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The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans

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ABSTRACT Ghrelin is a novel growth hormone-releasing peptide, originally identified in the rat stomach as the endogenous ligand for the growth hormone secretagogue-receptor (GHS-R1a). Ghrelin is involved in the regulation of GH release, but it has recently been suggested that ghrelin may have other actions, including effects on appetite, carbohydrate metabolism, heart, kidney, pancreas, gonads, and cell proliferation. The distribution of ghrelin, its functional receptor (type 1a) and the unspliced, non-functional GHS-R type 1b mRNA expression was investigated in various human tissues using classical and real-time reverse transcription and polymerase chain reaction. GHS-R1a was predominantly expressed in the pituitary and at much lower levels in the thyroid gland, pancreas, spleen, myocardium and adrenal gland. In contrast, ghrelin was found in the stomach, other parts of the gut and, indeed, in all the tissues studied (adrenal gland, atrium, breast, buccal mucosa, esophagus, Fallopian tube, fat tissue, gall bladder, human lymphocytes, ileum, kidney, left colon, liver, lung, lymph node, muscle, muscle, myocardium, ovary, pancreas, pituitary, placenta, prostate, right colon, skin, spleen, testis, thyroid, and vein). GHS-R1b expression was also widespread in all tissues studied. The significance of the widespread tissue distribution of ghrelin remains to be determined. These data suggest that ghrelin might have widespread physiological effects via different, partly unidentified, subtypes of the GHS-R in endocrine and non-endocrine tissues.

Ghrelin is a 28 amino-acid acylated peptide which was first isolated from the rat stomach, where it was localized to the neuroendocrine X/A-like cells of the gastric mucosa (1,2). It has also been identified in the pituitary gland and hypothalamus, where the highest concentration of its receptor, the growth hormone secretagogue receptor (GHS-R), has been reported (3,4). Ghrelin shows a clear, dose-dependent GH-releasing effect *in vitro*. *In vivo* it also has a synergistic effect with GHRH, previously demonstrated with synthetic GHSs (1,5). Apart from its GH-releasing activity, ghrelin also significantly stimulates prolactin, ACTH and cortisol secretion. More recently, a number of different central and peripheral effects have been described: on appetite, carbohydrate metabolism, heart function, the gonadal axis, exocrine function, and cell proliferation (6-12). Ghrelin circulates in the bloodstream and shows pulsatile secretion, with levels higher on fasting and lower after food intake (13,14).

The growth hormone secretagogue receptor (GHS-R) type 1a, a 7 transmembrane domain G protein-coupled receptor, was shown to transduce the GH-releasing effect of the synthetic GHSs as well as ghrelin. Originally it was shown to be exclusively expressed in several nuclei of the brain, in particular in the hypothalamus and in the anterior pituitary gland, and at very low levels in the pancreas (15). A non-spliced, non-functional receptor mRNA variant was also identified (GHS-R1b) that contains all of exon 1 and an additional 74 bases from the intron. It is unknown as to whether this mRNA specifies a protein.

We have now studied the mRNA distribution of ghrelin and GHS-R1a & 1b, using specific and sensitive techniques, in a wide range of human tissues.

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Materials and methods

Tissue samples Normal human tissues were obtained at surgery (adrenal, atrium, bladder, breast, buccal mucosa, Fallopian tubes, esophagus, fat, gall bladder, kidney, liver, lymph node, lymphocytes, muscle, myocardium, ovary, pancreas, placenta, prostate, spleen, testis, thyroid, vein) endoscopy (stomach [fundus and antrum], duodenum, jejunum, ileum, left and right colon) and autopsy (pituitary, lung and skin). Informed consent was obtained from all patients and the local Ethics Committee approved the study. The surgical and endoscopy tissues were snap-frozen on dry ice and stored at –70°C. The autopsy tissues were collected 12-24h post mortem. Tissues were taken from areas with no hemorrhage or necrosis, and not contiguous with a tumor.

RNA extraction and RT-PCR The tissues were homogenized (Ultra-Turrax T25 homogenizer, Jake & Kruskal GmbH, Germany) and RNA was extracted using SV Total RNA Isolation System (Promega Corp., Southampton, UK), which includes a DNase incubation step. All RNA samples underwent conventional RT followed by single and duplex PCR reactions using GAPDH as a housekeeping-gene as well as a one-step TaqMan® real-time RT-PCR for the ghrelin and GHS-R type 1a and 1b genes, using intron-spanning primers for the ghrelin and GHS-R1a gene, and special reverse primers for the intronic sequence of GHS-R1b, according to protocols described previously (4,16,17). For the real-time RT-PCR, absolute mRNA copy numbers were calculated from amplicon-specific standard curves and normalised against total RNA (18). Each sample (1-6/tissue) was assayed in duplicates in at least 2 different reactions with each of the techniques applied.

Results

Type 1a GHS-R mRNA expression in normal human tissues

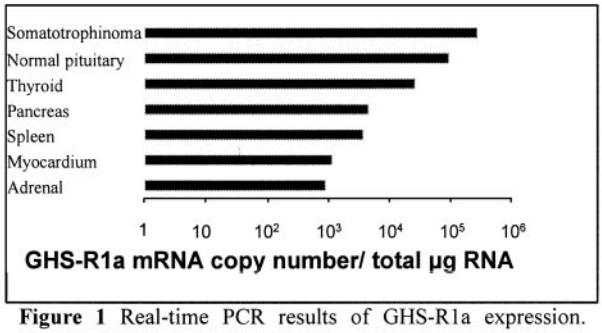

Figure 1 Real-time PCR results of GHS-R1a expression. Tissues studied but not shown on graph had no GHS-R1a mRNA expression

Type 1a GHS-R mRNA expression was found in the normal pituitary and at a much lower level in the thyroid gland, pancreas, spleen, myocardium and adrenal gland (Figure 1). There was no detectable receptor expression in the following tissues in PCRs performed at a high cycle number: antrum, atrium, bladder, breast, buccal mucosa, colon, duodenum, Fallopian tubes, fat, fundus, gall bladder, ileum, jejunum, kidney, liver, lung, lymph node, lymphocytes, muscle, esophagus, ovary, placenta, prostate, skin, testis or vein.

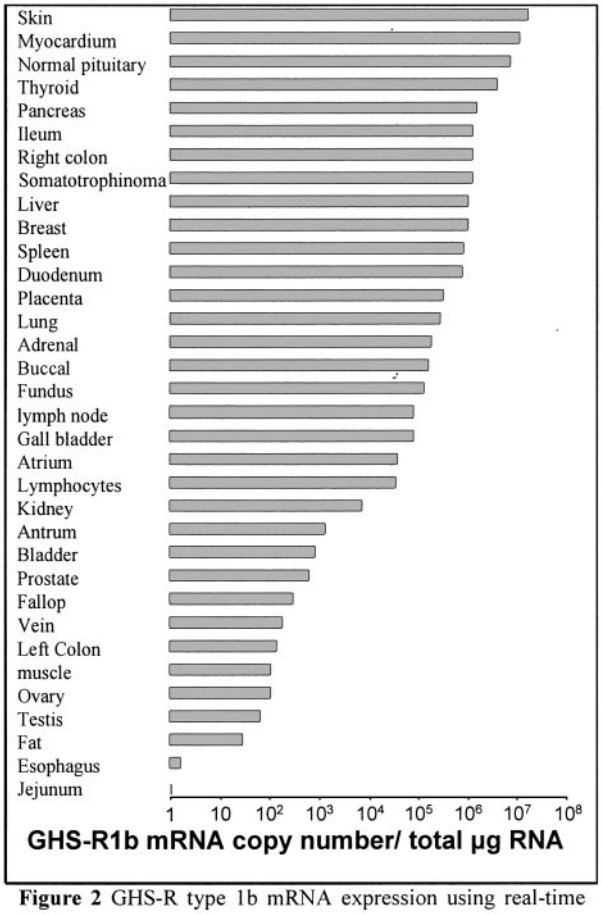

Figure 2 GHS-R type 1b mRNA expression using real-time PCR

Type 1b GHS-R mRNA expression in normal human tissues

By contrast, amplification of type 1b GHS-R was widely detected in all the tissues studied (Figure 2).

Human ghrelin mRNA expression in normal human tissues

There was widespread tissue expression of the ghrelin gene; ghrelin mRNA was present in all the tissues examined (Figures 3). The highest level was demonstrated in the fundus of stomach, then the small intestine (jejunum, duodenum), the antrum of the stomach, lymph node, pancreas, vein, gall bladder, lung, esophagus, left colon, buccal mucosa, pituitary, breast, kidney, ovary, prostate, right colon, adrenal gland, ileum, liver, spleen, Fallopian tube, human lymphocytes, testis, fat tissue, placenta, muscle, muscle, atrium, thyroid, skin and myocardium. The real-time and duplex PCR (not shown) data were concordant, as previously shown in pituitary tissue (4).

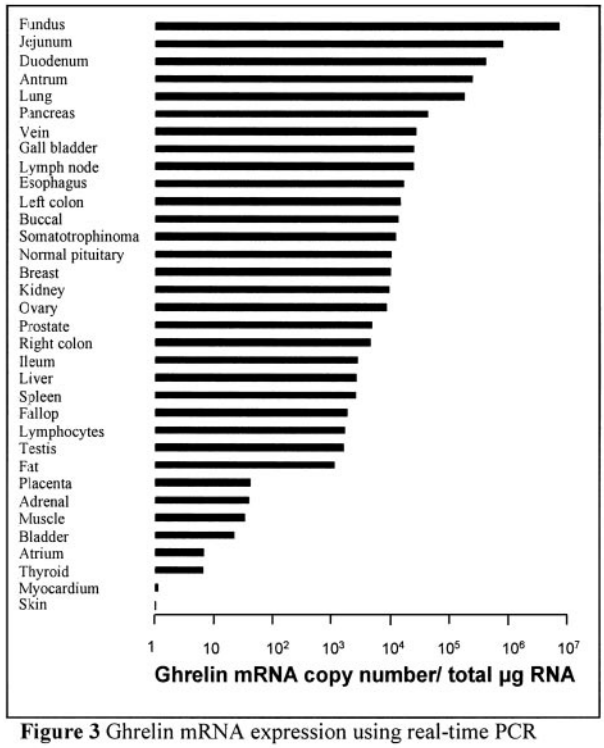

Figure 3 Ghrelin mRNA expression using real-time PCR

Discussion

In the present study, we have demonstrated type 1a GHS-R mRNA expression in the pituitary gland with lower level of expression in a few other tissues (thyroid, pancreas, spleen, myocardium and adrenal), with the majority of samples being negative. The tissue distribution of the mRNA of the

unspliced, non-functional 1b receptor variant was widespread; the physiological significance of this is unknown. All of the tissues we studied were positive for ghrelin mRNA expression. Our findings suggest that ghrelin may have an even wider role than previously thought, and that some of these effects may occur via a receptor different from the cloned GHS-R type 1a.

Recent studies have suggested that the GHS-R is expressed in a wider range of tissues than originally reported. Using RT-PCR, "GHS-R" was suggested to be expressed in the stomach, small intestine, colon, liver, kidney, testis, pancreas, kidney, and T cells (2,6,7,11,19). Papotti et al. reported widespread $^{[125I]}$Tyr-Ala-hexarelin binding in the adrenal gland, gonads, arteries, lung, liver, skeletal muscle, kidney, thyroid, adipose tissue, veins, uterus, skin and lymph node, but not in the stomach or colon (20). Using [^{125}I-His^{9}]-ghrelin, binding was observed in several cardiovascular tissues, and interestingly in atheromatic plaques (21). Shuto et al. identified the existence of the GHS-R protein in the rat stomach using a polyclonal antiserum against the receptor (22).

Northern blot analysis identified ghrelin mRNA most abundantly in the stomach, followed by the duodenum, jejunum, and lung, while no significant amount of ghrelin mRNA was detected in the esophagus, ileum, ileocecum, cecum, colon, rectum, liver, brain, heart, skeletal muscle, thymus, spleen, kidney, placenta or leukocytes using this technique (23). Using RT-PCR, ghrelin expression has been reported in the brain, placenta, kidney, small intestine, large intestine, adrenal, testis, adipose tissue, T and B lymphocytes, and neutrophils (1,2,6,24,25).

Previous studies therefore showed variable and controversial results regarding GHS-R and ghrelin mRNA expression. This might be explained by the use of different techniques which include Northern blotting, RNase protection assays, *in situ* hybridization and RT-PCR, that differ widely in specificity and sensitivity. Detection of the GHS-R is particularly contentious, with RT-PCR studies using non-intron spanning primers unable to differentiate between genomic DNA, 1a and 1b mRNAs (2,6,7,11,19). Cautious interpretation is necessary for receptor binding studies, as the demonstrated binding sites do not necessarily suggest the presence of the GHS-R type 1a receptor.

The present data demonstrate that the type 1a GHS-R was predominantly expressed in the pituitary and at much lower levels in the thyroid, pancreas, spleen, myocardium and adrenal gland. In contrast, the natural ligand ghrelin shows widespread expression in the periphery: we have previously also shown the presence of ghrelin protein centrally in the human hypothalamus, where it may not only be involved in GH regulation but also in appetite control via the agouti-related protein and NPY (4,26). The lack of detectable expression of type 1a GHS-R at the vast majority of the peripheral sites raises the possibility that ghrelin may be acting on other GHS-R subtypes in peripheral tissues. The role of the widespread expression of the non-functional, unspliced receptor mRNA transcript (type 1b) is unknown. What is, however, apparent from the current results as well as from previous data by other groups, is the complexity of ghrelin and GHS-R interactions, and the likelihood that ghrelin may have widespread effects independent of the known GHS-Rs.

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