SPANDIDOS PUBLICATIONS

Age-dependent decrease in the ghrelin gene expression in the human adrenal cortex: A real-time PCR study

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Abstract. Numerous lines of evidence indicate that ghrelin, an endogenous ligand of the growth hormone-secretagogue receptor, is expressed in the human and rat adrenal cortex. In this study, we examined whether ghrelin gene expression undergoes changes in the human adrenal cortex during aging. Semi-quantitative real-time reverse transcription-polymerase chain reaction demonstrated a highly significant negative correlation between ghrelin mRNA and age in adrenal cortexes of 27 patients (aged from 33 to 82 years), who underwent unilateral adrenalectomy/nephrectomy for kidney cancer. No significant differences in the level of adrenal ghrelin expression were observed between males and females. Since it has been previously shown that ghrelin exerts a marked growth-stimulating action on cultured adrenocortical cells, we hypothesize that the down-regulation of ghrelin gene transcription in adrenals could be associated with the reported decrease in adrenal DNA synthesis and mitogenic activity during aging.

Introduction

Ghrelin is a 28-amino acid peptide with an *n*-octanoyl modification on Ser^3, which acts as an endogenous ligand of the growth hormone (GH) secretagogue receptor (GHS-R) (1; and for review see refs. 2-7). Numerous lines of evidence indicate that ghrelin and its receptors are expressed in the human and rat adrenal cortex (8-13). Consistent findings show that ghrelin does not affect adrenal steroid-hormone secretion (8,10,11,13), but is able to enhance proliferative activity of cultured human and rat zona glomerulosa cells (10,13). Ghrelin was also found to decrease apoptotic deletion rate in cultured human adrenocortical cells (13), but to increase it in human aldosteronomas and adrenocortical carcinoma-derived cell lines NCI-H295 and SW-13 (14). In connection with the possible anti-tumor growth effect of ghrelin, we wish to recall that this peptide has been recently shown to exert a major antiangiogenic action in rats and humans (15,16).

Age-dependent changes in GH-RH and GH secretagogues have been demonstrated (17). Accordingly, it has been recently reported that the GH-releasing effect of ghrelin undergoes a gender-independent age-related decrease (18). Disappointingly, we were unable to observe appreciable age-related differences in human-adrenal level of ghrelin mRNA expression (12). However, these data were obtained in only eight patients (aged from 42 to 80 years) by conventional reverse transcription (RT)-polymerase chain reaction (PCR). Therefore, it seemed worthwhile to re-investigate the level of adrenal ghrelin mRNA expression in a larger number of subjects using semi-quantitative real-time PCR.

Materials and methods

Adrenal-gland specimens. Fragments of adrenal-gland tails, which contain only cortical tissue (19), were obtained from 27 adult patients (20 males and 7 females) aged from 33 to 82 years, undergoing unilateral adrenalectomy/nephrectomy for kidney cancer. Adrenal tails were immediately frozen at -30˚C by immersion in isopentane, and stored at -80˚C. Each patient gave written informed consent, and the study protocol was approved by the local Ethics Committee for Human Studies.

RT-PCR. Total RNA was extracted from frozen specimens, using an RNeasy Mini kit from Qiagen (Hilden, Germany). Briefly, after isopropanol precipitation, RNA was resuspended in RNase free water, then treated with RNase-free DNase to remove residual genomic DNA. RNA quantity and purity was determined as detailed earlier (20). Total RNA was then reverse transcribed to cDNA (21), and PCR was carried out in a Perkin-Elmer 480 DNA Thermal Cycler (Perkin-Elmer, Boston, MA), using the primers and PCR programs indicated in the legend of Fig. 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as the housekeeping gene. To rule out the possibility of amplifying genomic DNA, a PCR was carried out without prior RT of the RNA. Detection of PCR amplification products was performed by size fractionation on 1.5% agarose gel electrophoresis.

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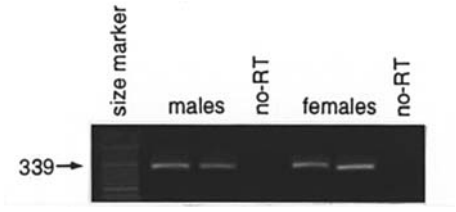

Figure 1. Ethidium bromide-stained 1.5% agarose gel showing cDNA amplified with human ghrelin specific primers from RNA of two exemplary human male and female adrenal cortexes. Primer sequences were: sense-58-5', 5'-AGC CTC CTG CTC CTC GGC AT-3'; and antisense-396-5', 5'-TGT GGG CGA TCA CTT GTC GGC T-3' (339 bp). PCR program was: 40 cycles at 95˚C for 30 sec, 62˚C for 30 sec and 72˚C for 30 sec; and additional extension step at 72˚C for 5 min was then performed. The first lane was loaded with Roche marker VIII (Roche, Mannheim, Germany). No amplification without previous RT of RNA is shown as a negative control.

Real-time PCR. Quantitative estimation of ghrelin mRNA in frozen specimens was carried out in a Bio-Rad I Cycler iQ Detection system (Bio-Rad Laboratories, Milan, Italy), as previously detailed (22,23). Briefly, reactions were carried out in 25 μl of final volume solution containing 800 nM specific primers (Fig. 1), 12.5 μl iQ SYBR-Green Supermix (Bio-Rad) and 2 μl of RT reaction solution. The PCR program was: denaturation step at 95˚C for 3 min, 35 cycles of two amplification steps at 95˚C for 15 sec and annealing at 60˚C for 30 sec, and melting curve at 60-90˚C with a heating rate of 0.5˚C/10 sec. During the exponential phase, the fluorescence signal threshold was calculated, and the fraction number of PCR cycles required to reach the threshold (Ct, cycle threshold) was determined. Ct values decreased linearly with increasing input target quantity, and were used to calculate relative mRNA expression (24). The specificity of amplification was tested at the end of each run by real-time PCR melting curve analysis, using the I-Cycler iQ software 3.0. All samples were amplified in duplicate, and GAPDH was used as a reference to normalize data.

Statistics. Data were averaged for male and female patients, and expressed as means ± SD. Their statistical comparison was done by the Student's t-test. The linear correlation between ghrelin mRNA expression and age was tested by the r coefficient of Pearson, and the regression lines were obtained by the least square method. A two-sided t-test was run to determine whether the slopes were significantly different in males and females.

Results

RT-PCR detected ghrelin mRNA expression in the adrenal cortexes of male and female patients (Fig. 1). A highly significant ($p<0.01$) negative linear correlation was observed between adrenal ghrelin mRNA and age. This occurred either when data were plotted without taking into account the sex (Fig. 2) or when data from male and female patients were disaggregated [r coefficient: males, -0.702 ($p<0.01$); females, -0.512 ($p<0.05$)] (data not shown). No significant differences between male and female slopes were observed ($p>0.1$). Likewise, relative ghrelin mRNA expression did not differ in male and female adrenals (Fig. 3).

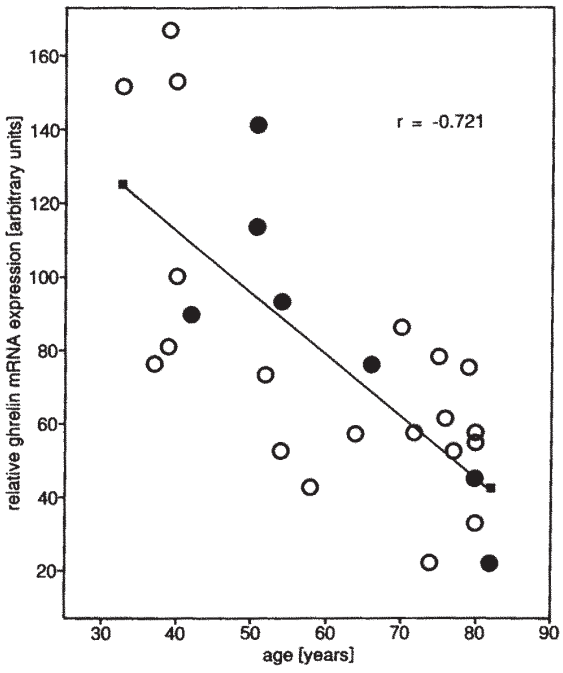

Figure 2. Negative linear correlation between adrenal ghrelin mRNA expression and age. Open circles, male patients; solid circles, female patients.

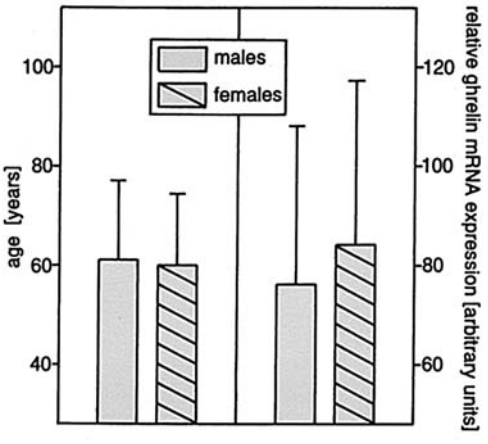

Figure 3. Lack of differences in the adrenal ghrelin mRNA expression between male and female patients. Bars are means ± SD.

Discussion

Evidence has been provided that GH response to the GH secretagogues, including ghrelin, decreases with aging (17,18). Despite the fact that GH exerts an evident growth-stimulating effect on adrenal cortex (for review see ref. 25), aging is normally associated with a rise in the weight and volume of adrenals, at least in the rat (26,27). This may be due to the aging-dependent impairment of adrenocortical secretion, which, by releasing hypothalamo-pituitary corticotrope complex from glucocorticoid negative feed-back, causes an increase in the level of circulating ACTH (for review see refs. 28-32). Although ACTH *in vivo* is known to exert a potent adrenal growth promoting action (for review see ref. 25), findings indicate that aging is associated with a net decrease

al DNA and RNA synthesis in rodent adrenocortical 35). This observations make it likely that during aging the proliferogenic effect of ACTH could be counteracted by other until-now unsettled mechanism(s).

Our present real-time PCR findings clearly show that adrenal ghrelin mRNA expression undergoes a clear age-related sex-independent decrease in humans. The bulk of findings reviewed in the Introduction raises the appealing possibility that ghrelin may act as an autocrine-paracrine stimulator of the growth of the normal rat and human adrenal cortex (10,13). Hence, the presently reported decrease in ghrelin expression could be considered one of the mechanism(s) by which basal proliferative activity of adrenal cortex decrease during aging (33-35). In contrast, since ghrelin does not affect steroid secretion from adrenocortical cells (8,10,11,13), the impairment in its expression cannot be associated with the age-related decrease in adrenal steroid-hormone production.

Further studies are underway to ascertain whether or not the aging-related down-regulation of ghrelin mRNA expression in the human adrenals is associated with changes in the expression of the GHS-R subtypes.

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