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Wierup, Nils; Sundler, Frank

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Ultrastucture of islet ghrelin cells in the human fetus.

Nils Wierup and Frank Sundler

Department of Physiological Sciences, Lund University, Lund, Sweden

Corresponding author:

Nils Wierup

Lund University

Department of Physiological Sciences

Section for Neuroendocrine Cell Biology

BMC F10

22 184, Lund

Sweden

Phone: +46 46 222 36 30

Fax: +46 46 222 32 32

e-mail: nils.wierup@mphy.lu.se

Abstract

Ghrelin is a peptide hormone predominantly produced in the stomach. Ghrelin expression has been reported also in other tissues including the pancreas. We reported that ghrelin cells constitute a novel endocrine cell type in the human and the developing rat islets. The cells are most numerous pre- and neonatally and in humans they constitute 10 % of all islets cells from mid-gestation to birth. Since gastric ghrelin expression is low before birth, the islets may during this time be the main source of circulating ghrelin. In the present investigation we performed ultrastructural analysis of pancreatic ghrelin cells in human fetuses using transmission electron microscopy and immunogold labelling. In addition, morphometrical analysis of secretory granules size was performed. Our data provide evidence for unique ultrastructural features of ghrelin cells vs other islet cells. Notably the secretory granules were of small size with a mean dense core diameter of 110 nm. We conclude that ghrelin cells constitute a novel islet cell type, distinct from the previously hormonally characterised islet cell types.

Introduction

Ghrelin is a novel hormone with multiple documented effects including metabolic regulation (Kojima et al. 1999; for review see Korbonits et al. 2004). Although ghrelin is predominantly a gastric hormone, expression has been reported in a number of tissues including the pancreas (Date et al. 2002; Volante et al. 2002; Wierup et al. 2002; Wierup et al. 2004); (for review see Korbonits et al. 2004) . We recently reported that ghrelin cells constitute a novel endocrine cell type in the human (Wierup et al. 2002) and rat (Wierup et al. 2004) islets; in human pancreas the ghrelin cells were devoid of any of the "classical" islet hormones. The pancreatic ghrelin cells display a

characteristic developmental regulation and constitute 10 % of all islets cells from mid-gestation to birth. In islets of adults the cells are much fewer, and constitute about 1% of all islet cells (Wierup et al. 2002). In rats pancreatic ghrelin cells are present from embryonic day 15 to one month postnatally and a minor subpopulation of the ghrelin cells coexpress glucagon or PP during a short period around birth (Wierup et al. 2004). These data were controversial since two groups presented data indicating ghrelin expression in glucagon- (Date et al. 2002) or insulin cells (Volante et al. 2002). However, recent studies on developing mice support our finding of an independent islet ghrelin cell (Prado et al. 2004). We recently reported that perinatally gastric ghrelin cells are few (Wierup and Sundler 2004; Wierup et al. 2002; Wierup et al. 2004) and others have reported that ghrelin gene expression in the fetal rat pancreas is higher than in the stomach (Chanoine and Wong 2004). Hence pancreas may be the main site of ghrelin production during development.

The ultrastructural identification of the gastric ghrelin cells in rodents has been facilitated by early detailed electron microscopy studies of gastric endocrine cells (for references see Sundler and Håkanson 1988). Thus, in rat and mouse it has became evident that A-like cells are equivalent with ghrelin cells (Dornonville de la Cour et al. 2001). Further, the ultrastructure of human gastric ghrelin cells has been investigated recently, using immunogold technique; the cells were characterised by small, round, compact to thin-haloed secretory granules (Rindi et al. 2002). Hitherto there is no information about the ultrastructure of pancreatic ghrelin cells. There are reports on islet cells with small secretory granules that have not yet been characterised with respect to hormone (Sundler and Håkanson 1988). Such cells include the P- and the D1-cell (Capella et al. 1978; Larsson et al. 1976; Solcia et al. 1987). The distinction between these two cell types is not very clear and cells with small granules have often been collectively classified as P/D1 cells (Bordi et al. 1986; Sundler and Håkanson 1988). To increase our knowledge about the cellular origin of pancreatic ghrelin in man, we examined ghrelin cells in human fetal pancreas using transmission

electron microscopy (TEM) and immunogold labelling.

Materials and Methods

Tissue

Since the ghrelin cell density is ten times higher in fetal pancreas compared to adult pancreas (Wierup et al. 2002), sampling of ghrelin cell profiles was facilitated by limiting our study to fetal pancreas. Pancreatic specimens from three human fetuses (gestational age 21-22 w) were from legal abortions and collected during the seventies, according to then used ethical guidelines.

Electron microscopy and immunogold

Specimens were fixed overnight in 0.075 M Sörensen buffer containing 3% parafomaldehyde and 1% glutaraldehyde, rinsed in Sörensen buffer, post-fixed for 1h with 1% OsO_4 in the same buffer, dehydrated in acetone and embedded in Epon. Alternatively, specimens were embedded without post-fixation in OsO_4 for immunogold. Ultrathin sections were cut and placed on copper grids, or gold grids for immunogold. For immunogold, sections were blocked with phosphate buffered saline (PBS) (pH 7.2) containing 0.5% bovine serum albumine (BSA), incubated overnight at 4°C with primary antisera (ghrelin, code H-031-31, dilution 1:100, Phoenix, Belmont, CA, USA; glucagon, code 8711, dilution 1:100, EuroDiagnostica, Malmö, Sweden; insulin, code 9003, dilution 1:100, EuroDiagnostica), diluted in PBS containing 0.25% bovine serum albumin and 0.25% Triton X-100, washed thoroughly in PBS, and thereafter incubated for 1h at room temperature with protein A-gold solution (10nm diameter dilution 1:20, Amersham-Pharmacia Biotech, Uppsala, Sweden), and again washed in PBS (Bottcher et al. 1986). All grids were contrasted with 0.5% lead citrate and 4% uranyl acetate before examination in a Phlips CM10 transmission electron microscope.

TEM-identification of immunoreactive cells.

The method has been described previously (Larsson et al. 1976). Briefly, semi-thin sections (1μm thickness) were cut and mounted on slides for immunocytochemistry (ICC). The immediately adjacent ultra-thin sections (50-70 nm thicknesss) were then processed for TEM as above. The semi-thin sections were deplasticized with KOH and ethanol, and used for indirect immunofluorescence. Briefly, the sections were incubated with two primary antibodies simultaneously (rabbit anti-ghrelin, code H-031-31, dilution 1:3200, Phoenix; guinea-pig anti-glucagon, code 8708, dilution 1:5120, EuroDiagnostica; guinea-pig anti- insulin, code 9003, dilution 1:100, EuroDiagnostica), diluted in the same buffer as above, overnight at 4° C, followed by rinsing in PBS with Triton X-100 for 2 x 10 min. Thereafter two different secondary antibodies with specificity for rabbit-, or guinea pig - IgG, and coupled to either fluorescein isothiocyanate (FITC), dilution 1:100 (Jackson, West Grove, PA, US) or Texas-Red , dilution 1:400 (Jackson) were applied on the sections. Incubation was for 1h at room temperature. Sections were again rinsed in Triton X-100 enriched PBS for 2 x 10 min and then mounted in PBS:glycerol, 1:1. The specificity of immunostaining was tested using primary antisera pre-absorbed with homologus antigen (100 μg of synthetic peptide per ml antiserum at working dilution), or by omission of the primary antibodies. In addition ghrelin antibodies were pre-absorbed with glucagon and insulin as above. The immunostained semi-thin sections were analysed in epi-fluorescence microscope (Olympus DP50) and images of immunoreactive cells were taken with a digital camera (Olympus BX60). The images were then used as guides for orientation and identification of immunoreactive cells in the adjacent ultrathin section using TEM.

Morphometry

Secretory granule diameter was measured on digitised images using NIH-image software. The analysis comprised 460 granules from 8 ghrelin cells and 430 granules from 7 glucagon cells. Results are shown as means ± SEM. Data were analysed by Student's un-paired t-test. Differences with a value of $p < 0.05$ were considered significant.

Results

Detection of ghrelin cells and morphometry

To locate ghrelin cells, semi-thin sections double immunostained for ghrelin and glucagon or ghrelin and insulin (glucagon and insulin also used as islet markers) were analysed in epi-fluorescence microscope and images of immunoreactive (IR) cells were captured with a digital camera. Using the image as a guide, ghrelin and glucagon IR cells were localised in ultrathin sections with TEM (Fig 1). The ghrelin cells were located at the islet periphery or in the exocrine tissue often in vicinity of glucagon cells. In the same way ghrelin and insulin IR cells were localised (not shown). Ghrelin IR was always in cells distinct from glucagon- or insulin IR cells. Ghrelin cell secretory granules were small, spherical, and displayed varying electron density, from dense to rather electron-lucent (Fig 1B). Further, the limiting membrane appeared to be tightly applied to the dense core of most of the granules. Morphometrical analysis revealed that the ghrelin granules (dense core) had a mean diameter of 110±3 nm, ranging from 70nm to 160nm in individual cells. By comparison, glucagon cell granules were highly electron dense and larger with a mean diameter of 185±7 nm, ($p<0.001$, vs ghrelin cell granules). Ghrelin cell granules were clearly different from those of insulin cells, recognised by their characteristic irregular, crystalline

dense core. Ghrelin cell granules were also clearly different from those of somatostatin cells, characterised by the large dense core of low electron density, and from those of PP-cells recognised by the irregularly shaped moderately electron dense granule core (Sundler and Håkanson 1988). Granules of the different islet cell types are illustrated in Fig 2.

Immuogold labelling

The identity of ghrelin cells was further verified with immunogold labelling for ghrelin, glucagon, and insulin. Within the cells labelling for ghrelin was found predominantly on the secretory granules (Fig 3A-C). Ghrelin cells were devoid of labelling for insulin or glucagon. In addition, insulin-, glucagon-, somatostatin, and PP-cells were devoid of labelling for ghrelin. The appearance of immunogold labelling for glucagon and insulin is illustrated in (Fig 3D-I). Weak, scattered, unspecific background labelling was seen with all three antibodies.

Discussion

The main islet cell types (insulin-, glucagon-, somatostatin, and PP-cells) have previously been characterised and they all have a unique ultrastructural appearance (for review see Sundler and Håkanson 1988). In the present study we provide evidence that also ghrelin cells have unique ultra structural features, which distinguish them from the previously characterised endocrine islet cells. Conflicting reports have appeared about the cellular origin of pancreatic ghrelin. Thus, Date et al (2002) presented results indicating that ghrelin is expressed in the glucagon cells of human and rat islets. Volante and coworkers on the other hand presented data indicating ghrelin expression in human insulin cells (Volante et al. 2002). There is no ready explanation for this discrepancy, but our present data together with our previous findings (Wierup and Sundler 2004; Wierup et al. 2002;

Wierup et al. 2004) and recent findings by others (Prado et al. 2004) do not support expression of ghrelin in glucagon- or insulin cells. Rather, our present data further support that ghrelin cells constitute a separate islet cell population.

Ultrastructurally, ghrelin cells were easily separated from insulin cells, recognised by their characteristic irregular crystalline granules (for references see Klöppel and Heitz 1984). Further, ghrelin cells were clearly distinct from somatostatin and PP-cells recognised by large electron lucent-, and small irregularly shaped, moderately electron dense granules, respectively, as previously described (Sundler and Håkanson 1988). Furthermore, although clearly different from the other cell types, the secretory granules of the ghrelin cells were most similar to those of glucagon cells; however, morphometrical analysis revealed that ghrelin cells had smaller secretory granules than glucagon cells.

In 1978 Capella and coworkers described endocrine cells in the human pancreas, closely resembling the previously classified gastric P-cells (Capella et al. 1978). Interestingly, the cells were numerous in fetal pancreas, but rare in adult pancreas. Clearly, this developmental pattern very much resembles that we recently reported for the islet ghrelin cells in human pancreas (Wierup et al. 2002). Further, they reported that the secretory granules of these cells had a mean diameter of about 130nm and the cells are classified as P/D1-cells (see Introduction). There is a great degree of resemblance between the P/D1-cells and the ghrelin cells as described in the present report. Together, these data suggest that the ultrastructurally defined cells with small granules (P/D1-cells) in human islets may actually be identical to ghrelin cells. The slight difference in granule diameter between the ghrelin granules in the present investigation and those described by

Capella et al (1978), could be due to different fixation used and/or the fact that we measured the dense core diameter, rather than the full diameter including the granule membrane.
Further, the ultrastructure of the pancreatic ghrelin cells displays a high degree of resemblance with the human gastric ghrelin cells recently characterised by Rindi and coworkers (Rindi et al. 2002), with the exception for slightly larger granules in the gastric ghrelin cells compared to the pancreatic (147 vs 110nm).

In conclusion this study provides evidence for a unique ultrastucture of the recently discovered islet ghrelin cells vs other hormonally identified islet cells and emphasizes the ghrelin cell as a fifth islet cell type.

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FIGURE LEGENDS

Figure 1. A: Fluorescence photomicrograph of semi-thin section of human fetal pancreas, double immunostained for ghrelin (red) and glucagon (green). B: Electron microscopy of the ghrelin IR cell within frame in A. Scale bars: In A = 5 μm, in B = 1 μm.

Figure 2. Secretory granules from islet cells. A: Ghrelin cell. B: glucagon cell. C: insulin cell. D: somatostatin cell. E: PP-cell. Note that ghrelin cell granules are distinct from other islet cell granules. Scale bar =250nm, in A for all images.

Figure 3. Immunogold labelling of human fetal pancreas. A-C: ghrelin cell with labelling for ghrelin. D-F, glucagon cell with labelling for glucagon. G-I: insulin cell with labelling for insulin. Scale bars: In A for A, D, G = 1μm, in B for B, E, H 150 nm, in C = 150nm, in F = 250nm, in I = 250nm.

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Fig1.

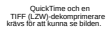
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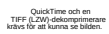
Fig 2.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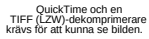
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Fig 3.