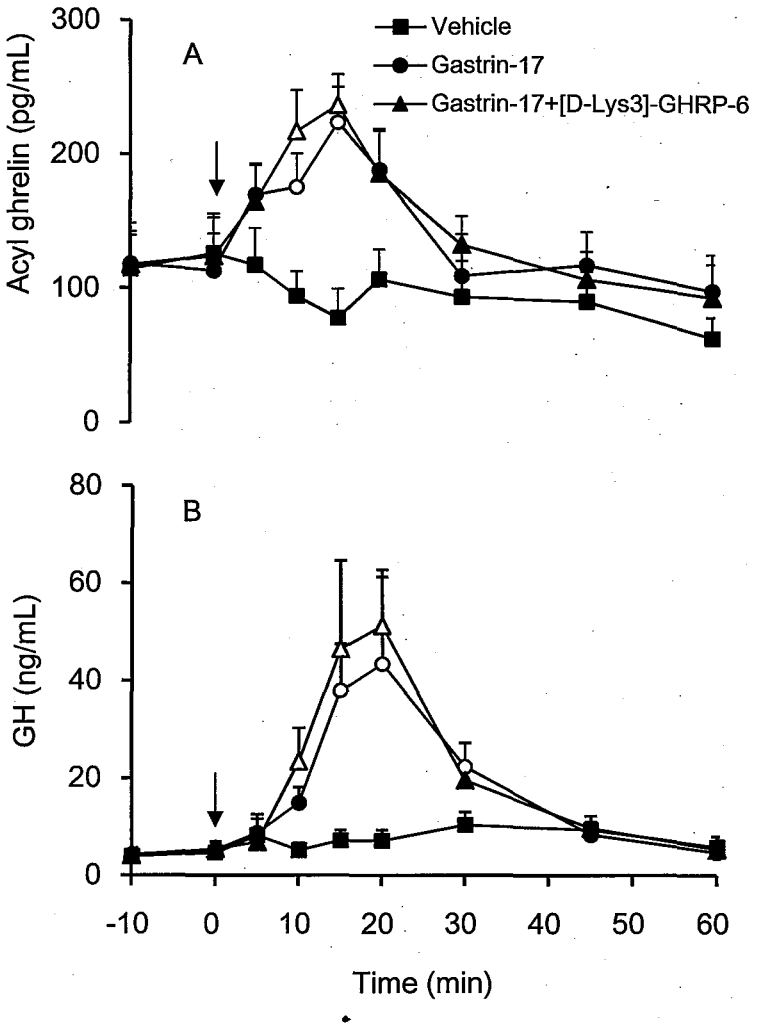

Fig. 5-4. Plasma concentrations of acyl ghrelin (A) and growth hormone (B). Animals were intravenously injected with 0.1% bovine serum albumin in saline (as vehicle), 0.53 μg/kg body weight of bovine sulfated gastrin-17 alone or combined with 20.0 μg/kg body weight of [D-Lys^3]-GHRP-6. The arrows show the injection time at 0 min. Each value represents the mean ± SEM for eight animals. Open symbols indicate that the value is significantly different from the corresponding levels of vehicle injection ($P < 0.05$).

Table 5-1. Effect of treatment and time on the mean of variables in steers injected with vehicle, gastrin-17 and gastrin-17 + [D-Lys^3]-GHRP-6.

Variables	Vehicle	Gastrin-17	Gastrin-17+ [D-Lys^3]-GHRP-6	SEM	*P* values	
					Treatment	Treatment×Time
Acyl ghrelin (pg/mL)	98.2^a	146.0^b	153.2^b	1.1	0.000	0.132
GH (ng/mL)	6.96^a	16.71^b	18.95^b	0.24	0.000	0.003

^{a,b} Values within a row with different superscript letters were significantly different ($P< 0.05$).
Time: at -10, 0, 5, 10, 15, 20, 30, 45, and 60 min relative to injection time.
Data were analyzed using two-way ANOVA.

4. Discussion

In this chapter study, we administered synthetic sulfated gastrin-17, -9, and non-sulfated gastrin-17 to investigate their effects on the secretion of ghrelin and GH in cattle. We found that the clearance rates of different forms of gastrin were different. The duration of total gastrin significantly higher than basal level in response to 1 nmol/kg BW of sulfated gastrin-34, -17 and -9 IV bolus injections was 60, 10 and less than 5 min, respectively (Fig. 4-2 and 5-3A). The clearance rate of non-sulfated gastrin-17 was faster than sulfated gastrin-17 since the concentration of total gastrin at 5 min in response to non-sulfated gastrin-17 injection was 3-fold lower than that of sulfated gastrin-17 injection (Fig. 5-3A). These results can explain why although there was no diffrence in the proportions of gastrin-34 and gasrin-17 in the tissues, there was an increase in the longer form of gastrin in the circulation of sheep [145]. A study in human showed that sulfated gastrin-17 had a 5-time longer half-life than non-sulfated gastrin-17 [123]. And also, in male subjects, disappearance half-times for natural human non-sulfated gastrin-17 after iv infusion (5.2 min) or rapid IV injection (6.4 min) were six to eight times shorter than those for natural human non-sulfated gastrin-34 (41.5 and 37.8 min, respectively) [171]. Although we did not measure the plasma concentration and ratio of gastrins in cattle, there was a report that the relative abundances of gastrin-34 and gastrin-17 were 32.9 and 67.1%, respectively, in Friesian heifer calves [126]. Andersen had shown a marked species variation in the degree of sulfated gastrins in the antral of ten mammalian species. The percentage of sulfated gastrin-17-like immunoreactivity (LI) and gastrin-34-LI varied from 80.1 in sheep and 50.1 in cows [5]. Rehfeld mentioned that due to gross differences in metabolic clearance rates, the distribution pattern of gastrins in peripheral plasma changed, so that larger gastrins with their long half-lives predominate over gastrin-17 and shorter gastrins. Hence, in peripheral blood, gastrin-34 was the predominant form of gastrin [133].

This chapter study revealed that both sulfated and non-sulfated gastrin-17 at same mole dose increased plasma ghrelin and GH levels in cattle (Fig. 5-3B and C). These results were in accordance with those of sulfated gastrin-34 (Fig. 4-3). And it seemed that the sulfation did not affect the potency of gastrin on ghrelin and GH secretion. But the duration of ghrelin elevation in response to sulfated gasrin-34 injection significantly longer than that of sulfated gastrin-17 at the same mole dose, 1 nmol/kg BW. Moreover,

sulfated gastrin-9 was not observed any effect on the elevation of ghrelin and GH as well as total gastrin during the sampling period. In fact, sulfated gasrin-9 may be not present in plasma or tissues. The purpose that we synthesized and used sulfated gasrin-9 was to explore the essential amino acid sequence required from C-terminus to the tyrosine-O-sulfation in the biological function. However, the metabolic clearance of this peptide was too fast and even within 5 min after injection we did not find the elevation of total gastrin compared with basal level of total gastrin by RIA for total gastrin using the antiserum 09G02 which had high affinity with sulfated gastrin-9 (Fig. 2-4 B). As the peptide chain was progressively lengthened towards the N-terminus the general potency of the molecule increased [163]. For example, acid secretion in response to rapid intravenous injection of human non-sulfated gastrin-34 reached a higher peak and lasted longer than in response to an equimolar dose of human non-sulfated gastrin-17; the total response to human non-sulfated gastrin-34 was about three times that to human non-sulfated gastrin-17 [171]. Increasing the peptide chain length of C-terminal gastrin increases its potency is likely to be due to enhanced the protection against elimination of the peptide.

In chapter 4 study, the duration of increment of plasma GH levels was shorter than that of ghrelin after sulfated gastrin-34 injection. Thereafter, in this chapter study, the selective antagonist of GHS-R1a did not block the elevation of GH when GHS-R1a combined with 0.25 nmol/kg BW of gastrin-17 administration. In a preliminary experiment, we found that GHS-R1a could not blocked the effect of 1 nmol/kg BW of gastrin-17 on GH release; therefore, we reduced 4 times dose of sulfated gastrin-17 in this chapter study. These results indicated that ghrelin/GHS-R1a system was not involved in the gastrin-induced GH release. On the other hand, significant amounts of gastrin peptide and the expression of gastrin gene are present in the pituitary of pigs, cows and mice [125,128,134]. It is possible that gastrin directly regulates GH secretion, since under physiological conditions significant amounts of gastrin as well as gastrin/CCK_B receptors are localized in the pituitary [117] where GH is secreted.

In this chapter study, sulfated gastrin-17 also increased plasma GH levels in pre-weaned calves; however, we could not determine whether ghrelin was changed by sulfated gastrin-17 due to the great variation of ghrelin levels among animals and time points. This variation indicated that the regulatory system of ghrelin in pre-weaned calves might not develop well.

5. Summary

The study in Chapter 5 was aimed to investigate the effects of different forms of gastrin on the circulating levels of ghrelin and growth hormone (GH), and whether the pre-weaning and post-weaning changed their effects, and whether endogenous ghrelin in respond to gastrin stimulated GH secretion in cattle. Three experiments were carried out by iv bolus injections: (1) seven pre-weaned claves were injected with 0.1% bovine serum albumin in saline as vehicle and 0.5 nmol/kg BW of bovine sulfated gastrin-17; (2) eight post-weaned steers were injected with vehicle, 1 nmol/kg BW of bovine sulfated gastrin-17, bovine non-sulfated gastrin and sulfated gastrin-9; (3) The same batch of eight steers were injected with vehicle, 0.25 nmol/kg BW of bovine sulfated gastrin-17 alone or combined with 20.0 μg/kg BW of [D-Lys^3]-GHRP-6, a selective antagonist for GHS-R1a. Blood samples were collected from -10 to 90 min relative to injection time. Plasma total gastrin, acyl ghrelin and GH levels were measured by radioimmunoassay. Plasma GH level significantly increased ($P < 0.05$) at 15 and 20 min after sulfated gastrin-17 injection compared with vehicle injection in pre-weaned claves; however, the changes of acyl ghrelin could not be analyzed due to the great variation of acyl ghrelin levels among animals and time points in both vehicle and gastrin injection groups. Both sulfated and non-sulfated gastrin-17 increased plasma ghrelin and GH levels though the duration of total gastrin elevation in response to sulfated gastrin-17 injection (10 min) was longer than that of non-sulfated gastrin-17 injection (5 min) in post-weaned steers. Sulfated gastrin-9 injection did not change the levels of any hormones assayed. Injection of [D-Lys^3]-GHRP-6 did not block the gastrin-induced GH increase. These results indicated that the main forms of gastrins in plasma, regardless of sulfation or not, may regulate the secretion of ghrelin and GH in ruminants, and the regulatory effect of gastrin on GH may be not dependant on ghrelin/GHS-R system.

Chapter 6

Effects of Bombesin-like Peptides on the Circulating Levels of Ghrelin, Growth Hormone and Insulin

1. Introduction

Bombesin and the related peptide ranatensin were first discovered in frog skin [32]. The first mammalian bombesin-like peptide (BLP) was isolated from porcine non-antral gastric and intestinal tissue and named gastrin-releasing peptide (GRP) because of its potent gastrin releasing activity [101,102]. Gastrin-releasing peptide was shown to be a 27-amino acid peptide and shares the same seven C-terminal amino acid with bombesin [101]. A decapeptide neuromedin B (NMB) was first purified from porcine spinal cord and named based on speculation that it could be involved in the neural communication system [107]. Neuromedin B was thought to be the mammalian homologue of ranatensin, as the C-terminal seven amino acid sequence is identical [30]. From a side fraction obtained in the isolation of NMB from porcine spinal cord, another decapeptide was found to be identical with the C-terminal sequence (18-27) of GRP and named neuromedin C (NMC) since it was closely related to NMB [108] .

Gastrin-releasing peptide and NMB are widely distributed throughout the central nervous system and gastrointestinal tract in mammals [120]. These peptides play many physiological roles, including stimulation of gastric acid secretion, inhibition of food intake, contraction of smooth muscle and regulation of the secretion of hormones, such as gastrin, CCK, growth hormone (GH), somatostain and insulin [120]. Binding studies and the development of highly selective antagonists established the existence of two main classes of receptors in mammalian tissues mediating the actions of BLPs. One class has a high affinity for GRP and a lower affinity for NMB and termed GRP-preferring receptor, GRP receptor or BB_2 receptor [140]. The other class has a higher affinity for NMB than for GRP and termed NMB-preferring receptor, NMB receptor or BB_1 receptor [169].

In Chapter 4 and 5, we found that gastrin stimulated the release of ghrelin and growth

hormone (GH) in cattle [181]. We hypothesize that GRP may also modulate the release of ghrelin and GH, since GRP was named because of its potent gastrin releasing activity. The effect of BLPs on GH secretion is much more a matter of debate. Most of reports supported that BLPs inhibited GH secretion in vivo in rats [72,76, 78,79,150]. In contrast, the stimulatory effect of bombesin on GH secretion in vivo was also demonstrated in rats and goldfish by two groups of researcher [19,137]. However, no effect of GRP on GH concentration in rats was reported [44]. Although the effect of BLPs on GH secretion has been investigated in monogastric animals, the effect has not been determined in vivo in ruminants, which have different digestive system from monogastric animals. Furthermore, which bombesin receptor subtypes, GRP-preferring receptor and/or NMB-preferring receptor, mediating the effect of BLPs on GH secretion remains to be clear.

Ghrelin, an endogenous ligand for the GH secretagogue receptor (GHS-R1a), was firstly purified from rat stomach in 1999 [84]. Ghrelin is a putative GH-releasing peptide in monogastic animals as well as in ruminants [160]. A microdialysis study using the rat stomach showed that human GRP reduced the microdislysate ghrelin by more than 30% [23]. Therefore, the possibility of endogenous ghrelin and GHS-R1a involved in the effect of BLPs on GH secretion should be considered.

The present study was designed (1) to investigate the effect of NMC on GH and insulin secretion; (2) to characterize bombesin receptor subtypes which mediate those effects using NMC, GRP_{1-27}, NMB and GRP-preferring receptor antagonist; and (3) to examine whether ghrelin and GHS-R1a are involved in those effects in cattle.

2. Materials and methods

All experimental procedures involving animals were approved by the Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine, Japan. Animal protocol numbers are 22-78, 22-88, 22-115, 22-131 and 22-132.

2.1. Peptides

Bovine GRP_{1-27} (APVTAGRGGALAKMYTRGNHWAVGHLM-NH_2, UniProt ID: Q863C3), Cys^1-bovine GRP_{2-27}, bovine NMC (GNHWAVGHLM-NH_2), NMB_{23-32} (GNLWATGHFM-NH_2, UniProt ID: Q2T9U8), [D-Lys^3]-GHRP-6 (HwkWfK-NH2, a putative GHS-R1a antaognist [180]), N-GRP-EE (N-acetyl-GRP_{20-26}-OCH_2CH_3, a GRP-preferring receptor antagonist [50]) were synthesized manually by Fmoc

(9-fluorenylmethoxycarbonyl) solid-phase peptide synthesis procedures. Synthesized peptides were purified by reverse-phase HPLC (TSKgel ODS-120A column; linear gradient of 0%-60% CH_3CN in the presence of 0.1% Trifluoroacetic acid), lyophilized and stored at -30 °C. Human GRP_{1-27} was obtained commercially from Phoenix Pharmaceuticals, Inc., USA. On the day of experiment, each administered peptide was freshly dissolved in sterilized double distilled water at a concentration of 10 mg/mL. Then, based on the desired dose of peptide for each animal, the 10 mg/mL of peptide was diluted with 0.1% bovine serum albumin in saline (BSA-saline) to obtain 5 mL of total injection volume.

2.2. Experimental design

2.2.1 Experiment 1: Studies on the effect of different doses of NMC on plasma GH levels in steers

Six Holstein steers aged 8 months and weighing 269 ± 7 kg were used. Each animal was fed with 3 kg/day of concentrate (crude protein 16.0%, crude fat 2.5%, crude fiber 9.0% and crude ash 10.0%, Nishin Marubeni, Japan) twice daily, at 09:00 and 16:00. Timothy hay, salt blocks and water were supplied *ad libitum*. One day before the experiment, a sterilized polyethylene catheter was non-surgically implanted into the left or right external jugular vein for injections and blood sampling. The patency of the catheter was maintained with heparin saline (10 IU heparin/mL saline, Wako, Japan). During the administration and sampling periods, animals were loosely chained to the stanchion and allowed hay and water *ad libitum*.

Animals randomly received an intravenous bolus injection of 0.1% BSA-saline (as vehicle), 0.2, 1.0, 12.5 and 50.0 μg/kg body weight (BW) of NMC at 11:20. Animals were allowed one day recovery between each treatment. The highest dose of NMC was decided based on our pilot studies in steers (unpublished data). Blood samples were withdrawn at -10, 0 (just prior to injection), 5, 10, 15, 20, 30, 45, 60, 75 and 90 min relative to injection time. Samples were put into pre-ice-chilled tubes containing heparin (8 IU/mL blood). Plasma was isolated by centrifugation at 1870×g at 4 °C for 30 min, and stored at -30 °C. For the ghrelin assay, 50 μL of 1M HCl was added to 1mL of plasma and stored at -30 °C until analyzed.

2.2.2 Experiment 2: Studies on the effect of NMC on plasma GH levels in pre-weaned calves

The results obtained from experiment 1 showed that plasma GH levels were elevated by NMC in ruminants; however, the results were opposite to most results obtained from rats, which are monogastric animals. We consider maybe the different digestive system contributes to this discrepancy. The function of forestomach is not developed well in pre-weaned claves [133], whose digestive function is closer to that of monogastric animals than mature cattle. Therefore, seven pre-weaned Holstein claves aged 27 ± 1 days and weighing 41.4 ± 0.4 kg were used. Each calf was raised in an individual pen under the natural light-dark conditions. Animals were bottle-fed with 300 g of milk replacer diet (crude protein 25%, crude fat 20%, crude fiber 1% and crude ash 8%; Snow Brand Seed, Japan) in 2 L of 39°C water per meal, twice daily (at 9:00 and 16:00). Calf starter (crude protein 25%, crude fat 2%, crude fiber 7% and crude ash 10%; Manna Pro Corp., St. Louis, MO, USA), timothy hay and water were supplied *ad libitum*. Each animal randomly received an intravenous bolus injection of 0.1% BSA-saline as vehicle and 1.0 μg/kg BW of NMC at 11:20. Other procedures and treatment of samples were identical to experiment 1.

2.2.3 Experiment 3: Studies on the mechanism of GRP on GH secretion in steers

In order to determine if the effect of GRP on GH secretion might be mediated via GHS-R1a, the antagonist of GHS-R1a was used. Moreover, in order to investigate which bombesin receptor subtypes mediating this effect, GRP_{1-27}, NMB and the antagonist of GRP-preferring receptor were administered. Eight Holstein steers aged 4 months and weighing 119 ± 3 kg were used. The animals were the same batch of animals as experiment 2 and weaned in 53 ± 1 days old and castrated in 84 days old. Animals were fed with 2 kg/head/day of concentrate (crude protein 16.0%, crude fat 2.5%, crude fiber 9.0% and crude ash 10.0%, Nishin Marubeni, Japan) twice daily, at 09:00 and 16:00. Each animal randomly received an intravenous bolus injection of 0.1% BSA-saline (as vehicle), 1.0 μg (equal to 0.9 nmol) /kg BW of NMC, 1.0 μg/kg BW of NMC combined with 20.0 μg/kg BW of [D-Lys^3]-GHRP-6, 1.0 μg/kg BW of NMC combined with 20.0 μg/kg BW of N-GRP-EE, 20.0 μg/kg BW of N-GRP-EE, 1.0 μg (equal to 0.9 nmol) /kg BW of NMB, 2.5 μg (equal to 0.9 nmol)/kg BW of $bGRP_{1-27}$ at 11:20. Animals were allowed one day recovery between each treatment. Blood sampling procedures and treatment of samples were the same as experiment 1.

2.2.4 Experiment 4: Studies on the effect of NMC on plasma GH levels in rats

To confirm the effect of NMC on the secretion of GH in monogastric animals, 12 male Wistar rats aged 72 days and weighing 317 ± 3 g were used. The animals were fed a standard rat chow (Clea Japan, Inc.) and water *ad libitum*. After 24-hour fasting, animals were anesthetized with pentobarbital sodium (60 mg/kg, IP; Schering-Plough Animal Health, USA). The right external jugular vein was cannulated for injections or sampling blood. Rats were randomly divided into three groups for three treatments and each group had 4 rats. After 30 min anesthetic injection, one of groups received an intravenous bolus injection of 0.1 μg/100 g BW of NMC or bGHRH (bovine growth hormone releasing hormone, as positive control) or 0.1% BSA-saline as vehicle in a volume of 300 μL. One milliliter of blood samples were withdrawn at 0 (just prior to injection), 5, 10, 20, 30, and 50 min relative to injection time. Treatment of blood samples was the same as experiment 1.

2.3. Measurement of hormones in plasma

2.3.1 Radioimmunoassay (RIA) for ghrelin, bovine GH and insulin

Concentrations of acyl ghrelin [160], bovine GH [180] and insulin [160] were quantified as described previously. Sensitivity, intra-assay coefficients of variation (CVs) and recovery rate were 77.5 pg/mL, 10% and 110% for acyl ghrelin assay; 0.36 ng/mL, 13%, and 106% for bovine GH assay; 0.05 ng/mL, 4% and 100% for insulin assay, respectively.

2.3.2 RIA for GRP

The general procedure of RIA for GRP was described at 2.3 of Chapter 2. In this chapter for measure GRP, the polyclonal antiserum (no. 09G05) against Cys^{1}-bovine GRP_{2-27} raised from a guinea pig was used at a 1:3000 final dilution. NMC was radioiodinated as tracer by the method of Tai et al. [153]. A cross-reactivity test of 09G05 was performed using bGRP, NMC, hGRP, NMB and N-GRP-EE ranged from 160 to 0.01 ng/mL. The specificity of the antiserum was determined by constructing a number of standard curves (Fig. 6-1). The half maximal inhibitory concentrations (IC_{50}) of bGRP, NMC, hGRP and N-GRP-EE to tracer were 1.66, 0.92, 3.95 and 0.39 ng/mL, respectively. Moreover, it displayed no cross-reactivity with NMB. For the assay of bovine plasma samples, NMC was used as cold standard in the range from 20 to 0.01 ng/mL and the results were designed as weight equivalents of NMC, termed as concentrations of

immunoreactive GRP (ir-GRP). The sensitivity of this RIA for GRP was 20.4 pg NMC/mL sample. The intra- and inter-assay CVs were 9% and 13%, respectively.

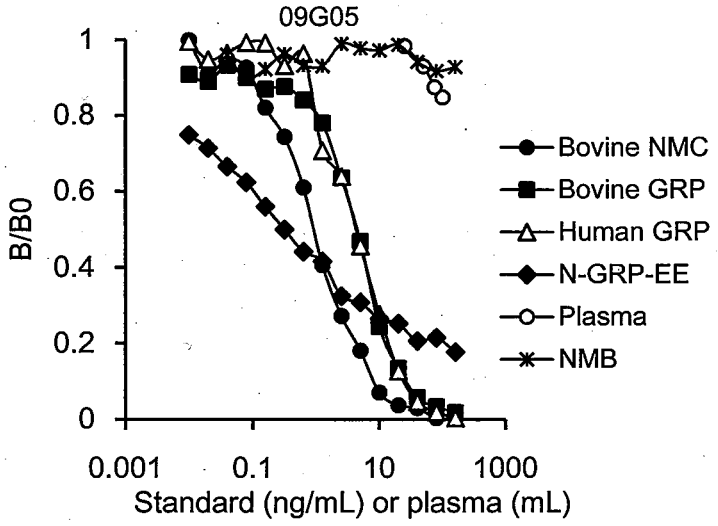

Fig. 6-1. Inhibition by various peptides on the antibody binding of ^{125}I-labeled bovine neuromedin C (NMC). The antibody was investigated with a serial dilution of bovine NMC, bovine gastrin-releasing peptide (GRP), human GRP, N-acetyl-GRP_{20-27}-OCH_2CH_3 (N-GRP-EE) and pooled bovine plasma. Each point is the mean of triplicate determinations. B/B0 represents bound/bound in zero standard.

2.3.3 RIA for rat GH (rGH)

The concentration of rGH in plasma was assayed as the procedure for RIA of rGH described by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Harbor-UCLA Medical center, California, USA. NIDDK-rGH-I-6 (AFP-5676B) for iodination, rabbit ant-rGH antiserum (AFP5672099, at a final dilution of 1:500,000 in 400 μL reactant) and NIDDK-rGH-RP-2 (AFP-3190B) for use as cold standard were obtained from Prof. Parlow, NIDDK, California, USA. Sensitivity and intra-assay CVs were 0.23 ng/mL and 14%, respectively.

2.4. Statistical analysis

The values are presented as mean ± SEM. Differences in the concentrations of control group and hormone treated group at each time point were analyzed using one-way ANOVA followed by post hoc multiple comparisons (LSD). SPSS for Windows version 16 was used. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Dose-response effects of NMC on the secretion of GH and insulin in steers

Concentrations of GH were significantly increased at 5 min compared with basal level by NMC at doses of 1.0, 12.5 and 50.0 μg/kg (Fig. 6-2A). The elevation of GH level was continued for 30 min after the highest dose of NMC injection. And, concentrations of GH were not changed by vehicle and 0.2 μg/kg BW of NMC throughout the sampling period. Plasma insulin concentrations increased from 5 min to 20 min after 0.2 and 1.0 μg/kg BW of NMC injections (Fig. 6-2B). Therefore, the minimal effective doses of NMC were 1.0 and 0.2 μg/kg BW on the release of GH and insulin, respectively.

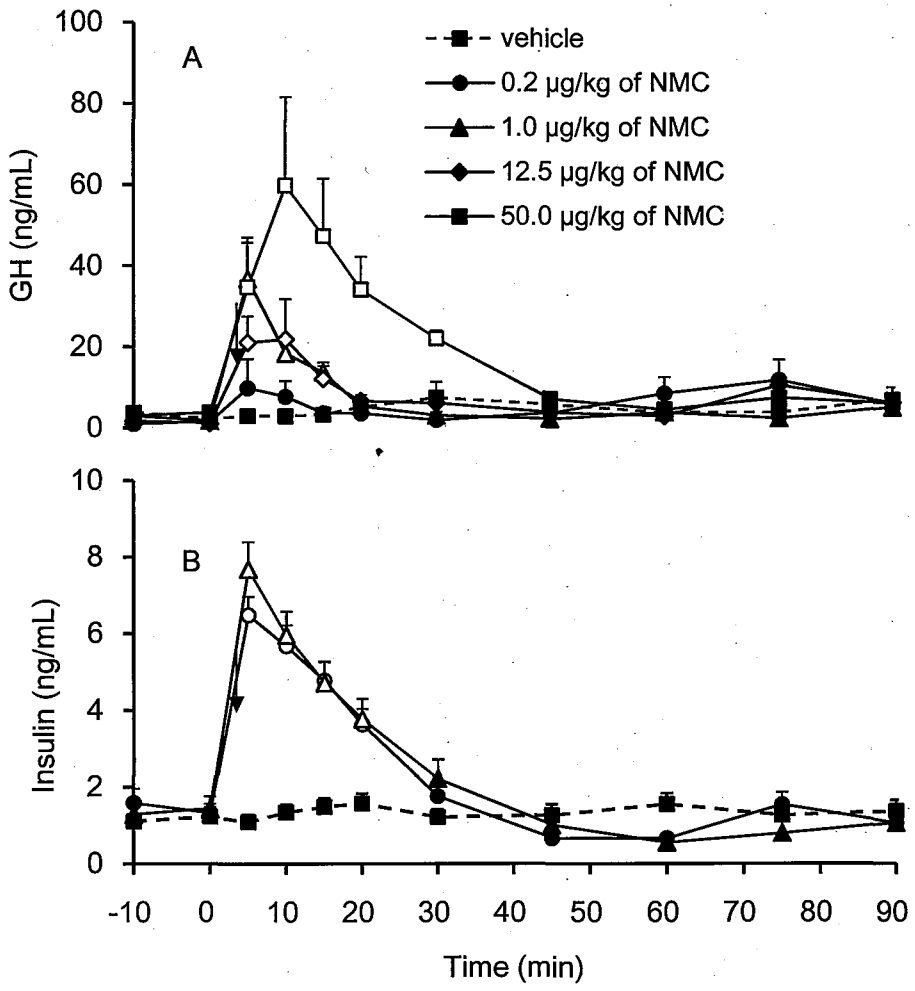

Fig. 6-2. Plasma concentrations of growth hormone (GH, A) and insulin (B) in response to neuromedin C (NMC) injection in steers. Animals were randomly received a bolus intravenous injection of 0.1% bovine serum albumin in saline (as vehicle), 0.2, 1.0, 12.5 and 50.0 μg/kg body weight of NMC. The arrow shows the injection time at 0 min. Each value represents the mean ± SEM for six animals. Open symbols indicate that the value is significant different from the corresponding levels of vehicle injection ($P<0.05$).

3.2. Effects of NMC on the release of GH, insulin and glucose in pre-weaned claves and post-weaned steers

Animals who came from the same batch, received 1.0 μg/kg BW of NMC injection during pre-weaning and post-weaning. Figure 6-3A shows that peak levels of ir-GRP at 5 min in response to NMC injection in the pre-weaned group (0.23 ± 0.03 ng/mL) was significantly lower ($P < 0.01$) than those in the post-weaned group (0.70 ± 0.12 ng/mL). Figure 6-3B presents that concentrations of GH approximately 6 folds increased compared with basal levels at 5 min after NMC injection in both periods; however, the duration of GH increase in pre-weaned group (20 min) was longer than that post-weaned group (10 min). Figure 6-3C reveals that concentrations of insulin were significantly increased with the peaks at 5 min after NMC injection in both periods, and the duration of insulin increase in pre-weaned group (25 min) was longer than that post-weaned group (20 min). Plasma glucose concentrations was found significantly increased ($P < 0.05$) at 5 min and after that significantly decreased ($P < 0.05$) at 45 and 60 min after NMC injection in post-weaned group (Fig. 6-3D); however, in pre-weaned group, plasma glucose concentrations was observed that the significant decrease was only at 20 min ($P < 0.05$) after NMC injection.

3.3. Roles of GRP-preferring receptor in the NMC-induced GH and insulin secretion

Concentrations of GH and insulin were significantly increased from 5 to 30 min (Fig. 6-4B and C) with the 5-fold elevation of ir-GRP level at 5 min in response to GRP injection (Fig. 6-4A). The peak level of insulin at 5 min (12.93 ± 1.17 ng/mL) was approximately 32-fold higher than the basal level. Plasma glucose levels also showed two phases which significantly increased at 5 and 10 min, and decreased from 30 min to 90 min, the last sampling point, after GRP injection compared with the basal level (Fig. 6-4D). On a same molar basis, effects of GRP on GH, insulin and glucose were more potent than that of NMC. Peak levels of ir-GRP at 5 min were 5-, 24- and 12-fold compared with the basal level (0.15 ± 0.01 ng/mL) after injections of NMC, NMC combined with N-GRP-EE and N-GRP-EE alone, respectively (Fig. 6-5A). The NMC-induced elevations of GH and insulin were blocked by N-GRP-EE (Fig. 6-5B and C). The NMC-induced two phases of glucose, increased followed with decreased, were blocked by N-GRP-EE. However, levels of GH, insulin and glucose were not affected by injection of NMB or N-GRP-EE alone (Fig. 6-4B, C and C).

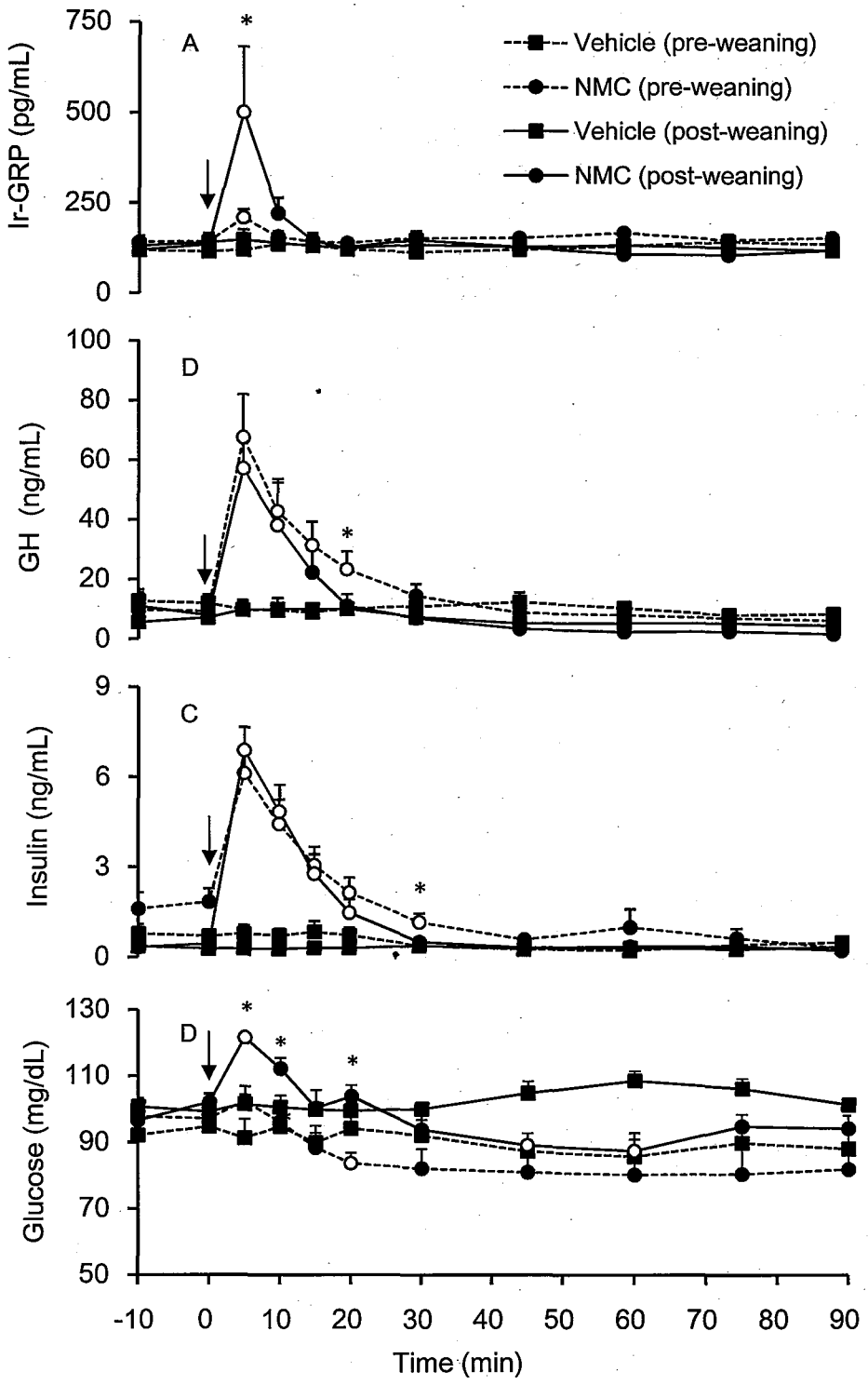

Fig. 6-3. Plasma concentrations of immunoreactive gastrin-releasing peptide (ir-GRP, A), growth hormone (GH, B), insulin (C) and glucose (D) in response to neuromedin C (NMC) injection in cattle. Animals were randomly received a bolus intravenous injection of 0.1% bovine serum albumin in saline (as vehicle) and 1.0 μg/kg body weight of NMC during pre-weaning (27 days old) and post-weaning (4 months old). The arrow shows the injection time at 0 min. Each value represents the mean ± SEM for six animals. Open symbols indicate that the value is significant different from the corresponding levels of vehicle injection ($P<0.05$). * indicates that the value is significant different between pre-weaned and post-weaned groups after NMC injection ($P<0.05$).

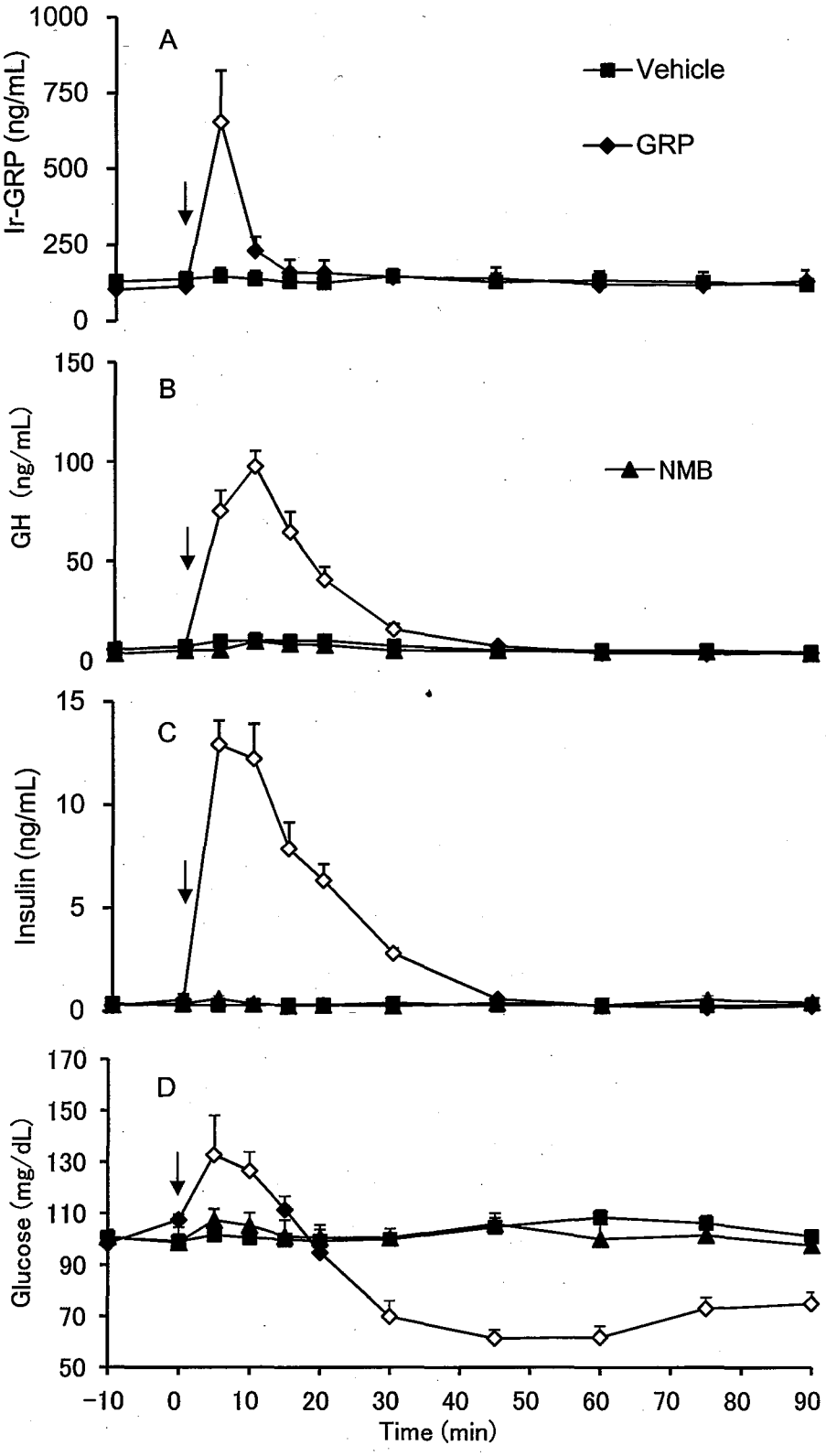

Fig. 6-4. Plasma concentrations of immunoreactive gastrin-releasing peptide (ir-GRP, A), growth hormone (GH, B), insulin (C) and glucose (D) in response to GRP and neuromedin B (NMB) injection in steers. Animals were randomly received a bolus intravenous injection of 0.1% bovine serum albumin in saline (as vehicle), 0.9 nmol/kg body weight of NMB and bovine GRP. The arrow shows the injection time at 0 min. Each value represents the mean±SEM for six animals. Open symbols indicate that the value is significant different from the corresponding levels of vehicle injection ($P<0.05$).

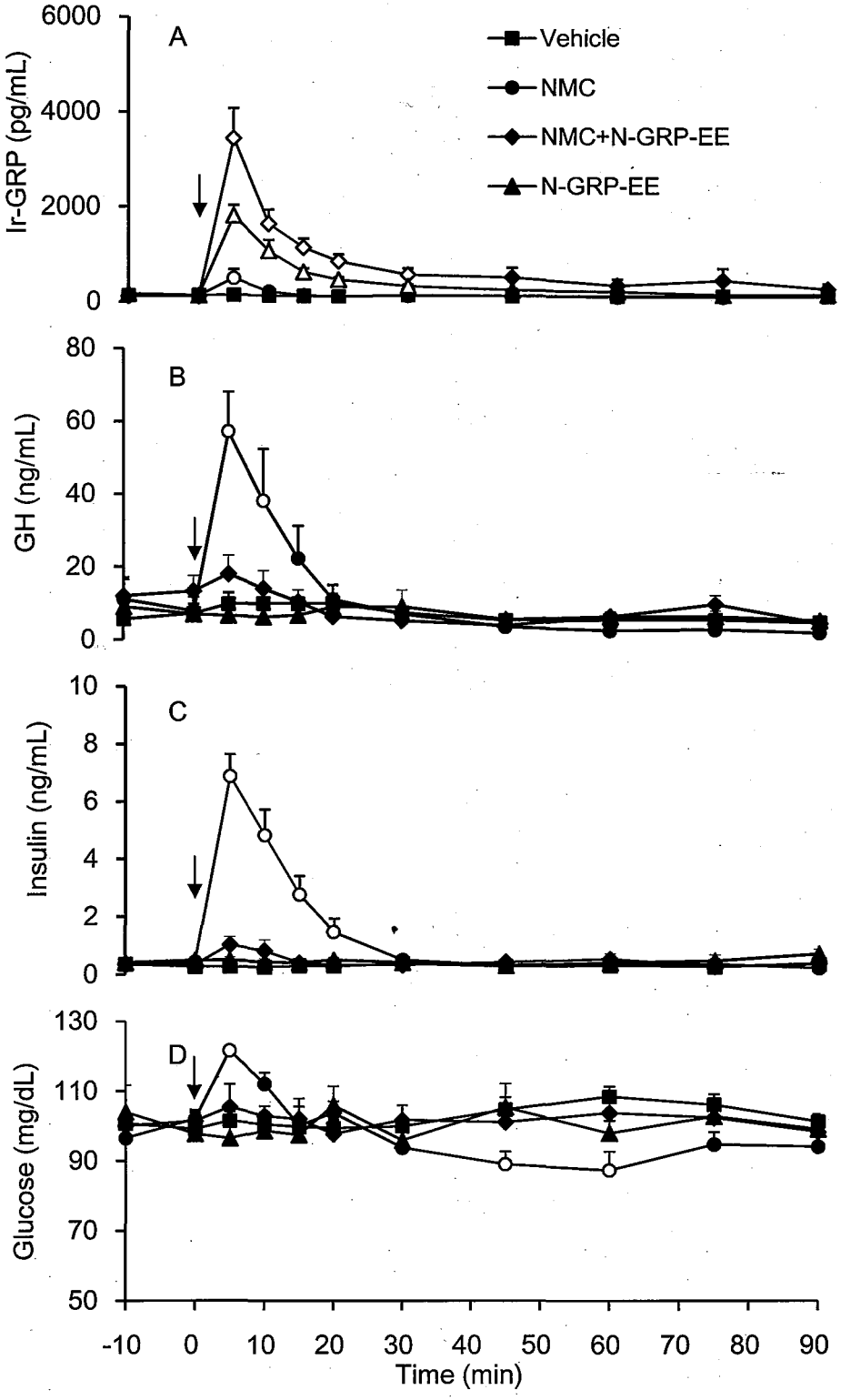

Fig. 6-5. Plasma concentrations of immunoreactive gastrin-releasing peptide (ir-GRP, A), growth hormone (GH, B), insulin (C) and glucose (D) in response to neuromedin C (NMC) and N-GRP-EE alone or combined injection in steers. Animals were randomly received a bolus intravenous injection of 0.1% bovine serum albumin in saline (as vehicle), 1.0 μg/kg body weight of NMC and 20.0 μg /kg body weight of N-GRP-EE (the selective antagonist of GRP-R) alone or combined. The arrow shows the injection time at 0 min. Each value represents the mean ± SEM for six animals. Open symbols indicate that the value is significant different from the corresponding levels of vehicle injection ($P<0.05$).

3.4. Roles of GHS-R1a in the NMC-induced elevation of GH, insulin and glucose

Concentrations of acyl ghrelin was not changed by any treatment in this study (data was not shown). Moreover, the elevated patterns of GH, insulin and glucose were no difference (P>0.05) between the injection of NMC alone and that of NMC combined with [D-Lys^3]-GHRP-6, the antagonist of GHS-R1a (Fig. 6-6).

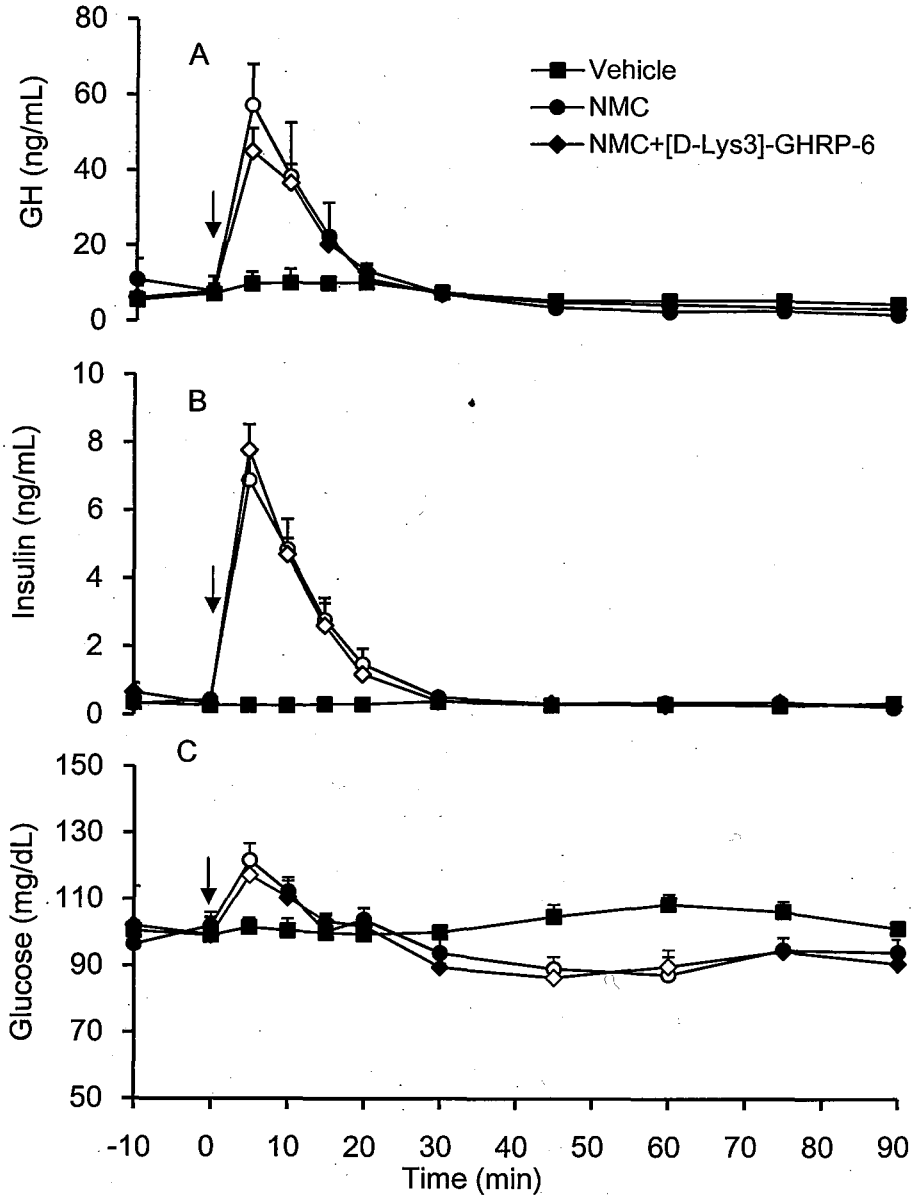

Fig. 6-6. Plasma concentrations of growth hormone (GH, A) insulin (B) and glucose (C) in response to neuromedin C (NMC) and [D-Lys^3]-GHRP-6 alone or combined injection in steers. Animals were randomly received a bolus intravenous injection of 0.1% bovine serum albumin in saline (as vehicle), 1.0 μg/kg body weight of NMC and 20.0 μg/kg body weight of [D-Lys^3]-GHRP-6 (the antagonist of GHS-R1a) alone or combined. The arrow shows the injection time at 0 min. Each value represents the mean ± SEM for six animals. Open symbols indicate that the value is significant different from the corresponding levels of vehicle injection (P<0.05).

3.5. Effects of NMC on the secretion of GH in rats

Figure 6-7 shows the changes of GH in response to the injection of NMC and bGHRH in rats. Concentrations of GH were found to significantly increase at 50 min (93.41 ± 8.52 ng/mL) after NMC injection compared with basal levels (2.53 ± 0.03 ng/mL). In the positive contral group, concentration of GH elevated from 5 to 50 min after bGHRH injection compared with the basal level.

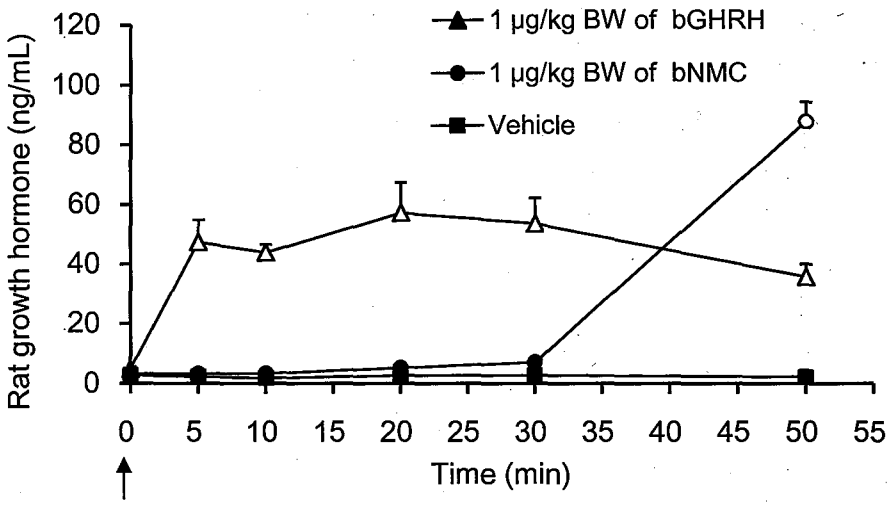

Fig. 6-7. Plasma concentrations of growth hormone in response to neuromedin C (NMC) and growth hormone releasing hormone (GHRH) injection in rats. Animals were randomly received a bolus intravenous injection of 0.1% bovine serum albumin in saline (as vehicle), 0.1µg/100 g body weight of bovine GHRH (as positive control) or bovine NMC. The arrow shows the injection time at 0 min. Each value represents the mean ± SEM for four animals. Open symbols indicate that the value is significant different from the corresponding levels of vehicle injection ($P<0.05$).

4. Discussion

This chapter study showed that the circulating GH level increased within 5 min after intravenous administration of NMC and GRP in cattle. These results are in line with those of previous reports obtained in rats and goldfishes [19,137]. However, other reports are contradictory even in the same species and most of them suggested that BLPs may inhibit GH secretion, at least in part, by stimulating the release of somatostatin, which reduces GH secretion and blocks the response of pituitary to GHRH [72, 76 , 78,79,150]. For conforming whether the different effects of GRP on GH secretion were due to the

difference between monogastric animals and ruminants, we tested the effect of GRP on GH secretion in pre-weaned calves, whose digestive system is closer to monogastric animals than post-weaned cattle. The results presented that NMC also significantly increased plasma GH level even though the clearance rate of NMC was significantly faster in pre-weaned male claves than in post-weaned steers (Fig. 6-3A). For further confirming the effect of NMC on GH secretion, NMC was intravenously injected to pentobarbital-anesthetized rats. Growth hormone level was found to increase at 50 min, the last sampling time point, in NMC injected rats; and GH level was observed to increase from 5 min to 50 min in GHRH injected rats, the positive control group (Fig. 6-7). The results can explain why Güllner group could not find the effect of intravenous injection of GRP on circulating GH level in rats since they only sampled blood within 30 min after injection of the peptide [44]. The significant elevation of GH level after NMC injection was much faster in cattle (5 min) than in rats (50 min), suggesting the pathway through which NMC exerts this effect is different between cattle and rats.

Although the effects of bombesin-like peptides on GH secretion were reported previously, involvement of bombesin receptor subtypes in these effects was not clear. In this chapter study, three kinds of ligand of bombesin receptor were used. Gastrin-releasing peptide and NMC were found to significantly increase plasma GH level in cattle; however, NMB did not change plasma GH level. GRP-preferring receptors have a high affinity for GRP_{1-27} and NMC but a lower affinity for NMB; and NMB-preferring receptors have a higher affinity for NMB than for GRP, indicating that the GRP-preferring receptor may mediate the GRP-induced GH secretion. To further confirm our suggestion, the selective antagonist for GRP-preferring receptors, N-GRP-EE, was administered. The antagonist action of have been proved. N-GRP-EE blocks GRP-stimulated mitogenesis in Swiss 3T3 mouse fibroblasts, inhibits GRP-dependent release of gastrin in vitro, and blocks GRP-induced elevation of $[Ca^{2+}]i$ in H345 small cell lung cancer cells [50]. In this study, N-GRP-EE blocked the NMC-induced elevation of plasma GH levels, indicating that the GRP-preferring receptor mediated this effect in cattle.

Ghrelin is identified as an endogenous ligand of GHS-R1a and stimulates the secretion of GH in monogastics as well as in ruminants. However, in the present study, GRP_{1-27} and NMC had no effect on plasma ghrelin level, and the antagonist for GHS-R1a, $[_D\text{-Lys}^3]$-GHRP-6, did not block the NMC-induced elevation of GH level (Fig. 6-6A). Therefore, these results indicated that GHS-R1a was not involved in the GRP-induced

elevation of GH in cattle. It is well accepted that GH release is stimulated by GHRH and inhibited by somatostatin. If GRP has a physiological role to stimulate GHRH release in hypothalamus, it should be expected that there is present GRP or the GRP-preferring receptor localization. The high concentration of GRP-like immunoreactivity was reported to present in the hypothalamus [77,81]. Moreover, characteristic expression of the GRP-preferring receptor is observed in the hypothalamic region [120]. These data suggest a possible role of GRP in regulation of GHRH secretion, which induced GH release in pituitary.

In this chapter study, we also found that both GRP_{1-27} and NMC increased plasma insulin levels. Likewise, both GRP and NMC regulated glucose showed two phases, the first increased followed by the second decreased (Fig. 6-4D and -5D). We hypothesized that the first phase of glucose increased by GRP stimulated insulin release, then the elevated insulin motivated the tissue utilized blood glucose and led to the second phase of plasma glucose decrease. Moreover, GRP-preferring receptors may also mediate the GRP-induce changes of insulin and glucose in cattle since N-GRP-EE, the selective antagonist of GRP-preferring receptors blocked these effects induced by NMC (Fig. 6-5C and D). In addition, ghrelin/GHS-R1a system may not involve in GRP-induced changes of insulin and glucose since GRP did not affect on plasma ghrelin level and [$_D$-Lys^3]-GHRP-6, the selective antagonist for GHS-R1a did not block these effects when combined with NMC injection.

In conclusion, these results demonstrates that (1) intravenous injection of GRP increases circulating levels of GH and insulin in cattle, and the sensitivity of insulin to NMC was higher than that of GH ; (2) those effects may be mediated by GRP-preferring receptors but not through a ghrelin/GHS-R1a pathway; and (3) the regulatory effect of GRP on GH secretion may be through different pathways between ruminants and monogastic animals due to the significantly different elevation time of GH after administration of peptides in cattle and rats. These results indicated that circulating GRP may involve in regulation of GH secretion and glucose homeostasis in ruminants.

5. Summary

The study in Chapter 6 was designed to determine the effects of bombesin-like peptides on the circulating levels of ghrelin, growth hormone (GH), insulin and glucose in ruminants during pre-weaning and post-weaning, and to characterize the receptor subtypes mediating these effects. Three experiments were carried out by intravenous bolus injections: (1) six steers were injected with 0.1% bovine serum albumin in saline (as vehicle), 0.2, 1.0, 12.5 and 50.0 μg/kg body weight (BW) of neuromedin C (NMC); (2) seven pre-weaned claves were injected with vehicle and 1.0 μg/kg BW of NMC; and (3) six post-weaned steers were injected with vehicle, 1.0 μg/kg BW of NMC alone or combined with 20.0 μg/kg BW of [D-Lys^3]-GHRP-6 (an antagonist for GH secretagogue receptor type 1a [GHS-R1a]) or N-GRP-EE (an antagonist for GRP-preferring receptors), N-GRP-EE alone, 1.0 μg/kg BW of neuromedin B (NMB) and 2.5 μg/kg BW of bovine gastrin-releasing peptide (GRP). Blood samples were collected from -10 to 90 or 150 min relative to injection time. Concentrations of immunoreactive GRP (ir-GRP), ghrelin, GH and insulin in plasma were analyzed by radioimmunoassay. Both GRP and NMC injection increased GH and insulin levels from 5 min, and the minimal effective doses of NMC were 1.0 μg/kg for GH and 0.2 μg/kg for insulin in post-weaned steers. Plasma glucose was observed two phases, a significant rise followed a remarkable fall after NMC or GRP administration. N-GRP-EE completely blocked the NMC-induced changes of GH, insulin and glucose, but [D-Lys^3]-GHRP-6 did not block any of these changes. Administration of NMB or N-GRP-EE alone did not change the circulating levels of hormones assayed and glucose during all the sampling time points. Effects of NMC on GH and insulin in pre-weaned calves were approximately identical to those in post-weaned steers. But the duration of elevating both GH and insulin levels was longer in pre-weaned calves than in post-weaned steers though peak levels of ir-GRP at 5 min in response to NMC injection in the pre-weaned group (0.23 ± 0.03 ng/mL) was significantly lower ($P < 0.01$) than those in the post-weaned group (0.70 ± 0.12 ng/mL). Ghrelin levels were not changed by any treatments in this study. These results indicated that NMC and GRP may regulate the secretion of GH and insulin as well as glucose homeostasis mediated by GRP-preferring receptors but not by the ghrelin/GHS-R1a system in ruminants.

Chapter 7

General Discussion and Conclusions

Ghrelin was first discovered from rat stomach and found mainly secreting from the stomach of monogastrics [84] as well as the abomasum of ruminants [58]. Ghrelin are an putative endogenous growth hormone (GH) secretagogue and a regulator of appetite. Our laboratory has conducting research on ghrelin in ruminants for several years since the two putative roles of ghrelin are so important for growing and producing meat and milk. Firstly, the former undergraduate and graduate students had successfully established the radioimmunoassay (RIA) systems for acyl ghrelin and total ghrelin (all ghrelin with bovine C-terminal amino acid sequence 11-27) using the rabbit anti-[Cys^{12}]-ghrelin(1-11) serum and guinea pig anti-[Cys^{0}]-bovine ghrelin (11-27) serum, respectively [160]. The RIA systems provide us with opportunity to investigate the physiological and pharmacological levels of ghrelin in ruminants, such as cattle, goats and sheep. The previous studies of our laboratory in ruminants in vivo have demonstrated that: (1) ghrelin is the potent GH releaser, and the acyl modification is essential for the endocrine effects of ghrelin [158, 160]; (2) ghrelin has synergistic effect with GH-releasing hormone (GHRH) on GH secretion and the physiological stages of ruminant influence the effect of ghrelin on GH axis [159]; and (3) nutritional conditions affect not only ghrelin level but also the ratio of acyl ghrelin and total ghrelin [161]. After the putative roles of ghrelin were confirmed in ruminants, our direction of study was shifted to investigating the regulatory mechanisms of brain-gut peptides on ghrelin secretion in ruminants. Endothelin (ET), gastrin and gastrin-releasing peptide (GRP) were three of the candidate brain-gut peptides in this thesis study.

1. Roles of ET, gastrin and GRP in the secretion of ghrelin as well as GH

A microdialysis study in rat stomach [23] and our laboratory previous study in cattle [157] have found that ET increased microdialysate ghrelin and circulating ghrelin levels, respectively. In the cattle study, ET also elevated circulating GH levels. Therefore, we were interested in determining which ET receptor subtypes, ET_A and/or ET_B, mediated these effects, and whether the endogenous ghrelin is increased by ET stimulated GH

secretion. Unfortunately, we did not find a peptide ET receptor antagonist which could be synthesized in our laboratory. However, the truncated linear peptide IRL 1620 (Table 2-2), a high selective agonist for ET_B receptors, was synthesized reported in Chapter 2 and injected to steers as reported in Chapter 3. IRL 1620 mimicked ET to elevate circulating ghrelin and GH levels, indicating that ET_B receptors involved in ET-induced elevation of ghrelin and GH levels in cattle. But in Chapter 3 study, we could not exclude the role of ET_A receptors in those effects. In addition, [D-Lys^3]-GHRP-6, a putative antagonist for GHS-R1a, blocked the ET-induced elevation of GH, indicating that endogenous ghrelin in response to ET stimulated GH secretion.

Compared with the studies on the effect of ET on ghrelin, the studies on the effect of gastrin on ghrelin were more and the results were contradictory [23, 27, 35, 94, 113]. Gastrin is predominantly secreted by antrum G-cells in monogastrics [28,42] as well as in ruminants [14]. Gastrin was defined by its effect on gastric acid secretion [28]. Moreover, ghrelin and gastrin have a synergistic action on gastric acid secretion [35]. The reason choosing gastrin as the candidate was that we hypothesized gastrin might regulate ghrelin secretion since both peptides were mainly secreted from the stomach and both stimulate gastric acid secretion. The study in Chapter 4 proved that sulfated gastrin-34 dose-dependently increased circulating both ghrelin and GH levels. Whether non-sulfated and shorter forms of gastrin had the identical effects of sulfate gastrin-34? Did the physiological conditions, such as pre-weaning, affect those effects of gastrin? Whether endogenous ghrelin responded to gastrin increased GH? These questions were answered in Chapter 5 study. Both sulfated and non-sulfated gastrin-17 increased plasma ghrelin and GH levels, but sulfated gastrin had no effects, indicating that the circulating main forms of gastrin, regardless of sulfation or not, may regulate the secretion of ghrelin and GH in steers. The circulating GH level significantly increased after sulfated gastrin-17 injection in pre-weaned claves; however, the changes of acyl ghrelin could not be analyzed due to the great variation of acyl ghrelin levels among animals and time points in both vehicle and gastrin injection groups. Therefore, we suggested that the regulatory system for ghrelin might not be established well in pre-weaned calves. [D-Lys^3]-GHRP-6 did not block the gastrin-induced GH increase, indicating that ghrelin/GHS-R1a system may not be involved in the gastrin-induce elevation of GH.

Gastrin-releasing peptide (GRP) was initially named because of its potent gastrin releasing activity in monogastrics [101,102]. Gastrin-releasing peptide belonged to the

bombesin-like peptides (BLP), which also included neuromedin C (NMC, the C-terminal decapeptide of GRP) and neuromedin B (NMB) in mammalians. As gastrin regulated the secretion of ghrelin and GH, we hypothesized that GRP and its family peptides might have the same function. This hypothesis was investigated in Chapter 6 study. Both GRP and NMC increased circulating GH levels, while did not like gastrin affect ghrelin levels in pre-weaned male claves and post-weaned steers. NMB was not found any effects in this chapter study. Two main classes of receptors, GRP-preferring receptor and NMB-preferring receptor, in mammalian tissues mediate the actions of BLPs. Gastrin-releasing peptide-preferring receptors have high affinity for GRP and lower affinity for NMB [140]; Neuromedin B-preferring receptors have higher affinity for NMB than for GRP [169]. We found that N-GRP-EE, an antagonist for GRP-preferring receptors, completely blocked the NMC-induced elevation of GH, but [D-Lys^3]-GHRP-6 did not block this effect. Accordingly, the elevation of GH by GRP and NMC was mediated by GRP-preferring receptors, while ghrelin/GHS-R1a system might not be involved in this action in ruminants.

From studies on gastrin and GRP, we noticed that the action of gastrin and GRP in ghrelin did not coincide, and our hypothesis was denied. Thereafter, we assayed the plasma total gastrin concentrations in BLPs injected cattle. Unfortunately, we did not observed the measureable changes in total gastrin after administration. There were many studies supported the gastrin-releasing action of GRP in monogastric animals [11, 18, 61, 93]. However, BLPs, such as bombesin and GRP, were reported to have np effect on gastrin secretion in calves and sheep [13, 100], indicating that BLPs may play different roles in gastrin release between non-ruminant species and ruminants. This could be one of the explanations why the action of gastrin and GRP was different in ghrelin secretion in ruminants.

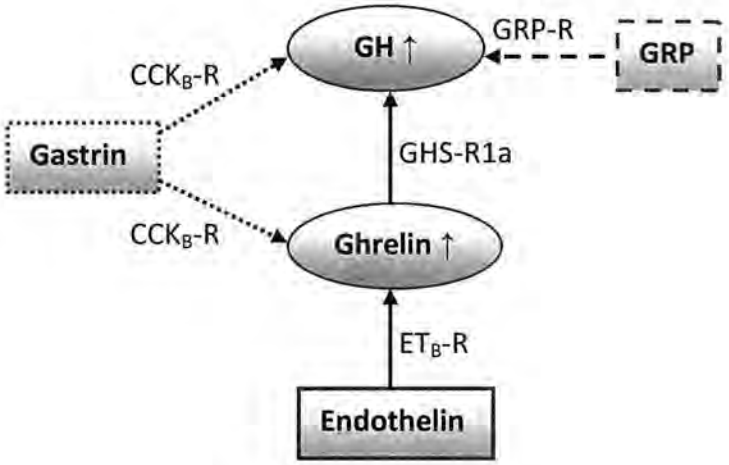

Fig. 7-1. Regulatory mechanisms of endothelin (⟶), gastrin (····▸) and gastrin releasing peptide (GRP, – – ▸) in the secretion of ghrelin and/or growth hormone (GH) in cattle.

2. Roles of ET, gastrin and GRP in glucose metabolism

In general, insulin causes cells to take up glucose from the blood, then glucose is stored as glycogen in the liver and muscle. In this thesis studies, ET, gastrin and GRP showed three obviously different behaviors in circulating glucose levels in cattle (Table 7-1). In Chapter 3 study, administration of ET-3 was found to have a significant effect on the increase of plasma glucose, while circulating insulin after administration of ET was reported to have a slight fall but without any significant effect in cattle [157]. In Chapter 4 study, gastrin did not change the circulating levels of glucose and glucagon, while circulating insulin level was decreased by gastrin over a long period of time. In Chapter 6 study, GRP increased circulating glucose level followed by a decrease in glucose, while circulating insulin level was sharply augmented after administration of GRP. We presumed that ET-induced increase of glucose may be related to weak insulin response to glucose. The actions of gastrin in glucose and insulin indicate that gastrin may regulate glucose homeostasis as a result of insulin resistance in the peripheral tissue. We also hypothesized that the first phase of glucose increased by GRP stimulated insulin release, then the elevated insulin motivated the tissue utilized blood glucose and led to the second phase of plasma glucose decrease. Endothelin and GRP-induced changes of glucose was suggested not to likely be related with ghrelin/GHS-R system, since [D-Lys^3]-GHRP-6 was not able to block ET and GRP-induced changes of glucose. This thesis studies reveal that many complicate mechanisms involved in maintaining glucose homeostasis in ruminants.

Table 7-1. Actions of ET, gastrin and GRP on circulating glucose and insulin levels

Peptides administered	Glucose	Insulin
Endothelin	↑	–
Gastrin	–	↓
Gastrin-releasing peptide	↑ followed ↓	↑

↑ increased; ↓ decreased; – no changes

3. Conclussions

In summary, the main findings present in this thesis as follows:

(1) Endogenous ghrelin in response to ET-3 injection increase GH secretion, and ET_B receptors are involved in this process in steers.

(2) The two main forms of gastrin, gastrin-34 and gastrin-17, regardless of sulfation or not, stimulate both ghrelin and GH secretion in pre-weaned male calves and post-weaned steers; and gastrin-induced secretion of GH is independent to ghrelin/GHS-R1a system.

(3) Both GRP and NMC enhanced GH secretion mediated by GRP-preferring receptors in pre-weaned male calves and post-weaned steers

(4) Endothelin, gastrin and GRP are involved in maintaining glucose homeostasis through, at least in part, regulating insulin secretion and/or insulin resistant in ruminants.

4. Implications for future studies

This thesis studies have provided the evidence that ET, gastrin and GRP may regulate the secretion of ghrelin and/or GH and the involvement of some receptors in these processes in ruminants. However, whether these regulatory effects also physiologically happen? Therefore, the further studies will be of interest in the three peptide hormones.

In ET study, we have demonstrated that ET_B receptors were involved in ET-induced secretion of ghrelin and GH; however, we can not exclude the other ET receptor subtype, ET_A receptor. So, if possible, using the selective antagonist for ET_A or ET_B to determine the ET receptor subtypes, which mediate the effect of ET on the secretion of ghrelin and GH could also be examined in future studies. In addition, we can use immunohisto -

chemistry methods to explore whether ET receptor subtypes localize on ghrelin secretion cells (Gr cells) in ruminants. This study can provide the histological possibility that ET and its receptors regulate ghrelin secretion.

In gastrin and GRP studies, we just observed that both gastrin and GRP increase GH with a ghrelin/GHS-R1a independent manner. However, we were not sure whether gastrin and GRP directly act on pituitary to stimulate GH secretion or indirectly regulate GH-releasing hormone (GHRH) and/or somatostatin secretion from hypothalamus. Then, further two studies can be done in ruminants: (1) using sensitive and specific radioimmunoassay (RIA) for GHRH and somatostatin measure of the peripheral or central circulating levels of GHRH and somatostatin after administration of gastrin and GRP; and (2) using the selective antagonists for the receptors of GHRH and somatostatin to determine the involvement of the ligands.

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List of Abbreviations

ANOVA	Analysis of variance
B/T	Binding/binding in total
BBS	Bombesin
BLAST	Basic Local Alignment Search Tool
BLP	Bombesin-like peptide
BSA	Bovine serum albumin
BW	Body weight
CCK	Cholecystokinin
CCK-8	Octapeptide cholecystokinin
CCK_A	Cholecystokinin A receptor
CCK_B	Cholecystokinin B receptor
cDNA	Complementary DNA
CV	Coefficient of variations
DMF	N,N-dimethylformamide
EC	Endothelial cell
ECL cell	Enterochromaffin-like cell
ET	Endothelin
ET-1	Endothelin-1
ET-2	Endothelin-2
ET-3	Endothelin-3
ET_A	Endothelin A receptor
ET_B	Endothelin B receptor
Fmoc	9-fluorenylmethoxycarbonyl
G cell	Gastrin cell
G-17-I	Non-sulfated gastrin-17
G-17-II	Sulfated gastrin-17
G-34-I	Non-sulfated gastrin-34

G-34-II	Sulfated gastrin-34
GH	Growth hormone
GHRH	Growth hormone-releasing hormone
GHRP	Growth hormone releasing peptide
GHS	Growth hormone secretagogue
GHS-R1a	Growth hormone secretagogue receptor type 1a
G-protein	Guanine nucleotide binding protein
GRP	Gastrin-releasing peptide
HPLC	High performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
ICV	Intracerebroventricular
IgY	Immunoglobulin in yolk
IP	Intraperitoneal
ir-GRP	immunoreactive GRP
IRL1620	Succinyl-(Glu^{9}, $Ala^{11, 15}$)-ET-1(8–21)
IV	Intravenous
IVT	Intracerebroventricular
mcKLH	Mariculture Keyhole Limpet Hemocyanin
NEFA	Nonesterified fatty acid
NMB	Neuromedin B
NMC	Neuromedin C
PEG	Polyethylene glycol
PRL	Prolactin
RIA	Radioimmunoassay
SEM	Standard error of the mean
SPPS	Solid phase peptide synthesis
SPSS	Statistical Package for the Social Science
TFA	Trifluoroacetic acid
TM	Transmembrane

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Abstract in Japanese (要約)

ウシでのグレリン分泌と成長ホルモン分泌におけるエンドセリン、ガストリン、およびガストリン放出ペプチドの作用機構

グレリンは、単胃動物では主として胃から、また反芻動物では第４胃から分泌されることが見出されている。グレリンの機能は、成長ホルモン（ＧＨ）の内因性分泌促進物質（ＧＨＳ）であり、また採食の調節因子である。これらグレリンの２つの作用は、家畜の成長や肉と乳の生産に密接に関わっていることから、グレリン分泌の制御機構が注目されている。この博士論文は、ウシにおける３種類の脳・腸管ペプチドホルモン、エンドセリン（ＥＴ)、ガストリン、ガストリン放出ペプチド（ＧＲＰ）によるグレリン分泌制御、ＧＨとインスリンの分泌制御、さらにはグルコース代謝調節について研究した結果である。

第２章では、固相合成法（ＳＰＰＳ）による、２３種のポリペプチドの化学合成について述べる。ウシ・ペプチドホルモンやその特異的抗体は市販されていないものが多いため、本研究を遂行するために合成する必要があり、動物への投与実験では合成したペプチドホルモンが利用された。それらは、ウシ硫酸化ガストリン－３４、Ｎ－ＧＲＰ－ＥＥ（ＧＲＰ受容体アンタゴニスト)、[D-Lys^3]－ＧＨＲＰ－６（ＧＨＳ受容体１ａ型「ＧＨＳ－Ｒ１ａ」アンタゴニスト)、ＩＲＬ１６２０（エンドセリンＢ受容体「ET_B」の特異的アゴニスト）などである。また、合成ペプチドは一次抗体を作るためのハプテンとしても利用され、ラジオイムノアッセイ（ＲＩＡ）では希釈標準ペプチドおよび放射化標識ペプチドとして用いられた。また、免疫したモルモットやニワトリの特異的一次抗体を利用して、ＲＩＡ測定系を構築した。ＲＩＡ測定系により、血漿中の硫酸化ガストリン、全ガストリン、ＧＲＰ、Ｎ－ＧＲＰ－ＥＥ、[D-Lys^3]－ＧＨＲＰ－６などを測定した。

第３章から第６章では、ホルスタイン雄授乳仔牛および４～９ヶ月齢ホルスタイン去勢牛を用いた動物実験について述べる。第３章の研究目的は、エンドセリン－３（ＥＴ－３）投与によって引き起こされるグレリンとＧＨの分泌やグルコース代謝変化に関与する受容体を明らかにすることである。ＥＴ－３は、投与用量に依存してアシルグレリン、全グレリン、および、

グルコースの濃度を有意に上昇させ、体重kgあたり0.4、0.7、または1.0μgのＥＴ－３投与により、ＧＨ濃度が増加した。その増加の程度は、0.7μgの方が1.0μgより大きかった。また、ＩＲＬ１６２０もＥＴ－３と同様の効果を示し、血漿中のグレリン、ＧＨ、およびグルコースを上げることから、ＥＴ－３の反応にＥＴ$_B$受容体が関与していることが明らかになった。また、ＥＴ－３で誘起される血漿ＧＨ上昇は、[D-Lys^3]－ＧＨＲＰ－６により阻害されることから、ＥＴ－３によるＧＨ分泌刺激にＧＨＳ－Ｒ１ａが関与していることが確認された。

第４章では、去勢牛の静脈内に硫酸化ガストリン－３４を投与し、血漿中のグレリン、ＧＨ、インスリン、グルカゴン、およびグルコース等の濃度変化を調べた結果を述べる。アシルグレリンと全グレリン濃度は、ガストリンの投与量に依存して有意に増加した（$P<0.05$）。ＧＨ濃度もガストリン投与で増加するが（$P<0.05$）、高投与量20μg/kgでは、投与量4.0μg/kgよりＧＨの増加分が低かった。また、インスリン濃度は、３種の異なる投与量のガストリンでいずれも15分以内に減少し（$P<0.05$）、最終の血液採取時間まで低濃度が持続した。一方、ガストリンはグルカゴンおよびグルコースの濃度に何ら影響しなかった（$P>0.05$）。

第５章では、様々な種類のガストリンによる血漿グレリンとＧＨの濃度変化を調べ、それらの離乳前後での変化を研究した。また、ガストリンによる内因性のグレリン分泌とＧＨ分泌の関係を調べた。離乳前の仔牛における血漿ＧＨ濃度は、硫酸化ガストリン－１７の投与後、15－20分で有意に増加した（$P<0.05$）。しかし、アシルグレリン変化は仔牛間で変動が大きく、有意な結果は得られなかった。離乳後の仔牛では、血漿中のグレリンとＧＨの濃度は硫酸化型および非硫酸化型ガストリン－１７の投与によって、同程度に増加した。ガストリン－１７による増加は、硫酸化型では１０分間持続するが、非硫酸型では５分しか続かなかった。一方、硫酸型ガストリン－９は、血漿中のグレリンとＧＨの濃度になんの影響も起こさなかった。さらに、[D-Lys^3]－ＧＨＲＰ－６の同時投与は、ガストリンで誘起されるＧＨ分泌を阻害しなかった。最後の結果は、ガストリンによるＧＨ分泌増加がグレリン・ＧＨＳ－Ｒ系とは無関係であることを示唆している。

第６章においては、ボンベシン様ペプチドが離乳前後のウシ血漿中のグレリン、ＧＨ、インスリン、グルカゴン、およびグルコースの濃度に及ぼす影響を調査し、それらがどの受容体を介して起こるか調べた。ＧＲＰまたはニューロメジンＣ（ＮＭＣ）の離乳去勢仔牛における静脈投与は、両ペプチドとも５分以内にＧＨおよびインスリンの濃度を増加させた。ＮＭＣ投与

は、ＧＨ変化では1.0μg/kg、インスリン変化では0.2μg/kgで有意な結果が得られた。血漿グルコースは、ＮＭＣ投与直後あるいはＧＲＰ投与直後に基礎濃度以上に増加するが、すぐに基礎濃度以下に減少した。ＮＭＣとＮ－ＧＲＰ－ＥＥの同時投与は、ＮＭＣによるＧＨ、インスリン、およびグルコース等の変化を完全に阻害したが、ＮＭＣと[D-Lys^3]－ＧＨＲＰ－６の同時投与はＮＭＣによるＧＨ分泌を阻害しなかった。また、ニューロメジンＢ（ＮＭＢ）あるいはＮ－ＧＲＰ－ＥＥの単独投与は血漿ホルモンやグルコースの濃度に全く影響を及ぼさないことから、ＧＲＰおよびＮＭＣによる効果はＧＲＰ受容体を介していると予想される。ＮＭＣ投与が誘起するＧＨとインスリンの変化量は離乳前後で同程度だったが、変化の持続時間は離乳前が離乳後より長かった。一方、グレリン濃度の変化は認められなかった。

この論文では（１）去勢牛におけるＥＴ－３の投与は、ET_B受容体を介する内因性グレリン分泌が起こり、ＧＨの分泌を誘起する。（２）ガストリンの２つの主成分であるガストリン－３４とガストリン－１７は、グレリンとＧＨの分泌を増加させるが、その活性はガストリンの硫酸化と関係がなく、離乳前後で大きな違いはない。また、ＧＨの分泌にグレリンとＧＨＳ－Ｒ１ａは関与していない。（３）離乳前オス仔牛と離乳後の去勢牛において、ＧＲＰとＮＭＣはＧＲＰ受容体を介してＧＨ分泌を増加させる。

キーワード: グレリン、成長ホルモン、インスリン、エンドセリン、ガストリン、ガストリン放出ホルモン（ＧＲＰ）、仔牛、去勢牛、ウシ、ラジオイムノアッセイ（ＲＩＡ）、免疫グロブリンＹ（ＩｇＹ）ｃ