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Hexarelin modulates stress effects on ghrelin system activity in growing lambs

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Hexarelin is a synthetic ligand for the growth hormone secretagogue receptor 1a (GHSR-1a), also known as the ghrelin receptor. Ghrelin and hexarelin have been observed to influence the HPA axis by increasing the circulating concentrations of both adrenocorticotropin and adrenal glucocorticoid in rats and humans. The aim of this study was to assess the effects of hexarelin on the ghrelin system and the growth hormone (GH)–insulin-like growth factor 1 (IGF-1) axis in lambs stressed by an emotional factor – isolation. The isolation stress in three-month-old lambs was accompanied consistently by decreases in the plasma concentrations of ghrelin, hypothalamic ghrelin concentrations, *in vitro* hypothalamic ghrelin release and the ghrelin (GHRL) gene's expression. In addition, the isolation stress induced shifts in plasma concentrations of the insulin-like growth factor 1 (increase) and the *in vitro* release of the growth hormone (increase). An administration of hexarelin was followed by a decrease in the plasma concentrations of ghrelin, hypothalamic ghrelin concentrations and the *in vitro* release of ghrelin from the hypothalamic tissue. In contrast, there were increases in the plasma concentrations of IGF-1 and GH release *in vitro* from the pituitary tissue following the hexarelin administration. The present study provides proof of the interaction between hexarelin administration and isolation stress. The evidence put forth supports the capacity of the growth hormone secretagogues and exogenous ligand hexarelin as well as endogenous ghrelin to modulate some of the effects of stress on the growth hormone-insulin-like growth factor 1(GH/IGF-1) axis in growing lambs.

Key words: GHRL expression, hypothalamus, pituitary, GH release, GHS-R1a receptor binding, IGF-1 plasma level.

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Ghrelin is synthetised by the stomach (Kojima *et al*. 1999), hypothalamus, pituitary gland, adrenal glands and ovaries (human: Ueberberg *et al*. 2009; rat: Rucinski *et al*. 2009). The octanoylated 28 amino-acid residue peptide plays an important role in the control of appetite (Kojima *et al*. 1999; reviewed e.g. Kojima and Kangawa 2005; Bouillon-Minois *et al*. 2021). Apart from its orexigenic effect, research has shown that ghrelin has a regulatory role on the release of the growth hormone (Sato *et al*. 2012). Ghrelin is the endogenous ligand for the growth hormone secretagogue receptor – GHS-R1a (Kojima *et al*. 1999). GHS-R1a is found on multiple cell types such as the hypothalamus, pituitary gland, spinal cord, adrenal glands, heart, vascular smooth muscle, renal tissue and leukocytes (Kojima *et al*. 1999; Guan *et al*. 1997; Ueberberg *et al*. 2009). It is also noteworthy that ghrelin, the endogenous ligand for the GH secretory receptor (GHS-R1a), exerts an influence on the hypothalamo-pituitary-adrenal (HPA) axis.

Recently, there has been increasing interest in the relationship between ghrelin and stress (Fritz *et al*. 2020; Koziec *et al*. 2023). Based on a meta-analysis, circulating concentrations of ghrelin are increased by acute stresses, with a larger effect in people with a high BMI (Bouillon-Minois *et al*. 2021). In rats, chronic unpredictable mild stress induces anxiety- and depression-like behaviours, but also increases in the peripheral acylated ghrelin and elevated ghrelin expression in the stomach and hippocampus (Huang *et al*. 2017). Similarly, the circulating concentrations of ghrelin are elevated in lactating rats where the pups have been removed (Abizaid *et al*. 2008) and in juvenile mice separated from the dams (Schmidt *et al*. 2006). Restraint stress in mice is followed by increases in the circulating concentrations of corticosterone and des-acyl-ghrelin, but not of acyl ghrelin (Nahata *et al*. 2014).

Hexarelin is a potent, synthetic, peptidic, highly-selective agonist of the ghrelin/growth hormone secretagogue receptor (GHS-R1a), which based on its structural differences is assumed not to cross react in assays for ghrelin (Arvat *et al*. 1999). Hexarelin has a potent stimulatory effect on GH secretion, and it has been suggested that it likely acts *via* the inhibition of hypothalamic somatostatin release. However, in spite of extensive studies, there are conflicting reports on the ability of hexarelin to change the insulin-like growth factor 1 (IGF-1) level in humans, with some studies finding no increase and others finding a tendency for an elevation (Alexopoulou *et al*. 2010). Hexarelin also has a cardioprotective effect independent of the GHS receptor activity in rat (Locatelli *et al*. 1999). Hexarelin has been observed to elevate the circulating concentrations of both ACTH and cortisol, as reported in human studies by Korbonits *et al*. (1999). Similarly, in rats, another ligand for GHS-R1a, GH-releasing peptide-6, has been demonstrated to cause an increase in the circulating concentrations of corticosterone, as shown in the research conducted by Thomas *et al*. (1997).

A previous experiment showed that hexarelin affects the activity of the HPA axis by a modulation of hypothalamic opioid activity and the release of glucocorticoid from lambs' adrenal (Koziec *et al*. 2023). Since Met-enkephalin as an opioid neurotransmitter, ghrelin and hexarelin may act through the GHS-receptors, the question arises: Is there an interaction between hexarelin and ghrelin centrally and/or peripherally?

Therefore, the aim of this study was to examine the involvement of hexarelin on the ghrelin physiology at the level of the hypothalamus and periphery under both control and stressful conditions in growing lambs. The components of ghrelin physiology examined in the present study were the plasma concentrations of ghrelin, hypothalamic concentrations of ghrelin, *in vitro* ghrelin release from the hypothalamic tissue, hypothalamic GHS-R1a binding and the hypothalamic ghrelin gene (*GHRL*) expression. Additionally, aspects of the hypothalamo-pituitary growth hormone (GH) axis were examined for the GH release *in vitro* from the pituitary tissue and the plasma concentrations of insulin-like growth factor 1 (IGF-1).

Material and Methods

Most of the chemicals were acquired from Sigma Aldrich, while others are mentioned in the text. The specific substances included: sodium chloride (S9888), potassium chloride (P3911), naltrexone chloride (N3136), RNAlater (R090), Tween 20 (P1379), sodium carbonate (S7795), Krebs (K3753), heparin sodium (H3149), hexarelin (80666), Tris-HCl (T5941) and the Bicinchoninic Acid kit (BCA1).

Animals

The studies employed 24 three-month-old female lambs of the Polish Mountain Sheep breed from the National Research Institute of Animal Production in Kraków.

Before the experiment, the lambs were maintained in a herd with the ewes (total number of animals: 32 ewes and 40 lambs). The lambs were randomly assigned to the experimental groups; animals chosen for the experiment were single, while twins were excluded from the study. The lambs were kept within a herd alongside the ewes in a controlled environment (photoperiod with lights on from 7 a.m. to 7 p.m.) and were maintained at a room temperature of 20°C. The sheep received feed and water *ad libitum*. The animals were allowed to acclimate to these conditions for a period of 7 days before the start of the experiment.

Animal experimentation

The study was conducted according to the Animal Study Protocol 64/OP/2005 and received approval from the Institutional Review Board and the First Local Ethical Committee on Animal Testing in Kraków, Poland.

There were six lambs in each of four groups:

Group A: Control (i.v. injection of 0.9% NaCl)

Group B: Hexarelin (i.v. injection of 0.5 µg/kg b.w. hexarelin)

Group C: Stressed by isolation (60 min, from time 0 to 60 min)

Group D: Hexarelin and isolation stress (injection of hexarelin and isolation)

The lambs in Groups B and D were subjected to isolation stress without visual or acoustic contact with other lambs for 60 minutes i.e. 0-60 minute time points. Injections of 0.9% NaCl or hexarelin were immediately administered after the first blood sample, 15 min before the isolation period (-15').

Blood sampling

Blood samples were taken from the jugular vein and collected into heparinised tubes: 15' before stress; and at 15, 30, 45, 60 and 90 minutes from the start of stress. At the 90 minute time point (thirty min after the end of isolation), the lambs were euthanised through an intravenous injection of pentobarbital (Euthanimal, 0.25 mg/kg b.w.). The blood collection at the -15 min time point started at 9 a.m. The hypothalamic and pituitary glands were dissected and placed in oxygenated Krebs-Ringer media within 60 min from the end of the stress.

Hormone assays

Concentrations of total ghrelin in the plasma, hypothalamus and in the culture media (*in vitro* studies) were determined by radioimmunoassay (RIA-3967, DRG Instruments GmBH, Marburg, Germany). Concentrations of plasma IGF-1 and the culture media GH were determined, respectively, by a radioimmunoassay (RIA-CT R-22, KIP-1588, IBL USA) and ELISA (Sheep GH ELISA Cat. No: MBS7606164, MyBioSource.com, San Diego, USA). The interassay and intraassay coefficients of variance were as follows: 16.3 and 4.9% (ghrelin); 6.5 and 3.4% (IGF-1); and less than 10 and less than 8% (GH).

In vitro studies: Ghrelin and GH release from the tissues

Ghrelin secretion from the hypothalamus and GH secretion from the pituitary were assessed using a modified method based on Kowalski and Giraud (1993). In summary, coronally sliced tissue sections (30 µm) were positioned on filter inserts within 24-well plates containing 1 ml of Krebs-Ringer bicarbonate medium. Following a 30 min preincubation period, the tissues were incubated at 37°C for three consecutive 30 min intervals in a 1 ml medium: (1) basal medium; (2) experimental medium; and (3) stimulating medium with 56 mM KCl (to verify the tissue survival). The concentrations of ghrelin or GH in the basal media did not exhibit significant differences, leading to the pooling of the results and their presentation as the ghrelin/GH release under basal conditions.

Expression of the ghrelin gene (*GHRL*)

Expression of *GHRL* was determined by a quantitative PCR employing the following primers: 18 rRNA: F5'-CTTTGGTCGCTCGCTCCTC-3′, R 5′-CTGACCGGGTTGGTTTTGAT-3′; GHRL: F5'-GAAACTGCTCCCCTGGCTGGCTCTAG-3'; and R5'-GAAGACAGACAGGCGATGTGTGG-3' (Genomed S.A. Warszawa, Poland). The tissues were extracted with the TRIzol reagent (Invitrogen; Thermo Fisher Scientific), and the concentration and purity of the total RNA were determined by measuring the absorbance at 260 and 280 nm (A260/A280=1.60/1.89). Single-strand cDNA was synthesised through reverse transcription with a High Capacity RNA-tocDNA Kit (Thermo Fisher, USA). A quantitative PCR (qPCR) was conducted using the StepOnePlusTM RealTime PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) with the TaqMan® Gene Expression Master Mix (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) and TaqMan chemistry, as recommended by the following protocol: 50°C for 5 min and 95°C for 15 min, followed by 45 cycles at 95°C for 15 sec, 62°C for 20 sec, and 72°C for 20 sec. The expression levels of the target genes were calculated relative to that of the housekeeping gene 18S rRNA using StepOne Software v2.1 (Applied Biosystems) with the $^{2\text{-}\Delta\Delta}$Ct method (Livak & Schmittgen 2001) and were presented as RQ.

Growth hormone secretagogue receptor 1a (GHS-R1a, ghrelin receptor) binding

GHS-R1a binding was determined using the method reported by Hytrek *et al*. (1996) with modifications by Pierzchała-Koziec *et al*. (2018). In brief, the dissected tissues were homogenised in an ice cold buffer (50 mM TRIS-HCl, pH = 7.4), and the homogenate was centrifuged at 20,000 × g for 15 min. The cell membranes (1 ml, 1 mg of protein) were incubated at 30°C for 30 min with 7.30 mM of agonist specific to the GHS-R1a receptor: GHRP-6 / [His1, Lys6]-GHRP – I-^{125} Labelled (sequence His-Asp-Trp-Ala-Trp-Asp-Phe-Lys-NH2, Phoenix Pharmaceutical, INC, USA, Cat. No: T-031-21). Nonspecific binding was assessed with 10 µM of unlabelled ligand – ghrelin. The separation of the free ligand from the membrane-bound radioligand was

achieved by filtration under reduced pressure through GF/B Whatman glass filters. The protein concentrations were determined using the bicinchoninic acid (BCA) method (Olson & Markwell 2016).

Statistical analysis

Data for the four treatment groups was analysed by a two way ANOVA. In addition, data for the multiple plasma samples was analysed by a one way ANOVA for repeated measures. The means were separated by a Tukey's test. The results were presented as X ± SEM and were considered statistically different at $p < 0.05$.

Results

Effect of isolation stress and/or hexarelin on the plasma concentrations of ghrelin

The plasma concentrations of ghrelin in the control lambs did not significantly change during the experiment (Table 1). In the lambs subjected to isolation stress, the plasma concentrations of ghrelin steadily declined between the -15' (418.3 ± 28.4 pmol/l) and the 90 minute time points by 36.9% (decrease to 264.0 ± 16.2 pmol/l, $p < 0.05$, Table 1).

The administration of hexarelin alone had no effect on the plasma concentrations of ghrelin. The plasma concentrations of ghrelin were transiently depressed in the lambs following a hexarelin administration by 5.6% and 10.3% at the 30 and 45 minute time points, respectively (Table 1). However, hexarelin injected prior to isolation partially prevented a decrease of the plasma ghrelin at 60 minutes, when the hormone level reached the value of 471.1 ± 27.0 pmol/l, which was 37.7% higher compared to the effect of stress alone at the same time point ($p < 0.05$, Table 1).

Effect of stress and/or hexarelin on *GHRL* expression in the hypothalamus

The expression of *GHRL* (Fig. 1A) was decreased ($p < 0.05$) by stress (48.3%, from 2.34 ± 0.04 to 1.21 ± 0.03 RQ) or the administration of hexarelin alone (by 44.0%, decrease to 1.31 ± 0.02 RQ).

Unexpectedly, the administration of hexarelin before the isolation stress completely reversed the stress diminishing effect on the *GHRL* expression (Fig. 1A).

Effect of stress and/or hexarelin on hypothalamic ghrelin concentrations

Hypothalamic concentrations of ghrelin (Fig. 1B) were depressed ($p < 0.05$) by 25.4% in the lambs that had been subjected to isolation stress (decrease from 0.570 ± 0.006 to 0.425 ± 0.011 pmol/mg protein). The injection of hexarelin increased ($p < 0.05$) the hypothalamic ghrelin concentration to 0.771±0.009 pmol/mg protein (35.3%). Pretreatment of the lambs with hexarelin was accompanied by a further decrease (36.7%) in the hypothalamic concentrations of ghrelin in the stressed lambs (Fig. 1B).

Effect of isolation stress and/or hexarelin treatment on ghrelin release from the hypothalamus *in vitro*

The *in vitro* release of ghrelin (Fig. 2A) was markedly lower ($p < 0.05$) from the hypothalamus in the lambs that had been stressed (by 72.8%, from 36.3 ± 2.6 to 9.85 ± 0.9 fmol/100 mg tissue per 20 min) or that had received a hexarelin administration (by 63.2%, decrease to 13.3 ± 1.31 fmol/100 mg tissue per 20 min). In contrast, there appeared to be an antagonistic effect with both *in vivo* treatments on the *in vitro* release of ghrelin: the ghrelin concentration in the cultured media was 16.6 ± 1.34 fmol/100 mg tissue per 20 min (decrease by 55%, $p < 0.05$).

Table 1

Effect of isolation stress and/or hexarelin on plasma concentrations of ghrelin (pmol/l, X ± SEM)

Treatment	Plasma concentrations of ghrelin (pmol/l, X ± SEM)					
Time in minutes	-15'	+15'	30'	45'	60'	90'
Control	409.7 ± 17.3^{a}	428.3 ± 26.4^{a}	418.3 ± 22.8^{a}	425.1 ± 21.7^{a}	394.6 ± 16.3^{a}	440.5 ± 21.0^{a}
Stress[1]	418.3 ± 28.4^{a}	353.9 ± 16.7^{b}	313.9 ± 24.1^{c}	325.7 ± 22.6^{d}	293.4 ± 19.4^{c,e}	264.0 ± 16.2^{f}
Hexarelin[2]	402.6 ± 13.7^{a}	408.5 ± 22.5^{a}	380.0 ± 17.6^{a}	361.0 ± 23.2^{a}	439.3 ± 26.8^{a}	455.1 ± 27.1^{a}
Stress[1] + Hexarelin[2]	402.8 ± 21.4^{a}	470.6 ± 27.7^{b}	434.7 ± 22.8^{a}	327.8 ± 18.8^{c}	471.1 ± 27.0^{b}	433.4 ± 22.5^{a}

[1] Lambs were subjected to isolation stress between 0 and 60 minutes
[2] Lambs received hexarelin immediately after the first sample (-15 minute)
[a,b,c,d,e,f] Different superscript letters in the row (treatment) indicate a difference $p < 0.05$

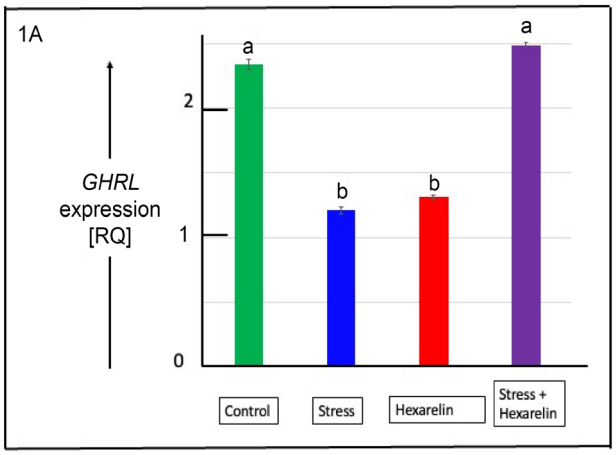

Fig. 1A. Effect of isolation stress and/or hexarelin on hypothalamic expression of ghrelin gene (*GHRL*) in 3-month-old lambs (RQ, X ± SEM, [a,b] $p < 0.05$ compared to the control).

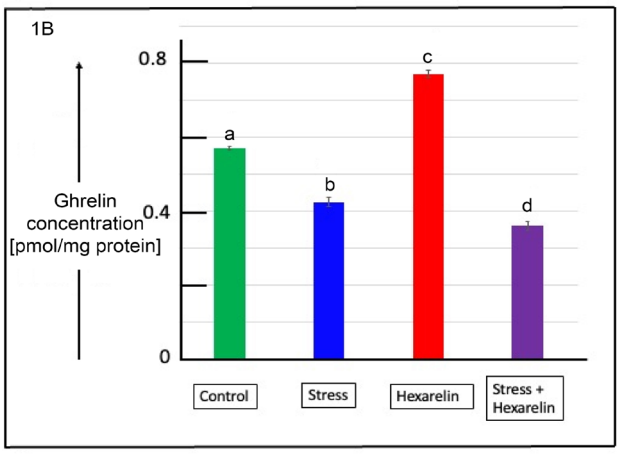

Fig. 1B. Effect of isolation stress and/or hexarelin on hypothalamic concentrations of ghrelin in 3-month-old lambs (pmol/mg protein, X ± SEM, [a,b,c,d] $p < 0.05$ compared to the control).

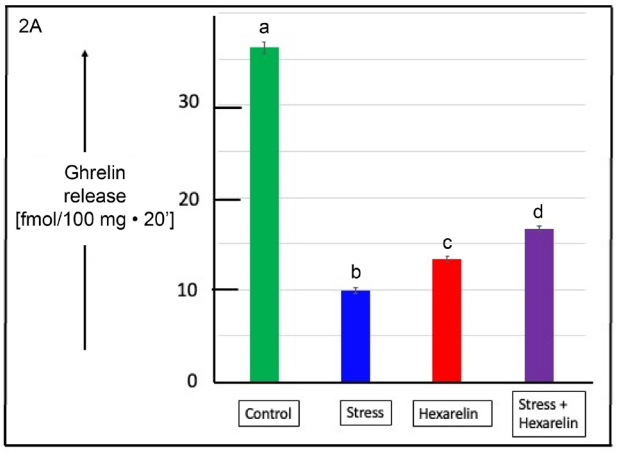

Fig. 2A. Effect of isolation stress and/or hexarelin on release of ghrelin from the hypothalamus *in vitro* in 3-month-old lambs (fmol/ 100mg tissue per 20 min, X ± SEM, [a,b,c,d] $p < 0.05$ compared to the control).

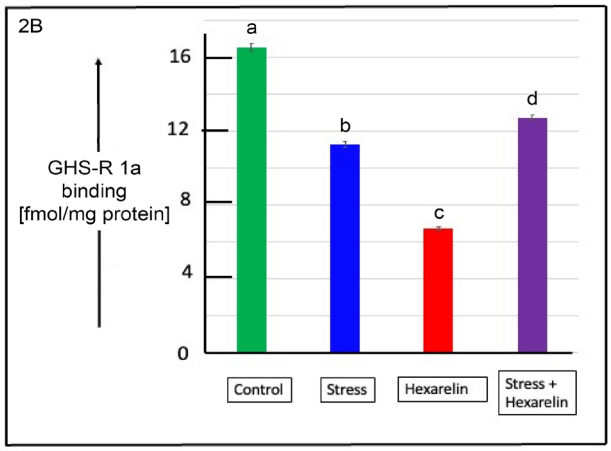

Fig. 2B. Effect of isolation stress and/or hexarelin on hypothalamic *in vitro* binding of GHS-R 1a in 3-month-old lambs (fmol/mg protein, X ± SEM, [a,b,c,d] $p < 0.05$ compared to the control).

Effect of isolation stress and/or hexarelin on *in vitro* hypothalamic GHS-R1a binding

Hypothalamic GHS-R1a bindings *in vitro* were reduced ($p < 0.05$) by 31.5% in the lambs subjected to isolation stress (Fig. 2B) and by 50.3% in the lambs receiving a hexarelin administration. Hexarelin followed by stress also decreased the receptor binding, but to less extent than when it was given alone (reduction by 23%, from control 16.5 ± 1.21 to 12.7 ± 0.85 fmol/mg protein).

Effect of stress and/or hexarelin *in vivo* on the pituitary GH release *in vitro*

There were effects of the *in vivo* treatment of the lambs on the subsequent *in vitro* release of GH from the pituitary tissue (Fig. 3). The *in vitro* release of GH was greater ($p < 0.05$) in the tissue from lambs subjected to isolation stress (by 115.8%) and the hexarelin-treated lambs, by 49.5% compared to the control animals. Hexarelin potentiated ($p < 0.05$) the stress stimulating effect on the GH release from the pituitary by 168% (increase from 0.95 ± 0.046 to 2.55 ± 0.048 fmol/mg protein per 20 min).

Effect of isolation stress and/or hexarelin on plasma concentrations of IGF-1

There were no differences of the plasma concentrations of IGF-1 in the control lambs during the experiment. Plasma concentrations of IGF-1 were expressed as the area under/over the curve (AUC) due to the shifting in the time responses to stress or hexarelin treatments (Fig. 4). The total AUC of the IGF-1 values was greater ($p < 0.05$) in the lambs receiving the administration of hexarelin, as well as in those that had been subjected to isolation stress for 60 minutes (Fig. 4). Interestingly, a hexarelin admini-

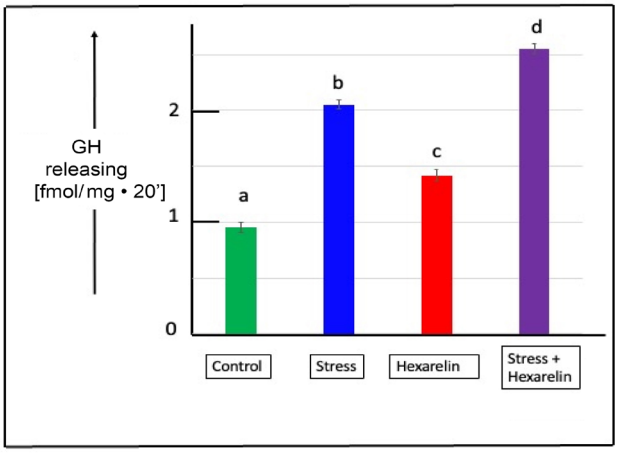

Fig. 3. Effect of isolation stress and/or hexarelin on *in vitro* release of GH from the pituitary gland in 3-month-old lambs (fmol/mg per 20 min, X ± SEM, ^{a,b,c,d} $p < 0.05$).

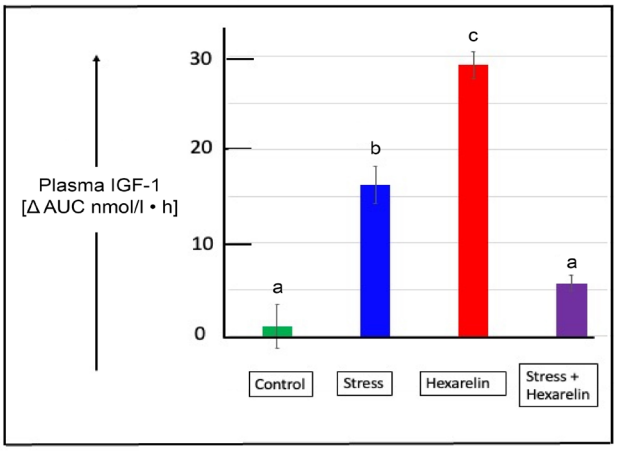

Fig. 4. Effect of isolation stress and/or hexarelin on plasma concentrations of IGF-1 [Δ AUC (delta area under/over the curve) between the -15'sample and 60' sample, nmol/l per hour] in 3-month-old lambs (^{a,b,c} $p < 0.05$, X ± SEM).

stration before the isolation reduced ($p < 0.05$) the magnitude of the increase in the plasma concentrations of IGF-1, as indicated by the area under/over the curve (Fig. 4).

Discussion

The obtained results demonstrate the acute effects of hexarelin and isolation stress on the synthesis, secretion, receptor binding and concentration of ghrelin, as well as on the concentrations of growth hormone and insulin-like growth factor-1 in growing lambs.

The summary of the effects of hexarelin and/or isolation stress in lambs is shown in Table 2. Isolation gradually depressed the circulating concentrations of ghrelin, starting from as early as 15 min of stress (Table 1). The lowest level of blood ghrelin was observed 30 min after the terminating the isolation, when lambs were again with the herd. It maybe speculated that the sources of circulating ghrelin were depleted (stomach, intestine) and/or the ghrelin bound to the blood protein was not enzymatically hydrolysed to the octanoylated form. Lower level of ghrelin after the stress also indicates the involvement of this hormone in the regulation of homeostatic mechanisms, such as faster glucose and lipids metabolism, as well as the re-synthesis of glucocorticoids (Mani *et al.* 2019).

The decrease in circulating concentrations of ghrelin in the stressed lambs differs from the effects of stress in rodents and humans, where stressors were found to cause an increase of this hormone (litters removed from rats: Abizaid *et al.* 2008; chronic unpredictable mild stress in rats: Huang *et al.* 2017; different acute stress in obese people: Bouillon-Minois *et al.* 2021). However, it must be mentioned

Table 2

Summary of the effects of isolation stress and hexarelin on the ghrelin system activity in lambs

	Stress	Hexarelin	Hexarelin & Stress
Plasma concentration of ghrelin	↓↓↓	↓	→
Hypothalamic concentrations of ghrelin	↓↓	↑↑	↓↓↓
In vitro ghrelin release from the hypothalamus	↓↓↓	↓↓	↓↓
GHRL expression	↓↓	↓↓	→
GHS-R1a binding	↓	↓↓	↓
In vitro GH release from the pituitary	↑↑	↑	↑↑↑
Plasma concentration of IGF-1	↑↑↑	↑↑	→

↑ increase; ↓ decrease; → unchanged

that these stressors in the abovementioned studies were acute, prolonged or even chronic, and the observed elevated concentration of ghrelin could be an effect of compensation mechanisms (obese people). On the other hand, when mice were exposed to restraint stress for 60 min, no change in the plasma acylated ghrelin levels was observed but a significant elevation of the plasma des-acyl ghrelin levels occurred (Nahata *et al*. 2014).

The tendency toward a transitory decrease in the circulating concentrations of ghrelin following the administration of hexarelin (decrease by 5.6 and 10.3% at the 30 and 45 minute time points, respectively) is consistent with hexarelin acting in a very short loop feedback mechanism following binding to the GHS-R1a. However, there is evidence that hexarelin can also act *via* a different receptor – the CD36 – which is a scavenger receptor class B member 3 (Mao *et al*. 2014). It has been suggested that effect of hexarelin on the heart is not mediated by a GH/ghrelin mechanism (Locatelli *et al*. 1999; Tivesten *et al*. 2000; Mao *et al*. 2014). There is, however, limited information on the effect of hexarelin on the circulating concentrations of ghrelin in other studies or species. An exception is the report finding no effects of hexarelin on the plasma concentrations of ghrelin in non-obese insulin-resistant MKR mice (Mosa *et al*. 2017).

Interestingly, hexarelin injected before the isolation caused biphasic changes in the blood ghrelin concentration. Firstly, an increase of the ghrelin concentration was observed after 15 min of stress, then a decrease occurred at 45 min followed by a second increase just before the termination of the stress. It may be speculated that the first peak of the ghrelin level was caused by the immediate release of hormones from peripheral sources, which were depleted during the next 30 minutes. A major portion of the circulating ghrelin pool is secreted in the unacylated form, which can also be derived by a rapid deacylation of the circulating acyl-ghrelin by the actions of enzymes including butyrylcholinesterase and acyl protein thioesterase 1 (Mani *et al*. 2019). Unacyl-ghrelin has a poor affinity for GHS-R1a, and it is uncertain how its actions, which are in some instances opposite to those of acyl-ghrelin, are mediated. We measured the total ghrelin (acyl- and unacylated forms), and it was impossible to prove which form was binding to the receptor. Our previous experiments (unpublished data) showed that the enzymatic hydrolysis of mice blood proteins increased the total pool of ghrelin. On that basis, it may be suggested that lambs' blood proteins also are able to release ghrelin, particularly during periods of stress.

The second elevation of the ghrelin level seems to be the effect of an additional secretion from stomach/jejunum cells. Maybe, similarly to other hormones (GH, gonadotropins, adrenocorticotropin), ghrelin is secreted in a pulsatile manner during the stress response.

It should be noted that ghrelin acts on the hypothalamo-pituitary-adrenal cortical axis. Ghrelin and/or des-acyl ghrelin have been demonstrated to increase adrenal glucocorticoid production, based on the circulating concentrations of ghrelin (mice: Cabral *et al*. 2016; Stark *et al*. 2016; humans: Lambert *et al*. 2011). Moreover, ghrelin stimulates glucocorticoid production from the adrenal cortical cells (rat: Rucinski *et al*. 2009). Similarly, serum concentrations of cortisol were found to be elevated by other GHS-R1a agonists, such as L-692,585 (Jacks *et al*. 1994) and L-692,429 in dogs (Hickey *et al*. 1994).

While the primary source of ghrelin is the stomach and small intestine, ghrelin is expressed in other organs including the pituitary gland, adrenal glands and ovaries (human: Ueberberg *et al*. 2009). In addition, ghrelin is expressed in other tissues including the hypothalamus (e.g. rat: Abizaid *et al*. 2008; goldfish: Sánchez-Bretaño *et al*. 2015).

In a consistent manner to the shifts in the plasma concentrations of ghrelin, both the hypothalamic expression of *GHRL*, the ghrelin gene and the *in vitro* release of ghrelin from the hypothalamic tissue was depressed by either hexarelin or isolation stress (Table 2). This decreased expression of the ghrelin gene could be an effect of negative feedback to an elevated hypothalamic concentration of ghrelin after the hexarelin injection. On the other hand, the decreased *in vitro* binding to GHS-R1a indicates that the *in vivo* condition receptors were active and bound the ligands. Thus, the question arises: What kind of ligands – endogenous ghrelin and opioid or exogenous hexarelin – were bound?

In the present study, while the hypothalamic concentrations of ghrelin in lambs were increased by hexarelin, they were decreased by isolation stress (Fig. 1B). As has been reported previously with the release of Met-enkephalin from the hypothalamic tissue (Koziec *et al*. 2023), hexarelin potentiated the release of this opioid during the stress response. However, the inhibition of the opioids receptor by naltrexone dramatically decreased the enkephalin secretion. Also, the results from previous experiments (Pierzchala-Koziec *et al*. 2018, 2019) showed that naltrexone potentiated the ghrelin release from the hypothalamus of stressed lambs. Thus, by analogy, is the hexarelin effect on the ghrelin release from

the hypothalamus conditioned by the presence of an additional factor (opioid, kisspeptin)? In a recent review presented by Devesa (2021), it was postulated that ghrelin cooperates with nesfatin and Klotho in the stimulation of GH from the pituitary.

In the present study, both the circulating concentrations of IGF-1 and the *in vitro* GH release were increased by either the administration of hexarelin or isolation stress (Table 2, Figs 3, 4). The effect of hexarelin is consistent with the concept of hexarelin acting *via* GHS-R1a to increase the GH release and, thereby, to elevate the circulating concentrations of IGF-1. However, is this finding in agreement with the relevant published studies? Hexarelin increased the circulating concentrations of GH in rats (Cattaneo *et al*. 1997). Moreover, hexarelin increased the circulating concentrations of IGF-1 in children of a short stature (Frenkel *et al*. 1995). However, while hexarelin increased the circulating concentrations of GH in aged dogs, it did not affect the circulating concentrations of IGF-1 (Cella *et al*. 1996). Moreover, while GH increased the hepatic IGF-1 expression, hexarelin had no effect on the hepatic IGF-1 expression in rats with experimental myocardial infarction (Tivesten *et al*. 2000).

Conclusions

The present study provides proof of the interaction between the administration of hexarelin and isolation stress. The basis for this interaction is unknown; however, the evidence put forth supports the capacity of the growth hormone secretagogues, exogenous ligand hexarelin as well as endogenous ghrelin to modulate some of the effects of stress on the growth hormone–insulin-like growth factor 1 (GH/IGF-1) axis in growing lambs. The present study also provides support for the utility of isolation stress in young sheep, as a very useful model for testing their metabolism as well as growth processes.

Author Contributions

Research concept and design K.P.-K., C.G.S.: Collection and/or assembly of data: K.P.-K.; Data analysis and interpretation: K.P.-K., C.G.S., A.G.; Writing the article: K.P.-K., C.G.S.; Critical revision of the article: K.P.-K., C.G.S., A.G.; Final approval of article: K.P.-K., C.G.S., A.G.

Conflict of Interest

The authors declare no conflict of interest.

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