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Ghrelin localization in the medulla of rat and human adrenal gland and in pheochromocytomas

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Summary. Objective: Ghrelin is predominantly produced by neuroendocrine cells of stomach and has been expressed in several normal and tumour endocrine tissues. It has been reported that the localization of ghrelin is exclusively in the cortex of human and rat adrenal gland and in adrenocortical tumours. This prompted us to analyze the expression of this peptide in medulla of human and rat adrenal glands and in human pheochromocytomas. Design and methods: Analysis of ghrelin mRNA expression in rat adrenal gland was conducted by means of semi-quantitative RT-PCR. Ghrelin localization was studied in medulla of human and rat adrenal gland by immunohistochemistry. In addition, we have carried out a double immunofluorescence with chromogranin A to determine the specific cell type expressing ghrelin immunoreactivity. Ghrelin expression was also analyzed in five cases of pheochromocytoma by immunohistochemistry. Finally, Western blotting analysis was performed with goat ghrelin antibody in the cortex and in the medulla of rat adrenal gland. Results: RT-PCR demonstrated expression of ghrelin mRNA in rat adrenal gland. We also detected ghrelin expression in virtually all rat pheochromocytes by immunohistochemistry and double immunofluorescence. Furthermore, we showed ghrelin immunoreactivity in the medulla of human adrenal gland and in pheochromocytomas. By Western blotting, we found the expression of ghrelin precursor, proghrelin and mature ghrelin in the medulla of rat adrenals. However, the cortex of rat adrenal gland only expressed ghrelin precursor. Conclusions: Our study is the first to demonstrate a medullar expression of ghrelin in human and rat adrenal gland; we also showed ghrelin expression in pheochromocytomas.

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Introduction

Growth hormone secretagogues (GHSs) are synthetic compounds discovered by Bowers et al. (1977) that are able to induce GH release (Bowers et al., 1984, 1990) by activation of GH secretagogue receptors (GHS-R) (Casanueva and Diéguez, 1999). After cloning the GHS-R (Howard et al., 1996), various cell lines that expressed the receptor (GHS-R) were used to follow endogenous ligand. The first natural ligand disclosed was ghrelin, a 28 amino acid peptide isolated from extracts of stomach tissue (Kojima et al., 1999). Octanoylation on serine 3 residue allows ghrelin to bind to its active receptor (GHS-R1a) (Kojima et al., 2001) which is required for its ability to alter endocrine function in rodents and man (Rosická et al., 2002; Gualillo et al., 2003; Van del Lely et al., 2004; Otto et al., 2005).

Ghrelin has been observed in various rat and human cell lines (Wei et al., 2005), and throughout rat and human tissues (Gualillo et al., 2001; Gnanapavan et al., 2002; Tena-Sempere et al., 2002; Caminos et al., 2003; Yabuki et al., 2006), including adrenal gland (Papotti et al., 2000; Barreiro et al., 2002; Gnanapavan et al., 2002; Andreis et al., 2003; Tortorella et al., 2003). These studies were conducted using RT-PCR and they only found ghrelin in the adrenal cortex (Carraro et al., 2004; Mazzocchi et al., 2004; Barzon et al., 2005).

Jeffrey et al. (2005) showed immunoreactivity for the GHS-R1a isoform in mouse adrenal medulla. The mRNA of this isoform was demonstrated by RT-PCR in human and rat adrenal cortex (Barreiro et al., 2002; Andreis et al., 2003). Moreover, it has been demonstrated that ghrelin does not affect the secretory activity of rat and human adrenocortical cells (Andreis et al., 2003) but exerts proliferogenic and antiapoptotic

effect via MAPK in human adrenal cortex. In addition, Nanmoku et al. (2003) observed ghrelin stimulation of dopamine secretion from rat pheochromocytoma PC 12 cells suggesting a possible role of ghrelin in tumour growth.

The aim of the current paper was to analyze ghrelin mRNA expression by RT-PCR in rat adrenal gland and to determine the expression and localization of ghrelin using Western blot, immunohistochemistry, and double immunofluorescence with chromogranin A. We also analyzed by immunohistochemistry five normal human adrenal glands and five cases of pheocromocytomas.

Materials and methods

Animals

Male Sprague Dawley rats (3-5 months) were bred in the vivarium from our institution. The animals were housed under constant conditions of light (14h of light; from 7:00 a.m.) and temperature (22°C), and had free access to standard pellet rat chow and tap water. The protocol of the experiments was approved by the Santiago de Compostela Medical School Animal Care Research Committee and was conducted in accordance with the European Union normative for care and use of experimental animals.

Ghrelin mRNA expression analysis by RT-PCR

Animals were killed by decapitation. The stomach wall (fundus) and adrenal gland were immediately removed, frozen at -80°C until ghrelin mRNA analysis.

Total RNA was extracted from the removed rat stomach and adrenal gland using Trizol® (Invitrogen, Inc., Carlsbad, CA) according to the manufacturer's instructions. The expression of ghrelin mRNA was assessed by RT-PCR, optimized for semi-quantitative detection using a specific primer pair (Table 1). As an internal control, amplification of mRNA was carried out in parallel in each sample using the primer HPRT.

Complementary DNA was synthesized using 1 nM random primers, 200 U M_0ML superscript II reverse transcriptase and 20 U ribonuclease inhibitor in total volume of 30 µL (Invitrogen). For amplification of the different genes, primers were selected to eliminate non-specific amplification of genomic DNA. RT reactions were incubated at 37°C for 1 h and at 42°C for 10 min and were terminated by heating at 95°C for 5 min.

PCR amplification of the generated cDNA was carried out in 50 µL of 1x PCR buffer in the presence of 1.25 U Taq-DNA polymerase (Invitrogen) and 1 nM forward and reverse primers.

The amplification profile for rat ghrelin was: denaturising at 95°C for 1 min, annealing at 62°C for 30 sec, and extension at 72°C for 1 min followed by 34 cycles. The RT-PCR products were confirmed by sequencing. As an internal standard, HPRT cDNA was amplified. PCR products were separated by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining with UV light using a Gel Doc 1000 Documentation System (Bio-Rad Laboratories, Inc., Richmond, CA). In all assays, liquid controls and reactions without RT resulted in negative amplification.

Immunohistochemistry

Rats (n=6) were decapitated and the adrenal glands were promptly removed and cleaned from adherent adipose tissue. We also took the fundic region of stomach and all specimens were fixed by immersion in 4% buffered formaldehyde for 24 h, dehydrated and embedded in paraffin by standard procedure.

Paraffin blocks of normal human adrenal glands were obtained from five patients ranging in age from 61-68 yr undergoing unilateral nephrectomy or adrenalectomy in cases of renal tumour. Samples of human pheochromocytoma were obtained from five patients ranging in age from 30-72 yr (one case was diagnosed as malignant pheochromocytoma). All human samples proceeded from the files of the Service of Pathology, University Clinical Hospital, Santiago de Compostela (Prof. J. Forteza).

Sections 5 µm thick were mounted on histobond adhesion microslides (Mariefeld, Laura-Konigshofen, Germany) and deparaffined. Antigen retrieval was carried out by heating in a microwave oven for 20 min at 750 W in 10 mM sodium citrate buffer (pH 6.0). Each sections were consecutively incubated in: (1) goat anti-ghrelin polyclonal antibody (sc-10368, Santa Cruz, CA) at a dilution 1/100 or rabbit anti-ghrelin polyclonal antibody (H-031-31, Phoenix, Belmont, CA) at a dilution 1/500 for 1 h. Both dilutions of ghrelin antibodies were prepared with a commercial antibody diluent (Dako, Glostrup, Denmark); (2) peroxidase blocking reagent (Dako) to eliminate endogenous peroxidase for 10 min; (3) LSAB+® detection system

Table 1. Primers used for semi-quantitative RT-PCR.

Primers	Sequence	Product (bp)	Genebank
Rat Ghrelin Forward	5´-AGCTAAACTGCAGCCACGAG-3´	151	AB029433
Rat Ghrelin Reverse	5´-GCCATGCTGCTGGTACTGAG-3´		
Rat HPRT Forward	5'-CAGTCCCAGCGTCGTGATTA-3'	139	NM013556
Rat HPRT Reverse	5'-AGCAAGTCTTTCAGTCCTGTC-3'		

composed of biotinylated anti-mouse, rabbit and goat Ig and streptavidin conjugated to horseradish peroxidase (Dako) was used for the goat antibody and horseradish peroxidase conjugated to dextran polymer system containing anti-rabbit and anti-mouse (Envision®, Dako) was used for the rabbit antibody, each one for 30 min; (4) 3,3'-diamino-benzidine tetrahydrochloride (Dako) was applied as chromogen for 10 min to reveal the immunostaining reaction. Between steps, sections were washed twice for 5 min with PBS (0.1 M phosphate, pH 7.4, containing 0.15 M NaCl) and after step 4, with distilled water. Counterstaining was done with haematoxylin for 1 min.

Controls for specificity of immunohistochemistry were performed by either: (1) omission of any essential step of the immunoreaction; (2) substitution of the primary antibodies with suitable dilution of normal (non-immune) rabbit or goat serum (Dako); (3) preadsorption of the anti-ghrelin antibody with the homologous antigen (ghrelin, 10 nmol/mL, from Global Peptide Services, Fort Collins, CO) (overnight at 4°C) as previously described (Gualillo et al., 2001).

Western Blot

Rat mucosa was obtained by scraping stomach lining in presence of saline buffer. The mix was pelleted by centrifugation and the tissue pellet finally frozen in liquid nitrogen. Rat adrenals were dissected and both cortex and medulla were obtained and frozen in liquid nitrogen. Lysis buffer (50 mM Hepes pH 7.4, 1% Triton X-100, 10 mM NaCl, 10 mM EDTA, 10 mM Na pyrofosfate, 2 µg/mL aprotinin, 10 µg/mL antipain, 0.5 µg/mL leupeptin, 5 µg/mL pepstatin and 17 µg/mL PMSF, all from Sigma) was added at 10 µL/mg tissue, and homogenisation was performed in ice with a Dounce homogenizer with B Pestle, followed by 15 min of incubation in ice. The homogenates were centrifuged in an eppendorf centrifuge at 14,000 rpm during 30 min at 4°C. Supernatants were kept at –80°C. 12.5% or 18% SDS-PAGE were run followed by western blot as described (Bravo et al., 2003) with anti-ghrelin from Santa Cruz. Ghrelin and unacylated ghrelin used as controls were from Sigma. Duplicate western blots using 10 µg of synthetic rat unacylated ghrelin (UAG), 30 µg of acylated ghrelin (G, Sigma) and 100 µg of lysates from rat stomach mucosa were hybridized with two different ghrelin antibodies.

The antibodies used were rabbit anti-ghrelin from Phoenix (obtained using human synthetic ghrelin as antigen) 1/200; goat anti-ghrelin from Santa Cruz, obtained using a peptide from amino acids 24 to 50 of the human ghrelin sequence (Swissprot, http://expasy.org/uniprot/Q9UBU3) 1/1000.

Double immunofluorescence

The adrenal sections were heated in a microwave oven for antigen retrieval during 20 min at 750 W in TE buffer (Tris-EDTA 10 mM pH 9.0). Sections were consecutively incubated in: (1) primary goat antibody to ghrelin Santa Cruz Biotecnnology at a dilution of 1/100 (overnight at 4°C); (2) anti-goat Ig conjugated with Alexa fluor 488 (A 21222, Molecular Probes, Eugene, OR) at a dilution of 1/200 for 1 h; (3) anti-mouse chromogranin A (Biogenex Laboratories, San Ramon, CA) at 1/200 for 1 h; (4) anti-mouse secondary antibody (Sigma, Saint Louis, MO) conjugated with Cyanin 3 prepared at a dilution of 1/100 for 1 h. All dilutions were made in PBS. This buffer was also used for washing baths. After step 4, the sections were washed in distilled water, and finally they were mounted with immuno-fluore mounting medium (ICN, Biomedicals, Aurora, OH). The sections were observed using 63x1.32 NA oil-immersion objective and photographed by a confocal laser-scanning microscope Leica TCS SP2 (Leica Microsystems, Mannheim, Germany) equipped with argon Laser (448-514 nm).

Results

RNA analysis by semi-quantitative RT-PCR

The expression of ghrelin mRNA in adrenal was detected by using specific primers for rat ghrelin. The products of the expected size (151 bp), were amplified from adrenal tissues by RT-PCR. For comparative purposes, RT-PCR amplification of ghrelin mRNA was also conducted in RNA samples from rat stomach as a control. The quality of mRNA has been corroborated by HPRT mRNA levels and a 100 bp molecular weight marker has been used (Fig. 1).

Immunohistochemistry

Positive and negative controls were performed in rat stomach (fundic region). Immunoreactivity for ghrelin was observed in endocrine cells of the oxintic glands (Fig. 2A), whereas no immunostaining was found when the anti-ghrelin antibody was preadsorbed with the homologous antigen (Fig. 2B).

In the rat adrenal gland we found immunoreactivity for ghrelin (using the antiserum from Santa Cruz) in the medulla (Fig. 2C). Controls performed by preadsorption of the antibody with ghrelin ascertain the specificity of the immunoreaction (Fig. 2D). Virtually all chromaffin cells were positive (Fig 2E) and the ghrelin signal was cytoplasmic (Fig 2F). Neurons scattered between pheochromocytes did not show any immunoreactivity for ghrelin (Fig. 2F). Ghrelin expression was also observed using a different ghrelin antiserum (Phoenix) but with this antibody we also found a weak immunoreactivity in the glomerulose zone (Fig. 2G).

In human samples, the results obtained with goat ghrelin antibody (Santa Cruz) were similar to those found in rats. Positivity was observed in the medulla (Fig. 2H). Chromaffin cells were immunostained with

ghrelin, whereas the neurons were negative (Fig. 2I). Negative controls were performed by omission of the biotinylated Ig (Fig. 2J positive, 2K negative). However, we failed to obtain immunostaining in human adrenals with the ghrelin antibody from Phoenix (data not shown).

The five human pheochromocytomas studied showed positivity for goat anti-ghrelin (Santa Cruz) with focal (3/5) or diffuse (2/5) immunostaining patterns (Fig. 2L, 2M). The intensity of immunoreactivity varied between different tumours and also between different cells of the same tumour (Fig. 2N).

Double immunofluorescence

Double immunofluorescence for ghrelin and chromogranin A was performed to confirm the results obtained by immunohistochemistry. Ghrelin and chromogranin A expressing cells were identified by green and red immunofluorescence, respectively. The colocalization of ghrelin and chromogranin A was confirmed by confocal laser visualization in virtually all chromaffin cells (Fig. 2O, 2P).

Western Blot

The ghrelin mRNA is translated to an initial long precursor, which enters the endoplasmic reticulum after losing the ER-signal peptide rendering pro-ghrelin. Mature ghrelin exists as two different modalities, either acylated or unacylated (UAG). Although initially it has been thought that UAG was inactive, recent data (Van der Lely et al., 2004) demonstrate a possible independent action through a different receptor. We wanted to study if the ghrelin was produced and secreted in the medulla or the immunoreactivity detected in the medulla was ghrelin uptaken from serum. Since the anti-ghrelin antibodies used in the immunohistochemistry have been obtained using different types of antigens, the question of what type of ghrelin was been recognized in the adrenal medulla remained. Rabbit anti-synthetic ghrelin (from Phoenix) have been obtained used acylated ghrelin as antigen; however, goat anti-ghrelin has been obtained using a peptide corresponding to 24-50 of the human ghrelin sequence (similar to the rat sequence Santa Cruz).

To investigate what type of ghrelin/s -acylated vs unacylated- was been recognized by the two anti-ghrelin antibodies a comparative Western blot was performed. Fig. 3A shows a comparison between both antibodies in replicate western blots loaded with commercial ghrelin, UAG and a protein lysate of mucosa lining of the stomach. In the ghrelin lane two bands can be seen, the lower one being coincident in molecular weight with UAG. We concluded that part of the commercial ghrelin was unacylated therefore UAG. Rabbit anti-ghrelin recognized only mature ghrelin both acylated and UAG either in the stomach or in the lanes where purified ghrelin or UAG were loaded. Goat anti-ghrelin recognized, as expected, both the precursor and the pro-

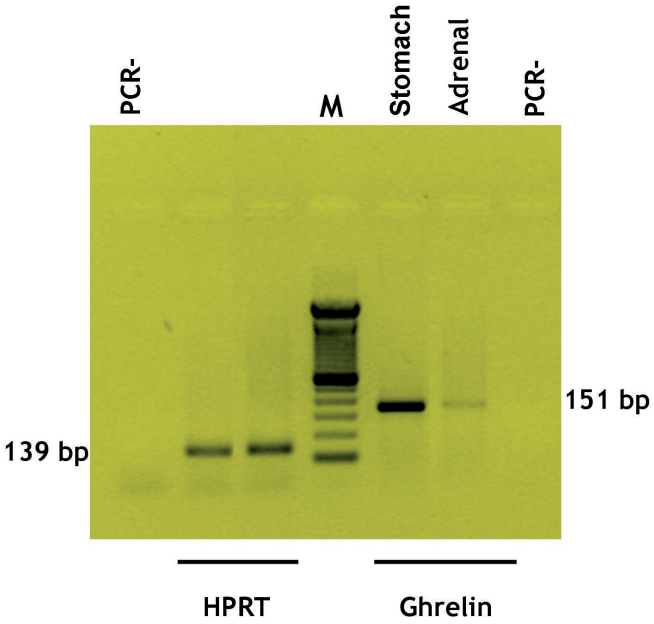
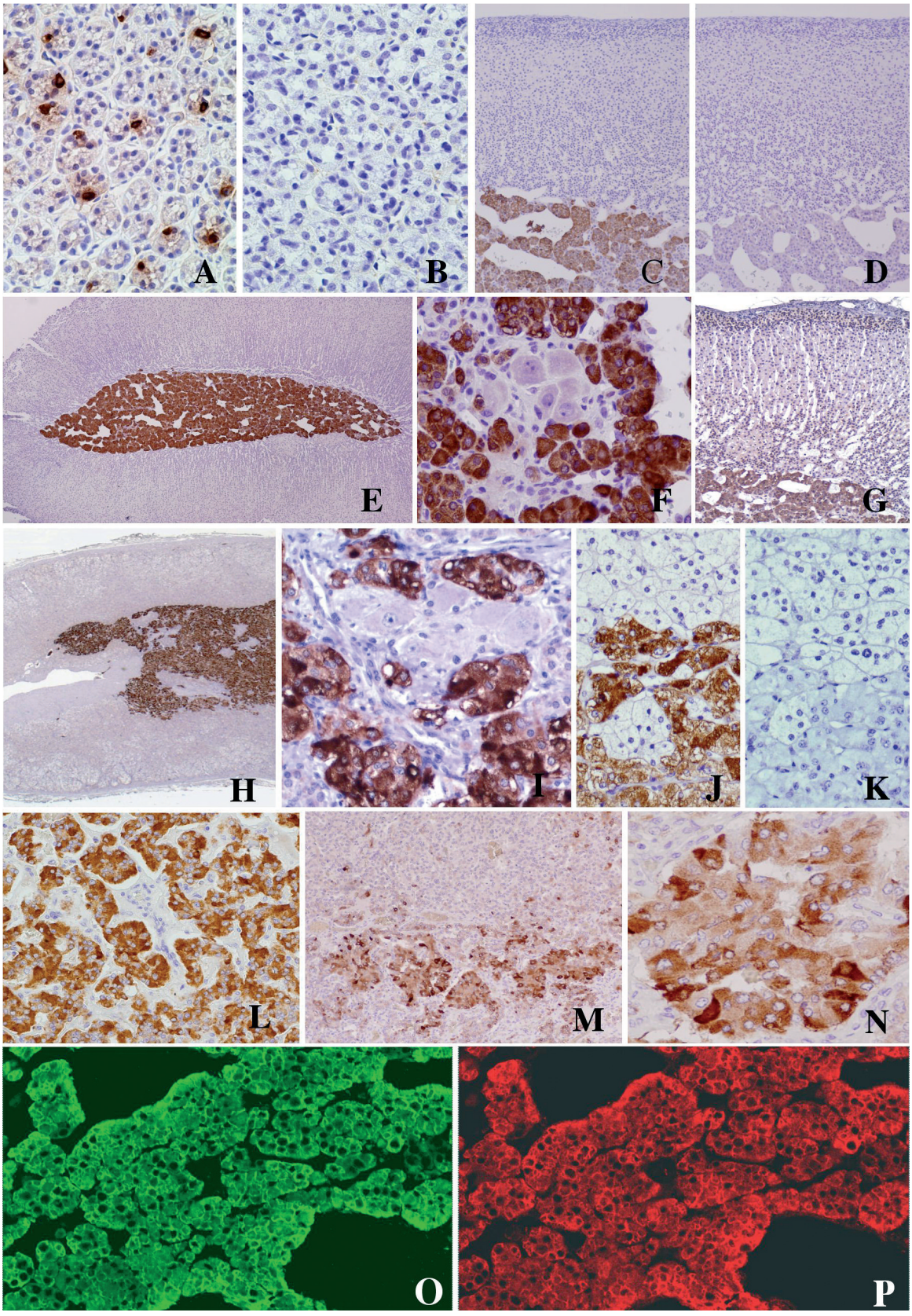


Fig. 1. Representative RT-PCR showed ghrelin mRNA expression in adrenal gland. Positive control was shown in stomach. A 100 bp molecular weight marker was used.

Fig. 2. Immunohistochemistry and immunofluorescence. **A, B.** Positive and negative controls of immunohistochemistry performed in normal rat gastric mucosa. **A.** Ghrelin immunoreactivity appeared in neuroendocrine cells of oxyntic gland (x 40). **B.** No immunostaining was found with goat ghrelin antibody preadsorption control (x 40). **C.** Immunoreactivity was found by anti-ghrelin from Santa Cruz in the medulla of rat adrenal gland (x 20). **D.** negative control was performed by preadsorption of anti-ghrelin with homologous peptide (x 20). **E-G.** Ghrelin immunostaining detected in the medulla of rat adrenal gland. **E.** Goat anti-ghrelin only immunoreacts with medulla of rat adrenal gland (x 4). **F.** The neurons rounding the chromaffin tissue remained negative (x 40). **G.** Rabbit anti-ghrelin immunostained medulla intensely however a weak staining was showed in glomerulose zone of the rat adrenal cortex (x 4). **H.** Goat anti-ghrelin immunostained the medulla of human adrenal gland while no immunoreactivity was observed in the cortex (x1,95). **I.** High magnification showed positivity in chromaffin cells and negativity in neurons (x20). **J-K.** Controls performed in serial adrenal sections (x20). **J.** The section incubated with goat anti-ghrelin showed intense immunopositivity. **K.** No immunoreactivity was found by omission of the biotinylated Ig. **L, M.** Pheochromocytomas showed two different patterns of immunostaining with ghrelin antibody from Santa Cruz. The diffuse pattern demonstrated a large expression in the tumour, but a negativity in the stroma (x 20) **(L)**. The focal one demonstrated groups of positive cells (x 20) **(M)**. **N.** Within the tumour, some cells present more immunoreactivity than others (x 40). **O, P.** Representative confocal images of double immunofluorescence with ghrelin and chromogranin A in medulla of rat adrenal gland (63x 1.32 NA oil immersion objective). **O.** Goat anti- ghrelin was immunolabelled by Alexa 488. **P.** Anti chromogranin A was immunolabelled by Cyanin 3. All chromaffin cells colocalize ghrelin and chromogranin.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ghrelin together with ghrelin in stomach mucosa (Fig. 3B). Since this antibody recognized the peptidic portion of either type of ghrelin, we use goat anti-ghrelin to compare lysates from adrenal cortex and adrenal medulla, using the same mucosa lining of the stomach as a positive control.

Discussion

Although the role of ghrelin in the regulation of food intake and GH secretion is undisputed, the wide expression of ghrelin synthesizing cells in different tissues indicates that it may play some other physiological roles. Indeed, Papotti et al., (2000) showed that human adrenal gland is the second tissue after myocardium that had a specific binding for synthetic growth hormone hexarelin. Furthermore, by real time RT-PCR, ghrelin mRNA was found in human adrenal gland with lower quantity than fundus (75 mRNA copy number per total µg RNA, 10^7 mRNA copy number per total µg RNA, respectively) (Gnanapavan et al., 2002). This finding explains the weak band of ghrelin that has been obtained by our semi-quantitative RT-PCR carried out in rat adrenal gland. However, Barreiro et al., (2002) have failed to find any amplification of ghrelin transcript in rat adrenal gland, despite a strong expression in the

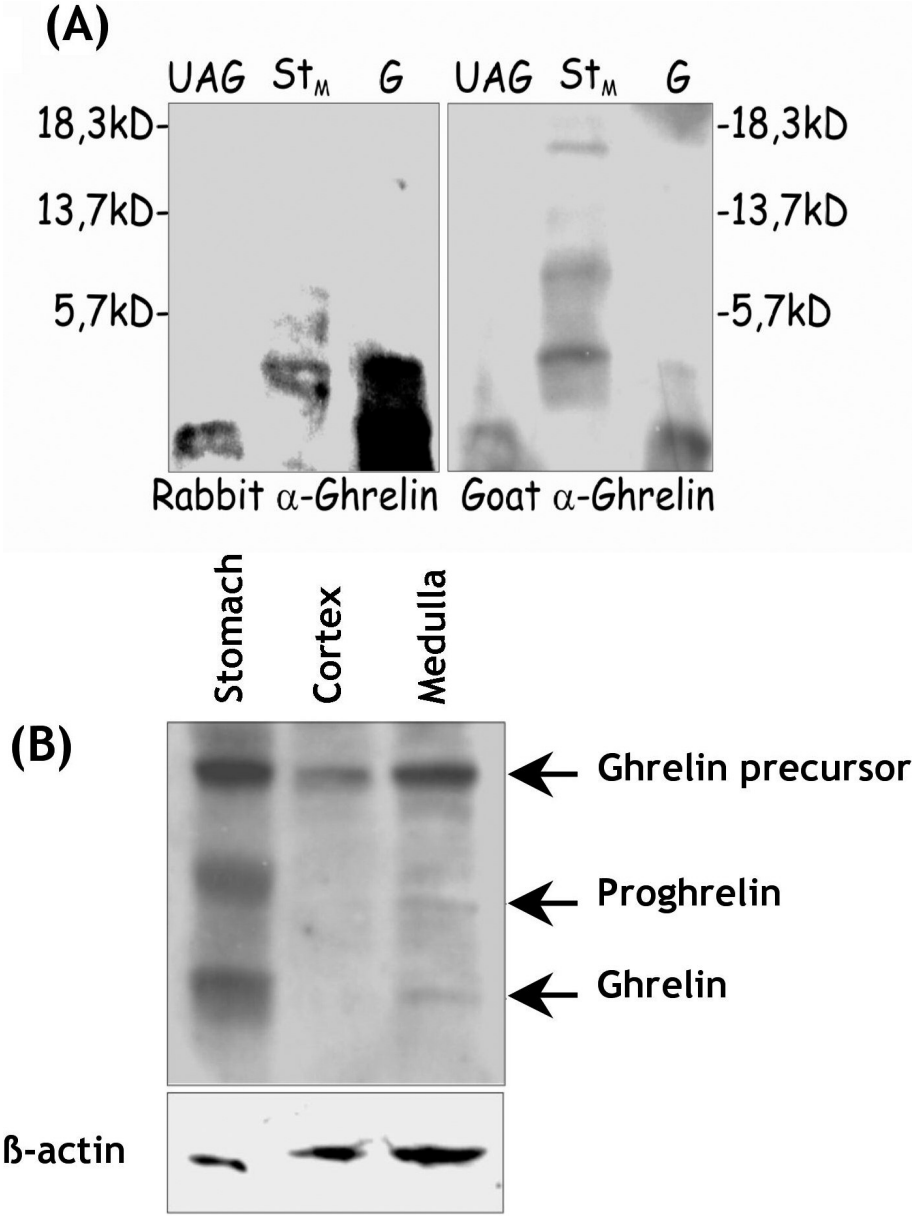

Fig. 3. Western blot. **A.** Synthetic or endogenous mature Ghrelin (acylated) is best recognized by rabbit anti-ghrelin (Phoenix) while goat anti-ghrelin (Santa Cruz Biotech.) recognizes better the peptidic portion of both synthetic and endogenous ghrelin. Duplicate Western blots using 10 µg of synthetic rat unacylated ghrelin (UAG) or 30 µg of acylated ghrelin (G, Sigma) and 100 µg of lysates from rat stomach mucosa (StM) were incubated with two different ghrelin antibodies. Rabbit anti-synthetic ghrelin recognized very well purified synthetic ghrelin and to a lesser extent the proteic part of ghrelin (either unacylated ghrelin or the endogenous ghrelin). Goat anti-ghrelin was made using a peptide corresponding to aa 24-50 of human ghrelin sequence and recognized well both the endogenous ghrelin (the ghrelin precursor, pro-ghrelin and mature ghrelin) and unacylated ghrelin but was less effective recognizing synthetic ghrelin. Note that in the commercial ghrelin preparation an important portion is unacylated ghrelin. **B.** The rat adrenal medulla expresses ghrelin in higher quantities than adrenal cortex. Western blot with 100 µg of total lysates from rat stomach mucosa, adrenal cortex and adrenal medulla incubated with goat anti-ghrelin. The medulla showed the expression of ghrelin precursor, pro-ghrelin and mature ghrelin. The adrenal cortex showed a faint band of the ghrelin precursor but in much less intensity than medulla.

stomach. This result may be explained by Wei et al., (2005) which have demonstrated the presence of two transcription initiation sites that influenced in ghrelin transcripts. The distal and proximal transcription initiation sites corresponding to long and short ghrelin transcripts, respectively, were assessed by densiometric analysis of RT-PCR in various cellular lines and tissues. Ghrelin mRNA was only detected with the short transcript in rat adrenal gland.

In agreement with previous papers, we found ghrelin mRNA expression, as assessed by RT-PCR, in the rat adrenal gland (Gnanapavan et al., 2002; De Vriese et al., 2005). Furthermore, our immunohistochemistry, achieved by two different ghrelin antibodies (from Phoenix and Santa Cruz), have demonstrated an intense ghrelin immunoreactivity in chromaffin tissue of rat adrenal gland. In addition, using ghrelin antibody from Phoenix, we noticed a weak immunoreactivity compared to that obtained in the medulla. For this reason, we carried out Western blot of both ghrelin antibodies in rat stomach to analyze the nature of the immunoreactive products detected by each one.

Our result obtained by Western blot in adrenal medulla, revealed expression of the ghrelin precursor, proghrelin and mature ghrelin as detected in stomach. This finding demonstrated that ghrelin is synthetized by the adrenal gland. However, in adrenal cortex a faint precursor band is detected but no smaller bands (mature ghrelin and proghrelin), suggesting reduced possibilities of ghrelin secretion by the cortex of adrenal gland.

In any event, whatever the reason, our data demonstrated the importance of careful characterization of the immunoreactive products detected by different antiserum. To our knowledge, our Western blot is the first one in assessing the ghrelin immunoreactive products detected by two commercial antisera most widely used. Moreover, these findings may explain the present discrepancies regarding ghrelin expression in adrenal tissues that was assessed by immunohistochemistry.

It is to be noted that ghrelin is unable to exert any sizeable effect on steroid hormone secretion from rat and human adrenal (Barreiro et al., 2002; Tortorella et al., 2003) and in cultured glomerulose zone and fasciculate-reticularis cells (Andreis et al., 2003), whereas ghrelin was capable of enhancing dopamine release from pheochromocytoma PC12 cells (Nanmoku et al., 2003). Lucidi et al. (2005) have demonstrated that a large amount of ghrelin in humans increases the concentration of several pituitary and adrenal hormones. Physiological increments in plasma ghrelin concentrations enhance only response in GH, but about three-fold increases in plasma ghrelin concentrations are required to obtain response of epinephrine, prolactin, ACTH, and nonesterified fatty acid (NEFA). By this means, ghrelin synthesized in the adrenal medulla could be involved in the autocrine/paracrine regulation of chromaffin cell secretion.

We also demonstrated by immunohistochemistry a positive signal for ghrelin in pheochromocytomas. There is evidence indicating that several cancer cells express the machinery of ghrelin/GHS-R axis which may have an important autocrine/paracrine role in regulating cancer cell biology. In previous reports, ghrelin has been observed to exert a proliferative effect in carcinoma cell line of breast (MCF7, T240, MDA, MB231) (Cassoni et al., 2001), of thyroid (ARO) (Ghé et al., 2002) and lung (CALU-1) (Cassoni et al., 2000) as well as invasive pancreatic adenocarcinoma (Duxbury et al., 2003). Cassoni et al. (2004) demonstrated that specific GHS binding sites, other than GHS-R1a and 1b, are present in human prostatic neoplasms and that ghrelin des-acylated exerts different effects on cell proliferation in prostate carcinoma cell lines (Jeffrey et al., 2002). Recently De Vriese et al., (2005), suggested that octanoylated ghrelin is involved in human erythroleukemic cell proliferation by autocrine pathway. Ghrelin expressed in pheochromocytomas could probably exert an autocrine/paracrine role in tumour growth.

Impaired glucose tolerance was observed in patients with pheochromocytoma with an incidence from 25-75% (Turnbull et al., 1980; Isles and Johnson, 1983) and it has been reported that reduced insulin was observed repeatedly in patients with pheochromocytoma. Wiesner et al. (2003) demonstrated that catecholamine overproduction in patients with pheochromocytoma led to insulin resistance. Recently, a clinical study carried out in overweight and obese children demonstrated an association between ghrelin secretion and postprandial insulin mediated glucose metabolism and proposed that maintenance of an adequate level of insulin sensitivity might affect appetite regulation by favouring a reduction of postprandial ghrelin (Maffeis et al., 2006). In another paper, circulating levels of catecholamines correlate positively with ghrelin levels in pathological states. Circulating norepinephrine and ghrelin were observed in patients with chronic obstructive pulmonary disease with underweight (Itoh et al., 2004; Mundinger et al., 2006). Furthermore, during coronial autopsies twelve of 32 patients with pheochromocytoma were overweight (body mass index >25 kg/m^2) and the weight of their heart was increased by 95%. The pathogenesis of the possible role of ghrelin in overweight and cardiohypertrophy observed in cases of pheochromocytomas is still to be investigated, since ghrelin and insulin are two hormones that play a relevant role in body weight regulation (Tschöp et al., 2000), it seems reasonable to speculate that high catecholamine levels, insulin resistance and ghrelin production in adrenal medulla could have a role in body weight regulation.

In conclusion, our study provides novel evidence for the expression of ghrelin in the rat adrenal medulla and in pheochromocytomas since the expression of the ghrelin receptor, GHS-R1a, has been shown in this region, and it has been shown that ghrelin can exert biological effects, namely dopamine release. Taken together, these data indicate that ghrelin produced in the adrenal medulla might play an important paracrine role

in the regulation of catecholamines release. Further studies are needed to ascertain the possible pathological implications of ghrelin in tumour growth in this tissue as well as its role in the development of insulin resistance and obesity in patients with pheochromocytoma.

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