Identification of a mouse ghrelin gene transcript that contains intron 2 and is regulated in the pituitary and hypothalamus in response to metabolic stress

Rhonda D Kineman^{1,2}, Manuel D Gahete^{1,2} and Raul M Luque^{1,2}

¹Section of Endocrinology, Diabetes and Metabolism, Department of Medicine, University of Illinois at Chicago, Chicago, Illinois, USA

²Research and Development Division, Jesse Brown Veteran's Administration Medical Center, Chicago, Illinois, USA

(Requests for offprints should be addressed to R M Luque who is now at Research and Development Division, Jesse Brown VA Medical Center, M.P 151, West Side, Suite #6215, 820 South Damen Avenue, Chicago, Illinois 60612, USA; Email: luque@uic.edu)

Abstract

The mouse ghrelin gene contains 5 exons (Ex), with Ex2–Ex5 encoding a 117 amino acid preproprotein that is processed to yield a 28 amino acid mature peptide. The current study examined if pituitary (PIT) and hypothalamus (HPT) ghrelin expression is up-regulated in response to fasting and down-regulated in obesity, as previously reported in the stomach. In the process of establishing a quantitative real-time RT-PCR system to accurately assess the changes in PIT and HPT ghrelin mRNA levels, we observed that primer sets located in Ex2 and Ex3 amplified a ghrelin transcript that contained the entire intron 2 (In2). Size and sequence analysis of RT-PCR products using multiple primer sets located throughout the ghrelin gene suggested that the In2-ghrelin variant contains Ex2 and Ex3, but lacks Ex1, Ex4, and Ex5. In2-ghrelin variant mRNA was not detected in stomach extracts, while expression levels were 10- and 50-fold greater than that of the native ghrelin transcript in the PIT and HPT respectively. In2-ghrelin variant mRNA levels increased in the PIT after 24 h fasting and decreased in the HPT and PIT of diet-induced obese mice. These changes may be due to the changes in circulating insulin or IGF-I, since both decreased In2-ghrelin variant expression in a mouse HPT cell line (N6) and in primary mouse PIT cell cultures. The fact that In2-ghrelin variant mRNA levels are dependent on energy intake in the PIT and HPT suggests that this transcript may encode a peptide important in coordinating the neuroendocrine response to metabolic stress.

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Introduction

Evidence is mounting that ghrelin is an endocrine link connecting physiological processes regulating nutrition, body composition, growth, and energy balance (Tschop *et al.* 2000, Horvath *et al.* 2001, Nakazato *et al.* 2001, van der Lely *et al.* 2004). Exogenous ghrelin stimulates food intake and promotes energy storage (Tschop *et al.* 2000, Asakawa *et al.* 2001, Nakazato *et al.* 2001). In a number of species including humans, endogenous ghrelin is down-regulated in obesity (Tschop *et al.* 2001, Sugino *et al.* 2002, Moesgaard *et al.* 2004, Luque & Kineman 2006, Martin *et al.* 2006) and up-regulated in response to food deprivation (Toshinai *et al.* 2001, Luque *et al.* 2006b, 2007).

Ghrelin was originally identified as a 117 amino acid preproprotein that is further processed to yield a 28 amino acid mature peptide (Kojima *et al.* 1999, van der Lely *et al.* 2004). The active core amino acid sequence comprises the first five residues of the mature peptide, with Ser3 being acylated (Gly-Ser-Ser(*n*-octanoyl)-Phe-Leu). This acylated peptide is required for the binding and activation of the growth hormone

secretagogue receptor 1a (GHS-R1a), which in turn stimulates food intake and energy stores (Bednarek *et al.* 2000, van der Lely *et al.* 2004). Non-acylated ghrelin does not bind to the GHS-R1a, but does have biological activities in multiple tissues, through a yet to be identified receptor that is distinct from GHS-R1a (Muccioli *et al.* 2004, Gauna *et al.* 2005, Toshinai *et al.* 2006). In addition to the modification by acylation, the preproghrelin peptide can be differentially processed by enzymatic cleavage (Hosoda *et al.* 2000a, 2003, Pemberton *et al.* 2003, Nishi *et al.* 2005, Zhang *et al.* 2005). For example, it is now appreciated that preproghrelin can be enzymatically cleaved to yield both ghrelin and obestatin, where obestatin is a 23 amino acid peptide that may act through distinct receptors to oppose the actions of ghrelin (Zhang *et al.* 2005). In addition to post-translational modification of ghrelin to produce biologically distinct peptides, it has also been documented that the ghrelin gene can produce multiple peptides by alternative splicing (Hosoda *et al.* 2000b, Tanaka *et al.* 2001b, Jeffery *et al.* 2002, 2005, Kawamura *et al.* 2003). For example, an alternative splice site in the In2 of the ghrelin gene results in the translation of a biologically active peptide identical to mature

ghrelin, except for the loss of a single glutamine residue at position 14 (des-Gln14-ghrelin; Hosoda *et al.* 2000b). Also, a testis-specific ghrelin gene-derived transcript (GGDT) has been identified which uses an alternative start site located in In3 of the ghrelin gene, resulting in a transcript containing a unique exon 1 (Ex1) in-frame with the Ex4 and Ex5 (Tanaka *et al.* 2001b). Therefore, it is now appreciated that the ghrelin gene is regulated at multiple levels to yield proteins of diverse function.

In the current study, we present data indicating the existence of a truncated spliced mRNA variant of ghrelin containing Ex2, In2, and Ex3, but lacking Ex1, Ex4, and Ex5, named 'In2-ghrelin variant'. This transcript was found to be dominantly expressed in the mouse hypothalamus (HPT) and pituitary (PIT), but was not detectable in the stomach. Physiologic relevance of In2-ghrelin variant is supported by the fact that mRNA levels are regulated in response to metabolic stress (fasting and obesity) in a tissue-dependent fashion.

Materials and methods

Animals and cell culture

All experimental procedures were approved by the Animal Care and Use Committees of the University of Illinois at Chicago and the Jesse Brown VA Medical Center. C57Bl/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA), while C57Bl/6J × FVBN mice were generated from an in-house breeding colony. All mice were housed under a ratio of 12 h light:12 h darkness (lights on 0700 h) with free access to standard rodent chow (LabDiet, Framingham, MA, USA, Cat#5008) and tap water unless specified. Mice were handled daily, at least 1 week prior to euthanasia (between 0800 and 1000 h) to acclimate to personnel and handling procedures. Tissues were collected and frozen in liquid nitrogen and stored at -80°C until analysis of mRNA levels by conventional or quantitative real-time RT-PCR (qRT-PCR, see below). The specific use of animals for each phase of the study is indicated below.

Identification of the In2-ghrelin variant

Initial amplification and sequencing of ghrelin gene transcripts were performed by standard and qRT-PCR using tissue extracts (HPT, PIT, and stomach) from C57Bl/6J or C57Bl/6J × FVBN male and females mice (2–6 months of age).

Comparison of In2-ghrelin mRNA levels between males and females

Male and female C57Bl/6J mice were killed at 10 weeks of age by decapitation under fed conditions.

Effect of fasting on In2-ghrelin expression

C57Bl/6J male mice at 10 weeks of age were weighed and food was withdrawn (0800–0900 h) from a subset of mice, while the remaining received food *ad libitum*. After 24 h, mice were killed by decapitation.

Regulation of In2-ghrelin variant in the obese state

To evaluate the effect of diet-induced obesity (DIO) on PIT and HPT ghrelin gene expression, tissue samples were used from mice fed a low-fat (LF) or a high-fat (HF) diet as previously reported (Luque & Kineman 2006). Briefly, C57Bl/6J male mice were placed on either a LF (10% kcal from fat) or HF (60% kcal from fat) diet at 4 weeks of age and killed at 20 weeks of age. HF-fed mice were found to be significantly heavier than LF-fed controls. The dramatic weight gain in HF-fed mice was associated with an increase in total calories consumed, an elevation in circulating insulin and glucose levels, as well as an increase in subcutaneous-, visceral-, and intra-abdominal fat mass (Luque & Kineman 2006).

Effect of insulin and insulin-like growth factor I (IGF-I) on In2-ghrelin expression in a mouse hypothalamic cell line (N6) and in primary PIT cell cultures

In order to investigate whether the In2-ghrelin variant mRNA levels could be directly regulated by metabolic hormones in hypothalamic cells, we used the hypothalamic cell line N6 (CELLutions Biosystems Inc., Toronto, Ontario, Canada), originally developed by Belsham *et al.* (2004) with a detailed expression profile provided at www.cellutionsbiosystems.com/n6.php. This cell line was maintained in our laboratory according to previously described methods (Belsham *et al.* 2004). Primary PIT cell cultures were prepared by enzymatic dispersion of 3–4 pituitaries/experiment from 8- to 10-week-old male mice, as previously described by our laboratory (Luque & Kineman 2006, Luque *et al.* 2006b).

For *in vitro* experiments, N6 cells and primary PIT cells were cultured in monolayer at 2×10^5 /well in α -minimum essential media (MEM; Invitrogen Life Technologies, Inc., Grand Island, NY, USA) containing 2.5% fetal bovine serum (FBS; Sigma), 0.1% BSA (Sigma), 6 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; Invitrogen), 125 nM transferrin (Sigma), 0.6 nM tri-iodothyronine (Sigma), 275 nM hydrocortisone (Sigma), and 1% penicillin–streptomycin antibiotic (Invitrogen) and maintained at 37°C in an atmosphere of 5% CO_2 . After 24 h incubation, cultures were preincubated in serum-free medium for 2 h and subsequently the medium was replaced with serum-free medium containing 0 (control group) or 10 nM insulin or IGF-I (3–4 wells/treatment group). Cultures were incubated for an additional 24 h and total RNA recovered (see below).

RNA isolation and reverse transcription

Tissues or cell cultures were processed for recovery of total RNA using the Absolutely RNA RT-PCR Miniprep kit (Stratagene, La Jolla, CA, USA), with DNase treatment as previously described (Luque & Kineman 2006, Luque *et al.* 2006*a,b*). The amount of RNA recovered was determined using the Ribogreen RNA quantification kit (Molecular Probes, Eugene, OR, USA). Total RNA (1 µg for whole tissue extract and 0.25 µg for primary cell cultures) was reverse transcribed, in a 20 µl volume using random hexamer primers, with enzyme and buffers supplied in the cDNA First-Strand Synthesis kit (Fermentas, Hanover, MD, USA). cDNA was treated with ribonuclease H (1 U; MRI Fermentas).

Primer selection and standard or qrtRT-PCR

Primers used for standard and real-time RT-PCRs (Table 1) were selected using the mouse ghrelin gene sequence as template (Genbank accession #AB060078) and primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi; Steve Rozen, Whitehead Institute for Biomedical Research, Cambridge, CA, USA). Sequences of selected primers were used in Basic local alignment search tool (BLAST; NCBI, Bethesda, MD, USA) searches to check for potential homology to sequences other than the designated target. Primers were then used in a standard PCR with the 2× Master Mix PCR reagent (MRI Fermentas) and cDNA from the tissue of interest as template. Amplification was performed using the Perkin–Elmer

GeneAmp PCR System 9600 (Perkin–Elmer, Downers Grove, IL, USA). The final volume of the PCR was 25 µl:1 µl RT sample (50 ng cDNA), 12.5 µl of the 2× Master Mix, 1 µl of each primer (20 µM stock solution; 1.25 µM), and 9.5 µl dH₂O. The thermocycling profile consisted of one cycle of 95 °C for 10 min, followed by 30–40 cycles of 95 °C for 1 min, 61 °C for 1 min, and 72 °C for 1 min, and a final cycle of 72 °C for 10 min. Products were run on agarose gels and stained with ethidium bromide to confirm that only one band, of the expected size, was amplified. These PCR products were then column-purified (Qiagen, Valencia, CA, USA) and sequenced to confirm target specificity.

Initial screening of primer sets for qrtRT-PCR amplification was performed by amplifying twofold dilutions of RT products where optimal efficiency was demonstrated by a difference of 1 cycle threshold (CT) between dilutions. Following confirmation of primer efficiency and specificity, the concentration of purified products (generated by standard PCR and purified for sequencing as described above) was determined using Molecular Probe’s Picogreen DNA quantification kit and the PCR products were serially diluted to obtain standards containing 10¹, 10², 10³, 10⁴, 10⁵, and 10⁶ copies of synthetic cDNA template per microliter. Standards for native ghrelin were prepared using stomach and PIT cDNA as template (Sn1432, As3499), while standards for In2-ghrelin variant were prepared using PIT cDNA as template (Sn1221, As1444). Standards were then amplified by real-time and standard curves generated by the Stratagene Mx3000p Software. The slope of a standard curve for each template examined was approximately 1, indicating that the efficiency of amplification was 100%, meaning all templates in each cycle were copied. For real-time PCRs, Brilliant SYBR Green QPCR Master Mix (Stratagene) was used, where thermocycling and fluorescence detection were performed using a Stratagene Mx3000p real-time PCR machine. The final volume of the PCR was 25 µl:1 µl RT sample (50 ng cDNA from the tissue of interest), 12.5 µl QPCR Master Mix, 0.375 µl of each primer (10 µM stock solution; 0.67 µM), 0.375 µl reference dye, and 10.375 µl dH₂O. Thermal cycling profile consisted of a preincubation step at 95 °C for 10 min, followed by 40 cycles of denaturation (95 °C, 30 s), annealing (61 °C, 1 min), and extension (72 °C, 30 s). Final PCR products were subjected to graded temperature-dependent dissociation to verify that only one product was amplified. PCR products were then column-purified and sequenced again to confirm target specificity. To determine the starting copy number of cDNA, RT samples were PCR amplified and the signal compared with that of synthetic cDNA standard curve run on the same plate as previously described (Luque & Kineman 2006, Luque *et al.* 2006*b*). In addition, total RNA samples that were not reverse transcribed and a

Table 1 Mouse-specific primers for ghrelin used for standard RT-PCR or quantitative real-time RT-PCR

Primer sequence	Nucleotide position
Sense: GCTGTCTTCAGGCACCATCT	Sn 1221
Sense: CTCAGCATGCTCTGGATGGA	Sn 1255
Sense: GTCATCTGTCTCCACACCAA	Sn 1194
Sense: ACATCCCCAGGCATTCCAG	Sn 766
Sense: TCCAAGAAGCCACCAGCTAA	Sn 1432
Sense: TCAGGTTCAATGCTCCCTTC	Sn 1532
Sense: CAATGCTCCCTTCGATGTTG	Sn 3481
Antisense: GTGGCTTCTTGGATTCCTTTC	As 1444
Antisense: AGAGCTCGTGGCTGCAGTTTA	As 1469
Antisense: GTAGATGTGGGGGCTTAGGG	As 1362
Antisense: TTCTCTCTCTCACACACACAC	As 1395
Antisense: CTCTCTCTCTCACACACACACA	As 1393
Antisense: CCTTCTCTGCTGGGCTTTC	As 1429
	(Spanning Ex2/Ex3)
Antisense: AACATCGAAGGGAGCATTGA	As 3499
Antisense: GCGCCTCTTTGACCTCTTC	As 4376
Antisense: AGGCCTGTCCGTGGTTACTT	As 4404

non-DNA control were run on each plate to control for genomic DNA contamination and monitor potential exogenous contamination respectively. In addition, to control for variations in the amount of RNA used in the RT reaction and the efficiency of the RT reaction, mRNA copy number of the transcript of interest was adjusted by that of cyclophilin A (a peptidyl isomerase) as previously described (Luque & Kineman 2006, Luque *et al.* 2006b). Cyclophilin A mRNA levels did not significantly vary between experimental groups within tissue type.

Statistical analysis

Samples from all groups within an experiment were processed at the same time and therefore the *in vivo* effects of gender and diet and the *in vitro* effects of insulin and IGF-I on native ghrelin and In2-ghrelin variant were assessed by Student's *t*-test. $P < 0.05$ was considered significant. All values are expressed as means $\pm$ S.E.M. All statistical analyses were performed using the GB-STAT software package (Dynamic Microsystems, Inc. Silver Spring, MD, USA).

Results and discussion

Identification of the In2-ghrelin variant using standard RT-PCR

The mouse ghrelin gene contains Ex5 with Ex2–Ex5 encoding the preproghrelin as illustrated in Fig. 1A. A set of PCR primers located in Ex2 (Sn 1221) and Ex3 (As 1444) of the mouse ghrelin gene were initially selected to study the regulation of expression in the PIT and HPT under different physiological conditions. Using stomach cDNA as a template, this primer set generated a PCR product that was found to be of the expected size (131 bp) and sequence (Fig. 1B, primer set 1 and C). However, when this same primer set was used to amplify PIT and HPT cDNA, a single PCR product was generated that was approximately 100 bp larger than the expected size (226 bp; Fig. 1C). This PCR product did not appear using RNA samples that were not reverse transcribed or in PCRs that did not contain samples, indicating that it was not the result of genomic or reagent contamination respectively (Fig. 1C). DNA sequencing revealed that this product contained the entire In2 of the mouse ghrelin gene. This larger product could also be amplified using an additional set of primers located within Ex2 (Sn 1255) and Ex3 (As 1469; Fig. 1B, primer set 2). It should be noted that at 30 cycles in a conventional PCR, both Ex2 and Ex3 primer sets only generated a visible band corresponding to the In2-ghrelin variant in the PIT and HPT. However, by increasing the cycle number (≥ 35

cycles), a second band appeared corresponding to native ghrelin (i.e., lacking In2), thus suggesting that the In2-ghrelin is the dominant ghrelin gene transcript in the PIT and HPT. The In2-ghrelin variant could also be amplified using two different sets of primers where the sense primer was located in Ex2 and the antisense primer was located in In2 (see Fig. 1B, primer sets 3 and 4 and Fig. 1D). This PCR product could not be amplified if stomach cDNA was used as a template (data not shown). When an antisense primer spanning Ex2/Ex3 (As 1429) was paired with a sense primer located in Ex2 (Sn1194; Fig. 1B, primer set 5), only a faint band could be detected in HPT and PIT cDNA samples, corresponding to the expected size and sequence of the native ghrelin (not containing the In2; data not shown), confirming that native ghrelin is expressed in the HPT and PIT, but only at low levels.

Sequence analysis was performed on PCR products generated from multiple PIT and HPT cDNA samples. This analysis consistently revealed that, in the HPT and PIT, the dominant ghrelin transcript included In2, where the intron sequence differed from that originally reported by Tanaka *et al.* (2001a; Genbank accession # AB060078) by 2 bp. Specifically, our sequence included eight TG nucleotides repeated in tandem in the middle of the In2 of the mouse ghrelin gene, starting at nucleotide position 1370 (Fig. 1E), while Tanaka *et al.* (2001a) reported seven TG repeats. The sequence of the In2-ghrelin variant was submitted to Genbank (accession # DQ993169). Our sequence data proved to be identical to that of a mouse chromosome 6 clone that contained the ghrelin gene (Genbank accession # AC117596). Therefore, if we assume that the start of transcription is unaltered in the In2-ghrelin variant, inclusion of In2 would encode a premature stop codon (TGA) in position 1384 (Fig. 1E). A truncated transcript is supported by our observations that sense primers located in Ex2, Ex3, Ex4, or Ex3–Ex4 and antisense primers located in Ex4 or Ex5 only amplified the native ghrelin transcript (see Fig. 1B, primer sets 6–10 and Fig. 2B). It should also be noted that using sense primers located in Ex1 (Sn 766) combined with antisense primers located in Ex3 (As 1444), Ex4 (As 3499), or Ex5 (As 4404; Fig. 1B, primers sets 11–13) only amplified the native ghrelin transcript. Also, no PCR product was generated if sense primers located in Ex1 (Sn766) were combined with antisense primers located in In2 (As1362 and As1395; Fig. 1B, primers sets 14 and 15). Collectively, these findings indicate that the In2-ghrelin variant does not include Ex1. However, it should be noted that Ex1 has been identified as a 5' untranslated region (Tanaka *et al.* 2001a), where its exclusion would not modify the predicted start codon located at nucleotide position 1219 (Genbank # AB060078). Therefore, we speculate that the In2-ghrelin variant would encode a protein that includes

Figure 1 (A) Schematic representation of mouse ghrelin gene. Exons are shown by boxes (black boxes indicate the 5' and 3' non-coding regions and white boxes indicate the coding region). This scheme is based on Genbank accession # AB060078 (Tanaka *et al.* 2001a). (B) Nucleotide numbers indicating the boundaries of the introns/exons in the mouse ghrelin gene (exons are indicated by shaded boxes). Arrow heads are used to indicate the relative positions of primer sets. Closed arrows indicate primers within exons, while open arrows indicate primers spanning exons. (C) Representative agarose gel showing PCR products amplified for 30 cycles using primers located in Ex2 (Sn1221) and Ex3 (As1444) with standard PCR conditions. (D) Representative agarose gel showing PCR products amplified for 35 cycles using primers located in Ex2 (Sn1194) and In2 (As1362) with standard PCR conditions. All products were size separated on an agarose gel containing EtBr, column-purified, and sequenced to confirm target specificity. (E) Nucleotide sequence of the In2-ghrelin variant (accession # DQ993169) with the putative start and stop codons underlined and the In2 sequence shaded.

Figure 2 (A) Dissociation curves of qRT-PCR products (40 cycles) with identical T_m of dissociation (83.2°C), generated from the stomach and PIT, using primers located in Ex3 (Sn1432) and Ex4 (As3499). (B) Agarose gel separation of qRT-PCR products generated from stomach, HPT, and PIT using primers located in Ex3 (Sn1432) and Ex4 (As3499). (C) Dissociation curves of qRT-PCR products (40 cycles) generated from the stomach and PIT with different T_m of dissociation (86.6°C in stomach vs 84.7°C in PIT), using primers located in Ex2 (Sn1221) and Ex3 (As1444). (D) Agarose gel separation of qRT-PCR products generated from stomach, HPT, and PIT using primers located in Ex2 (Sn1221) and Ex3 (As1444).

the first 36 amino acids of the preproghrelin, where residues 24–28 (Gly-Ser-Ser-Phe-Leu) represent the first five amino acids of the mature peptide, while the C-terminal tail would be a novel 19 amino acid sequence encoded by In2 (VSQSVSLSPHIYPDLVCV). If this is indeed the case, obestatin, which is thought to oppose the GHS-R mediated actions of ghrelin (Zhang *et al.* 2005), would not be produced. Therefore, if the protein product of In2-ghrelin has similar functions to that of native ghrelin, we might speculate that its

actions would go unopposed in the mouse HPT and PIT. However, we cannot say at this time if the putative protein of the In2-ghrelin variant can be acetylated and appropriately activate GHS-R1a (Kojima *et al.* 1999, van der Lely *et al.* 2004).

It is well known that regulation at different levels of the ghrelin gene contributes significantly to protein diversity in several species, including human and mouse (Hosoda *et al.* 2000a,b, 2003, Tanaka *et al.* 2001b, Jeffery *et al.* 2002, 2005, Kawamura *et al.* 2003, Pemberton *et al.*

2003, Nishi *et al.* 2005, Zhang *et al.* 2005, Gualillo *et al.* 2006). Consistent with the alternative usage of In2 observed in the current report, utilization of an alternative splice site in In2 has previously been reported to yield 'des-Gln¹⁴-ghrelin' in several species (Hosoda *et al.* 2000b, 2003, Kawamura *et al.* 2003; Genbank #: AB089202). In addition, consistent with the current study, Jeffery *et al.* (2005) recently reported the expression of a ghrelin variant that retains the coding sequence for mature ghrelin but has a novel C-terminal tail due to the exclusion of Ex4 resulting in a cDNA frameshift that generates a premature stop codon. The same group previously identified a human homolog of this isoform that is expressed in a wide range of human tissues (Jeffery *et al.* 2002). Another example of the alternative usage of an intron within the ghrelin gene is the report by Tanaka *et al.* (2001b), who identified an alternative splice variant of the ghrelin gene in the mouse testis, GGDT, that is derived from a start codon located in the In3. Therefore, evidence is accumulating that the ghrelin gene can be differentially regulated at multiple levels (transcriptional, translational, and post-translational).

Verification and quantification of the In2-ghrelin variant and native ghrelin expression using standard and qrtRT-PCR

Next, we wanted to accurately compare the level of expression of the In2-ghrelin variant with that of native ghrelin. As discussed above, our data indicate that the In2-ghrelin variant lacks Ex4 and Ex5. Therefore, a set of primers located in Ex3 and Ex4 were used to amplify native ghrelin (Fig. 1B, primer set 8). Using this primer set in a qrtRT-PCR, products were obtained in the stomach and PIT (Fig. 2A) and HPT (data not shown) that had identical temperature-dependent dissociation ($T_m \sim 83.2^\circ\text{C}$). When the same samples were size separated in an ethidium bromide containing agarose gel, single bands of similar size were visualized (Fig. 2B), which proved to have identical sequences. To amplify the In2-ghrelin variant, a primer set located in Ex2 and Ex3 was selected (Fig. 1B, primer set 1). Using this primer set in a qrtRT-PCR, a single product was obtained in all tissues; however, the temperature of dissociation varied (86.6°C in stomach vs 84.7°C in HPT and PIT), indicating a different product was generated in the HPT and PIT compared with the stomach (Fig. 2C), as confirmed by gel separation of the qrtRT-PCR products (Fig. 2D) and sequence analysis. These data demonstrate that under the conditions tested, the qrtRT-PCR primer sets for native ghrelin and In2-ghrelin variant were appropriate to perform quantitative analysis of ghrelin isoform expression in the HPT and PIT. Therefore, using qrtRT-PCR analysis, we found that mRNA levels for the In2-ghrelin variant were in the order of

Table 2 Absolute cDNA copy number/0.05 μg total RNA of ghrelin gene transcripts in the hypothalamus, pituitary, and stomach of male and female C57Bl/6 mice, as determined by quantitative real-time RT-PCR. Values represent means $\pm$ S.E.M. ($n=5-12$ tissues/group)

	Males	Females
Hypothalamus		
Native ghrelin	26 $\pm$ 2	27 $\pm$ 6
In2-ghrelin variant	948 $\pm$ 166	1336 $\pm$ 300
Pituitary		
Native ghrelin	18 $\pm$ 3	19 $\pm$ 7
In2-ghrelin variant	139 $\pm$ 24	122 $\pm$ 31
Stomach		
Native ghrelin	197 933 $\pm$ 4627	190 880 $\pm$ 18 293
In2-ghrelin variant	Not detected	Not detected

50- and 10-fold greater in the HPT and the PIT than that of the native ghrelin transcript in both male and female mice (Table 2) respectively, confirming that In2-ghrelin variant is the dominant ghrelin isoform in the HPT and PIT and that the expression of this transcript is not gender-dependent under fed conditions.

Metabolic regulation of the In2-ghrelin variant

We have previously reported that fasting up-regulates the expression of native ghrelin in the mouse PIT, but has no effect on HPT mRNA levels (Luque *et al.* 2006b, 2007). Consistent with these findings, in the current report, we observed that the expression of both native ghrelin (Fig. 3A) and In2-ghrelin variant (Fig. 3B) increased after 24 h fasting in the PIT, whereas HPT expression was resistant to this metabolic stimulus. In contrast to the parallel regulation of native ghrelin and In2-ghrelin variant in the PIT in response to fasting, excess nutrient intake (HF-fed mice) which was previously reported to increase fat mass and elevate circulating insulin levels (Luque & Kineman 2006), led to a significant suppression of only the In2-ghrelin variant in the HPT and PIT (Fig. 4A and B), indicating that native ghrelin and In2-ghrelin variant mRNA can be differentially regulated depending of the metabolic insult. Differential metabolic regulation of stomach native ghrelin transcript (the main transcript produced in the stomach) has also been reported by us (Luque *et al.* 2006b, 2007) and others (Moesgaard *et al.* 2004). Specifically, fasting does not alter the expression of native ghrelin in the stomach (Moesgaard *et al.* 2004, Luque *et al.* 2006b, 2007), while HF diet does significantly suppress stomach mRNA levels (Moesgaard *et al.* 2004).

The question arises, which factors may contribute to the regulation of HPT and PIT ghrelin expression in response to metabolic stress? One factor may be insulin, since it is down-regulated in response to fasting and up-regulated in states of obesity and has previously been

Figure 3 Tissue-dependent expression levels of native ghrelin (A) and In2-ghrelin variant (B) in fed and 24-h fasted mice ($n=7$ mice per group). Both ghrelin transcripts mRNA copy numbers were corrected by cyclophilin A mRNA copy number and values are shown as the mean $\pm$ S.E.M. mRNA values are expressed as percent of fed controls (set at 100%). *Values that differ from fed controls. $P<0.05$ was considered significant.

Figure 4 Hypothalamic and pituitary native ghrelin (A) and In2-ghrelin variant (B) mRNA levels of C57BL/6J male mice fed a low-fat (LF) or high-fat (HF) diet for 16 weeks (starting at 4 weeks of age; $n=6-7$ mice per group). Both ghrelin transcripts mRNA copy numbers were corrected by cyclophilin A mRNA copy number and values are shown as means $\pm$ S.E.M. mRNA values are expressed as percent of LF diet control mice (set at 100%). Asterisks (* $P<0.05$, ** $P<0.01$) indicate values that differ from fed controls.

reported to negatively regulate the synthesis and release of ghrelin from the stomach (Kamegai *et al.* 2004). Therefore, we sought to determine if insulin could also have a direct inhibitory effect on PIT and HPT ghrelin expression. In addition, we examined the direct effect of IGF-I because the levels of 'free' IGF-I have been reported, by some, to be elevated in obesity (Frystyk *et al.* 1999, Ricart & Fernandez-Real 2001), where the insulin and IGF-I receptors share significant structural homology, are activated by their respective ligands at high doses, and can signal through common intracellular signal transduction pathways (for review, see Nakae *et al.* 2001).

To this end, we utilized a mouse HPT cell line (N6) developed by Belsham *et al.* (2004) using retroviral transfer of SV-40 T-antigen into primary fetal mouse hypothalamic cell cultures. In our hands, these cells expressed native ghrelin and In2-ghrelin variant mRNA by conventional PCR (Fig. 5A). Use of qRT-PCR indicated that In2-ghrelin variant is expressed in N6 cells at levels comparable with those in whole mouse HPT extracts (N6 cells vs mouse HPT: 24 ± 4 vs 20 ± 4 copies/ $0.05 \mu\text{g}$ total RNA for native ghrelin and 629 ± 33 vs 1147 ± 198 copies/ $0.05 \mu\text{g}$ total RNA for In2-ghrelin variant). It should be noted that both primary PIT cell cultures and N6 cells also express insulin and IGF-I receptors at levels comparable with those in whole mouse PIT and extracts (data not shown). Insulin and IGF-I (24 h) inhibited In2-ghrelin variant mRNA in N6 cells (Fig. 5B). However, native ghrelin expression levels were not significantly altered (Fig. 5B). To examine the direct effect of insulin and IGF-I on the PIT, we utilized primary mouse PIT cell cultures which expressed In2-ghrelin variant mRNA at levels comparable with whole PIT extracts (primary cell culture vs mouse PIT: 203 ± 85 vs 130 ± 19 copies/ $0.05 \mu\text{g}$ total RNA); however, native ghrelin was not detectable in cultured cells. The lack of native ghrelin expression in primary PIT cell cultures is not surprising based on the low levels of native ghrelin (< 20 copies) observed in whole PIT extract and the fact that the amount of total RNA used to generate cDNA in culture experiments was 75% less than that used in the studies of whole tissue extracts. Nonetheless, we cannot exclude the possibility that enzymatic dispersion and culture of primary cells does not provide the same environment as found *in vivo* to support native ghrelin expression. Similar to that observed in the HPT cell line, insulin and IGF-I (24 h) inhibited In2-ghrelin variant mRNA in PIT cell cultures (Fig. 5C). Taken together, these results suggest that a rise in circulating insulin, which is observed in DIO mice (Luque & Kineman 2006), may be directly responsible for suppressing In2-ghrelin expression in both the HPT and PIT.

In summary, we report for the first time the existence of a spliced mRNA variant of the ghrelin gene containing Ex2, In2, and Ex3, but lacking Ex1, Ex4,

Figure 5 (A) Agarose gel of RT-PCR products generated using cDNA from a mouse hypothalamic cell line, N6, as template and primers located in Ex2 (Sn1221) and Ex3 (As1444). The upper band (226 bp) corresponds to mRNA for In2-ghrelin variant, the middle band (131 bp) corresponds to mRNA for native ghrelin, and the lower band corresponds to primer dimers. It should be noted that this PCR was performed with 40 cycles of amplification in order to visualize the native ghrelin transcript. (B) Effect of insulin and IGF-I treatment (24 h; 10 nM) on In2-ghrelin variant and native ghrelin mRNA levels in mouse N6 cultures. Data represent the mean $\pm$ S.E.M. of three separate experiments performed on different cell preparations. (C) Effect of insulin and IGF-I treatment (24 h; 10 nM) on In2-ghrelin variant mRNA levels in mouse pituitary cell cultures. Data represent the mean $\pm$ S.E.M. of four separate experiments performed on different cell preparations. Native ghrelin transcript was not detectable in primary pituitary cell cultures. All values are expressed as absolute mRNA copy number of In2-ghrelin variant or native ghrelin adjusted by cyclophilin A copy number. Copy number was determined by qRT-PCR using primers located in Ex2 (Sn1221) and Ex3 (As1444) to detect In2-ghrelin variant and primers located in Ex3 (Sn1432) and Ex4 (As3499) to detect native ghrelin. **Values that differ from controls. $P < 0.01$ was considered significant.

and Ex5 which is dominantly expressed in the PIT and HPT of the mouse. In2-ghrelin variant may be of biological relevance because it is regulated in extreme metabolic states (fasting and obesity) and its regulation is tissue-dependent. The fact that the levels of In2-ghrelin variant mRNA are dependent on energy intake in the PIT and HPT suggests that this transcript may

encode a peptide important in coordinating the neuroendocrine response to metabolic stress.

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