Ghrelin and its unacylated isoform stimulate the growth of adrenocortical tumor cells via an anti-apoptotic pathway

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Ghrelin and its unacylated isoform stimulate the growth of adrenocortical tumor cells via an anti-apoptotic pathway. Am J Physiol Endocrinol Metab 293: E302–E309, 2007. First published April 3, 2007; doi:10.1152/ajpendo.00377.2006.—Ghrelin is expressed in normal human adrenocortical cells and induces their proliferation through growth hormone secretagogue receptor 1a (GHS-R1a). Consequently, it was of interest to us to determine whether acylated ghrelin and its predominant serum isoform, unacylated ghrelin, also act as factors for adrenocortical carcinoma cell growth. To examine a potential ghrelin-regulated system in adrenocortical tumors, we measured proliferative effects of acylated and unacylated ghrelin in the adrenocortical carcinoma cell lines SW-13 and NCI-H295R. We also examined the expression of ghrelin, GHS-R1a, and corticotropin-releasing factor receptor 2 (CRF-R2). Acylated and unacylated ghrelin in the nanomolar range dose-dependently induced adrenocortical cell growth up to 200% of untreated controls, as measured by thymidine uptake and WST1 assay. The proliferative effects of acylated and unacylated ghrelin in SW-13 cells was blocked by [D-Lys3]growth hormone-releasing peptide 6 (GHRP6), but a CRF-R2 antagonist had no effect on unacylated ghrelin growth stimulation. Cell cycle analysis suggests that acylated and unacylated ghrelin suppress the sub-G1/apoptotic fraction by up to 50%. Measurement of DNA fragmentation and caspase-3 and -7 activity in SW-13 cells confirmed that acylated and unacylated ghrelin suppress apoptotic rate. SW-13 cells express preproghrelin mRNA and secretes ghrelin, and [D-Lys3]GHRP6 suppresses their basal proliferation rate, strongly suggesting that ghrelin could act as an auto/paracrine growth factor. Acylated and unacylated ghrelin are potential auto/paracrine factors acting through an antiapoptotic pathway to stimulate adrenocortical tumor cell growth. Unacylated ghrelin-stimulated growth is suppressed by an antagonist of GHS-R1a, suggesting either that unacylated ghrelin is acylated before its action or that ghrelin, unacylated ghrelin, and [D-Lys3]GHRP-6 bind to a novel receptor in these cells.

unacylated ghrelin

Ghrelin is an acylated 28-amino acid peptide hormone and is the ligand for the growth hormone secretagogue receptor 1a (GHS-R1a) (25, 38). The predominant form of ghrelin in serum is unacylated, occurring at a concentration of 150–200 pM in plasma, ~10 times the concentration of ghrelin (25). GHS-R1a signals via Goq11 to stimulate inositol 1,4,5-trisphosphate generation and Ca2+ release and has constitutive activity (18, 19). GHS-R1a gene expression is limited to the hypothalamus and pituitary and a few other peripheral tissues (15, 33, 37). In contrast, ghrelin gene expression is widespread among tissues in humans, including the adrenal cortex (15, 35). Acylated ghrelin stimulates the release of growth hormone (GH), as well as prolactin and ACTH, from the anterior pituitary gland. However, much recent evidence suggests numerous other functions for ghrelin at the periphery, including effects on cell proliferation (1, 3, 20, 22, 23, 32). Interestingly, there is also evidence suggesting that unacylated ghrelin can modulate cellular function and body growth. For example, transgenic mice that overexpress unacylated ghrelin are smaller than their wild-type counterparts (2), direct treatment with unacylated ghrelin alters adipogenesis in the bone marrow of rats (34), and in vitro unacylated ghrelin inhibits apoptosis of human cardiomyocytes and endothelial cells (3). We also have demonstrated effects of unacylated ghrelin in primary hepatocytes (14) and fetal osteoblasts (13) in vitro. Because unacylated ghrelin cannot activate the GHS-R1a at physiological concentrations, it has been suggested that these effects are mediated by an alternative receptor. This possibility has recently been substantiated in vivo, whereby centrally administered unacylated ghrelin was shown to stimulate feeding in GHS-R1a gene-deleted mice (36).

Recent work has demonstrated the expression of preproghrelin mRNA in the human adrenal cortex (9, 35). In addition, transcripts for ghrelin’s cognate receptor, GHS-R1a, have been identified in human adrenocortical tissues (8, 30). A functional result of this coexpression was found to be the proliferative effect of acylated ghrelin on adrenocortical cell growth, as shown in primary cell cultures (30). The effects of exogenous acylated ghrelin were blocked by the GHS-R1a antagonist [D-Lys3]growth hormone-releasing peptide 6 (GHRP6), suggesting the involvement of GHS-R1a in the proliferative effect.

Adrenocortical carcinoma is a relatively rare disease in the human population (24). However, since the majority of tumors do not cause clinical manifestations (so-called nonfunctional tumors), with only a small proportion causing endocrine disorders, adrenocortical carcinomas are usually diagnosed late, by which time they have already metastasized (11, 27). Thus the prognosis for patients with this type of adrenocortical tumor is very poor, with a mean survival rate of 20% at 5 yr (29). Surgical and chemotherapeutic approaches have proved relatively ineffective treatments, and the identification of new means of treatment seems necessary (24). The development of adrenocortical carcinomas probably involves autocrine overexpression of particular growth factors, such as insulin-like growth factor II (IGF-II) (11). However, the biology of these
tumors is not completely characterized, since they are heterogeneous and may not all express high levels of these factors. The findings that the adrenal cortex expresses ghrelin and its cognate receptor and that ghrelin appears to be a growth factor for other cell types suggested to us that acylated and unacylated ghrelin may be relevant growth factors for adrenocortical tumor cells.

The aims of this study were to determine whether acylated and unacylated ghrelin could be proliferative auto/paracrine factors for adrenocortical tumor cell growth and, if so, to determine whether receptors relevant to this growth stimulatory effect are expressed in these cells. The model we have chosen is the SW-13 cell line, since it is so-called nonfunctional (derived from the most poorly diagnosed form of adrenocortical tumor). Moreover, under basal conditions, SW-13 cells, unlike the other commonly utilized adrenocortical carcinoma cell line, NCI-H295R (28), express relatively low levels of the potent growth factor IGF-II, meaning that the potential growth effects of acylated and unacylated ghrelin can be distinguished.

MATERIALS AND METHODS

Culture of cell lines. The SW-13 (26) cell line was obtained from the European Collection of Cell Cultures, and the NCI-H295R cell line was obtained from the American Type Culture Collection. The vimentin +/- subclones of SW-13 were kindly provided by Dr. L. Borradori (University Hospital of Geneva, Geneva, Switzerland). All cell lines were maintained in DMEM-F-12 (Invitrogen, Breda, The Netherlands) supplemented with 5% fetal calf serum (FCS), 10 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2-95% O2.

Cell growth experiments. Cells were seeded in a volume of 100 μl at a density of 2,000 cells/well in 96-well plates and allowed to attach overnight, and then their medium was replaced with media containing the various treatments described in RESULTS. The peptides used were as follows: acylated ghrelin and [o-Lys3]GHRP-6 were obtained from NeoMPS (Strasbourg, France), unacylated-ghrelin was synthesized by Thera Technologies (Montreal, QC, Canada), and astressin2B was synthesized by NeoMPS (Strasbourg, France). Glutamate was added to each well, and the cells were incubated for some experiments were terminated at the basis of this analysis, we used an assay time of 6 days, although the cellular DNA content was measured using the fluorescent dye Hoechst 33258 (Boehringer Diagnostics, La Jolla, CA).

To assess changes in DNA content, we seeded cells in 24-well plates at a density of 10,000 cells/ml in 96-well plates and allowed to attach overnight, and then their medium was replaced with media containing acylated or unacylated ghrelin (quadruplicate wells). Three days later, the cellular DNA content was measured using the fluorescent dye Hoechst 33258 (Boehringer Diagnostics, La Jolla, CA).

To examine whether the SW-13 cells secrete ghrelin, we measured its release into serum-free medium in vitro. Briefly, 3.5 × 105 SW-13 cells were seeded into the wells of six-well plates. After 48 h, the cells were washed twice with 0.9% saline and once with serum-free medium. The cells were then incubated for 24 h in 1 ml of medium containing 0.1% bovine serum albumin (BSA fraction V; Roche). The medium was then collected, and 50 μl were assayed for acylated ghrelin by radioimmunoassay (active ghrelin kit; Linco Research, St. Charles, MO) following the manufacturer’s protocol. Nonglucocorticoid medium (50 μl) containing 0.1% BSA was assayed as a control.

Cell cycle analysis. Cells (4 × 105) were plated in six-well plates (Corning Costar, Amsterdam, The Netherlands). After 10 h of incubation, medium was changed with medium containing (control group) or incubated with 10 nM acylated or unacylated ghrelin. Treatments were performed in duplicate in two independent experiments. After 24 h, cells were harvested by gentle trypsinization and prepared for cell cycle determination, as previously described (39), using propidium iodide for DNA staining. The stained cells were analyzed by FACScalibur flow cytometer (Becton Dickinson, Erembodegem, Belgium) and CellQuest Pro Software (Becton Dickinson; Macintosh version). Cell cycle progression was measured with corresponding absorbances for G0/G1, S, and G2/M phases, whereas apoptosis was assessed by quantifying the sub-G0 peak.

Measurement of apoptosis. We first substantiated the effects of acylated and unacylated ghrelin on apoptosis by measuring DNA fragmentation. Cells were seeded at 1 × 105 cells/well in 24-well plates. Three days later, the cell culture medium was replaced with 1 ml/well of medium containing acylated or unacylated ghrelin at 10 nM. After 24 h, apoptosis was assessed using a commercially available ELISA kit (Cell Death Detection ELISAPlus; Roche Diagnostic, Penzberg, Germany) following the manufacturer’s protocol. Data are from three independent experiments (n = 4), expressed as percentages of untreated control.

To assess caspase activity, we measured the ability of lysates from treated SW-13 cells to cleave a luminogenic substrate (containing the tetrapeptide DEVD) that is specific for the effector caspases-3 and -7 by using the Caspase Glo 3/7 kit (Bio-Rad, Madison, WI). To do this,
we seeded white-walled 96-well plates with 1 x 10^4 cells/well. After 24 h, their growth medium was replaced with 100 µl of medium containing either 10 or 100 nM acylated or unacylated ghrelin. After 24 h of treatment, 100 µl of reconstituted Caspase Glo 3/7 reagent was added to each well, and the plate was mixed for 30 s on a plate shaker. Thirty minutes later, luminescence was measured using a Wallac Victor2 multiplate reader. Data are reported from two independent experiments (n = 4), expressed as percentages of untreated control.

Data analysis. All data were analyzed using ANOVA followed by Fisher’s protected least significant difference post hoc test (Statview, v.5, Macintosh).

RESULTS

Acylated and unacylated ghrelin induce cell growth in adrenocortical tumor cells. Ghrelin caused a dose-dependent increase in thymidine uptake with a maximum of 200% of controls at 10 nM following 3 days of treatment (Fig. 1A). This effect was essentially dose dependent. Similarly, unacylated ghrelin dose-dependently increased thymidine uptake by this cell line, reaching 175% of control values with 100 nM peptide (Fig. 1B). Acylated and unacylated ghrelin also significantly increased cellular DNA content by day 3 to 125% (P < 0.001) and 115% (P < 0.01) of controls, respectively (Fig. 1C).

We also tested another indicator of cell proliferation, WST1. This reagent showed linearity of response over the range of cell densities that we observed in the wells of 96-well plates (data not shown). We used this assay to confirm the thymidine uptake data for acylated and unacylated ghrelin dose responsiveness of the SW-13 cells at 6 days of culture. Over several experiments (3–5 independent experiments, 5 replicates per experiment), acylated and unacylated ghrelin caused a growth effect reaching ~150% of control levels at 10 nM of each peptide (Fig. 2, A and B).

Under the conditions used to culture the adrenocortical cells, we found that ~90% of the added ghrelin was lost from the medium within 24 h, as measured by radioimmunoassay. Since ghrelin can be rapidly deacylated and proteolyzed in cell culture conditions, we tested a stabilized form, [Dap-octanoyl]ghrelin (5), and hexarelin to determine whether the cells responded in a manner similar to other GH secretagogues. [Dap-octanoyl]ghrelin and hexarelin had potent effects on SW-13 cell growth, again peaking at ~200% of controls at 10 nM of each peptide (Fig. 2, C and D). The growth effect of these peptides was also dose dependent.

SW-13 cells in regular culture consist of two subtypes (16), vimentin positive (vim+) and vimentin negative (vim−). Since a previous report suggested a different growth response to ghrelin in SW-13 cells (4), we examined whether these cell subtypes responded differentially to acylated and unacylated ghrelin, perhaps explaining the conflicting results. However, we found that these sublines both showed increased proliferation in response to acylated and unacylated ghrelin and that there was no significant differential response by either subtype to acylated and unacylated ghrelin (Fig. 3, A and B). Furthermore, their response was relatively blunted compared with our standard SW-13 cell-line. Again, in concordance with our findings in SW-13 cells, another human adrenocortical cell line, NCI-H295R, also showed a proliferative response to acylated and unacylated ghrelin (Fig. 3C), but this was again blunted and apparently biphasic, with a decreased response to 100 nM peptide and a maximal response at about 1 nM.

NCI-H295R cells express IGFI receptor (IGF-IR) and IGF-II mRNAs; therefore, we examined whether ghrelin or unacylated ghrelin might modulate IGF-IR and IGF-II gene expression in a way that could explain the increase in proliferation. However, after 24 h of treatment with 10 nM peptide, we were unable to detect any effect of ghrelin or unacylated ghrelin on the level of expression of these genes (data not shown).

GHS-R1a and CRF-R2 gene expression. Since GHS-R1a mRNA has been reported to be expressed in adrenocortical cells, we next examined the expression of this gene in SW-13 cells. GHS-R1a was not readily detected by TaqMan assay using the primer/probe combination described by Gnanapavan et al. (15); therefore, we used a locked nucleic acid (LNA)
probe from Exiqon (13) and intron spanning primers and obtained variable expression levels, with the cycle numbers at which amplification became detectable (Ct) at or above 33. However, reactions lacking reverse transcriptase did not generate a signal, so it was considered possible that the high Ct values represent real expression levels. Therefore, we ran acylated and unacylated ghrelin dose-response experiments in SW-13 cells. However, the variability of the extremely low GHS-R1a gene expression made it impossible to detect any effect (data not shown). Qualitative PCR assays using alternative intron-spanning primers (23) confirmed the sporadic nature of the quantitative RT-PCR results (Fig. 4A), expression of a poorly defined amplicon was detectable only in SW-13 sample 1a), and suggest that GHS-R1a mRNA is not expressed in these cells at physiologically meaningful levels.

Qualitative RT-PCR also was used to examine whether SW-13 cells express the CRF-R2 mRNA. Using our intron-spanning primers, we found detectable expression of the mRNA for CRF-R2 in SW-13 cells, as well as in HEK-293, a human embryonic kidney cell line (Fig. 4A).

Antagonism of GHS-R1a and CRF-R2. We next examined the effects of well-characterized antagonists of GHS-R1a and CRF-R2, [d-Lys³]GHRP-6 and astressin2B, respectively. Using [d-Lys³]GHRP6, we attempted to block the growth response of the cells to acylated and unacylated ghrelin. In this separate set of experiments, 10 nM acylated and unacylated ghrelin again stimulated SW-13 growth measured using WST1 (Fig. 4B). We found that at 100 µM, [d-Lys³]GHRP6 completely abrogated the growth response to both of these peptides and suppressed the growth of untreated cells. However, we found that the selective CRF-R2 antagonist astressin2B, at 2 µM (IC₅₀ = 1.3 nM), had no significant effect on unacylated ghrelin-stimulated proliferation of SW-13 cells (Fig. 4C).

Ghrelin expression. Preproghrelin mRNA was readily detectable using our TaqMan-based real-time RT-PCR assay. Preproghrelin gene expression was found to be significantly downregulated by ghrelin treatment to ~50% of control levels at 10 and 100 nM (P < 0.05 and 0.01, respectively; Fig. 5A). In contrast, unacylated ghrelin had an inductive effect, almost doubling expression relative to controls at 100 nM (P < 0.01) and having a significant effect down to a concentration of 1 nM (Fig. 5B). These results could not be explained by regulation of HPRT, the housekeeping gene we used to normalize the ghrelin data.

Since SW-13 cells express preproghrelin mRNA, we also examined whether they secreted ghrelin. RIA of conditioned
medium with the use of the Linco active ghrelin kit (limit of detection, 2.5 pM) confirmed that these cells secreted ghrelin, which reached a concentration in the medium of 6.8 pM at 24 h. Medium that had not been conditioned by cells contained no detectable ghrelin.

Effects of acylated and unacylated ghrelin on cell cycle and apoptosis. The effects of acylated and unacylated ghrelin on cell proliferation would be expected to be linked to modulation of some aspect of the cell cycle. Cell cycle analysis using flow cytometry (4 experiments with 20,000 cells per run) demonstrated that although neither ghrelin nor unacylated ghrelin (10 nM) significantly altered the proportion of cells in the G0/G1, S, or G2/M phases, they caused a consistent decrease in the number of cells in sub-G0, indicative of cells undergoing apoptosis (Fig. 6A).

Apoptotic deletion rate can have an important influence on the overall growth rate of cells in culture. To confirm our cell cycle analysis data, we used a cell death ELISA as a direct measure of apoptosis in cells treated with the peptides. Substantiating our cell cycle analyses, we found that acylated and unacylated ghrelin (10 nM) significantly suppressed the level of apoptosis in SW-13 cells by 15 and 20%, respectively, in three independent experiments (Fig. 6B).

To further substantiate, and provide a more mechanistic explanation for, our cell cycle analysis and cell death ELISA results, we examined the effects of acylated and unacylated ghrelin on effector caspase activity in SW-13 cells. We found that the rate of cleavage of a luminogenic substrate specific for the effector caspases-3 and -7 containing the tetrapeptide DEVD was suppressed following treatment of the SW-13 cells with 10 and 100 nM acylated and unacylated ghrelin (Fig. 6C). At 10 nM, unacylated ghrelin suppressed caspase-3/7 activity by 20%, whereas 10 nM acylated ghrelin suppressed caspase activity by 10%. This confirms the cell cycle analysis and cell death ELISA data, which indicate that unacylated ghrelin is more potent at blocking apoptosis of SW-13 cells than acylated ghrelin.

Fig. 5. AG and UAG differentially regulate ghrelin gene expression. A: AG dose-dependently suppressed ghrelin gene expression, reducing mRNA to 50% of control levels at 10–100 nM. B: UAG dose-dependently induced ghrelin gene expression, reaching 200% of control levels at 10–100 nM. Data are expressed as the ΔΔCt relative to hypoxanthine phosphoribosyltransferase expression (see MATERIALS AND METHODS) and are derived from 4 replicate samples in duplicate. *P < 0.05; **P < 0.01.
DISCUSSION

SW-13 cells are derived from a small-cell, nonfunctional (nonsteroidogenic) adrenocortical tumor (26). Unlike the other well-characterized adrenocortical cell line, NCI-H295R, they express relatively little IGF-II, making them a tractable model for examining growth effects. As for primary adrenocortical cells (1), we found that SW-13 cell proliferation is increased when they are treated with ghrelin in the nanomolar range. However, this carcinoma cell line may be more responsive to ghrelin and unacylated ghrelin in SW-13 cells and carcinomas, which appear to express low levels of this gene (4). However, SW-13 cells were still capable of responding to ghrelin. To address this issue, we attempted to block the growth response to ghrelin using the GHS-R1a antagonist [D-Lys³]GHRP6. We found that this antagonist blocked ghrelin-induced proliferation of SW-13 cells, which is consistent with a GHS-R1a-mediated response. However, [D-Lys³]GHRP6 also blocked the growth response to unacylated ghrelin. This could be explained by acylation, or activation, of unacylated ghrelin before its activity in stimulating growth. Currently, there is no evidence either way that unacylated ghrelin can be modified in this way outside the cell. Another possible explanation is that there is an alternative receptor that mediates the response to ghrelin and unacylated ghrelin in SW-13 cells and whose activity is antagonized by binding [D-Lys³]GHRP6.

Acylated ghrelin is inactivated in culture medium over time, and this process is probably dependent on cell-derived proteases (21) or the removal of the octanoyl side chain by esterases (12). A study described by Muccioli et al. (31) using adipocytes suggests a very rapid removal of ghrelin from cell culture medium, with levels declining to 1% of starting values within 3 h of incubation. However, our finding that the stabilized ghrelin analog [Dap-octanoyl³]ghrelin had similar, if not more potent, effects on cell growth than native ghrelin suggests that the induction of proliferation that we have observed cannot be attributed to acylated ghrelin.

Intriguingly, acylated and unacylated ghrelin regulate preproghrelin gene expression, but with opposing effects. Acy-
lateral ghrelin downregulates its own transcription, perhaps via a classic negative feedback loop. Potentially, this would suppress both acylated and unacylated ghrelin expression. On the other hand, we found that unacylated ghrelin stimulated ghrelin gene expression. This suggests that failure of the cells to acylate ghrelin could lead to overexpression of the ghrelin gene, which in turn might contribute to tumorigenesis and/or tumor-like growth rates in these cells. Thus inactivation of the mechanism for acylation of ghrelin and/or an increase in the rate of deacylation could be an important tumorigenic signal(s). Our finding that SW-13 cells express the proghrelin gene complements similar findings in the adrenal cortex (1). Importantly, we also found that these cells secrete ghrelin, and together with the suppressive effects of a GHSR antagonist on basal proliferative rate, this strongly suggests an auto/paracrine role for this peptide.

Both acylated and unacylated ghrelin increase SW-13 cell growth through reduction of apoptotic rate, at least partly by suppressing effector caspase-3/7 activity. The growth of adrenocortical cells is modulated by a potent antiapoptotic hormone, IGF-II, and it was possible that acylated and unacylated ghrelin could modulate the expression and/or function of this growth factor. Therefore, we examined whether the gene expression of the IGF-1R and/or IGF-II were regulated by these peptides in SW-13 and NCI-H295R cells. SW-13 cells express low, whereas H295 cells express high, levels of the IGF-II gene, and both express IGF-1R mRNA. Nevertheless, we were unable to demonstrate any regulation of these genes by ghrelin or unacylated ghrelin (data not shown), suggesting that their effects are not mediated by the auto/paracrine IGF system in these cells.

The mechanism for unacylated ghrelin signal transduction is not currently understood. Unacylated ghrelin is known not to activate the GHS-R1a at physiological concentrations (5). Recent evidence from an in vivo model of unacylated ghrelin action suggests the involvement of the CRF-R2. In a recent report, Chen et al. (10) showed that specific antagonism of central CRF-R2 can block at least some of the effects of unacylated ghrelin, such as decreased food intake and the regulation of motor activity in the gastric antrum. Since this receptor may also mediate the proliferative response to unacylated ghrelin, we examined its gene expression in SW-13 cells and found that it was present. However, the effects of unacylated ghrelin on cell growth could not be blocked with the potent selective antagonist astressinB, suggesting that CRF-R2 does not mediate unacylated ghrelin induced proliferation in these cells. Recently, additional compelling evidence for an alternative receptor has been demonstrated in GHS-R1a knockout mice (36). The feeding response of these mice is augmented by central administration of unacylated ghrelin, indicating the presence of an alternative receptor for this peptide hormone, at least centrally. However, currently there is no direct proof of a specific receptor for unacylated ghrelin in these cells.

In conclusion, we have shown a growth stimulatory effect of both acylated and unacylated ghrelin on the adrenocortical carcinoma cell line SW-13. Our findings fit well with observations on the proliferative effects of ghrelin on primary adrenocortical cells (30, 35) and the local expression of ghrelin in the majority of adrenocortical tumors (4), although contrasting with two other reports (4, 6). The expression of preproghrelin mRNA and ghrelin protein by SW-13 cells suggests that ghrelin, and perhaps also unacylated ghrelin, may act as auto/paracrine factors in adrenocortical tumor growth, perhaps even tumorigenesis, and this is substantiated by our finding that [α-Lys3]GHRP6 antagonizes not only ghrelin-stimulated but also basal cell growth. The proliferative response to unacylated ghrelin suggests at least one new receptor-mediated signaling pathway in these cells. The absence of consistently expressed GHS-R1a and the ability of [α-Lys3]GHRP6 to block both acylated and unacylated ghrelin effects suggest that this receptor could bind all three peptide ligands. Finally, the finding that astressinB does not block the unacylated ghrelin effect suggests that its mechanism of action in these cells does not involve the CRF-R2. Further work is required to determine the significance of acylated and unacylated ghrelin as growth factors and potential tumorigenic agents in adrenocortical cancer.

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REFERENCES


