Ghrelin and obestatin inhibit enucleation-induced adrenocortical proliferation in the rat

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Abstract. Studies involving the role of ghrelin (GHREL) in regulating the proliferative activity of various cell types have obtained variable results depending primarily on the experimental model applied. It was recently reported that neither GHREL nor obestatin (OBS) affected the proliferative activity of cultured rat adrenocortical cells. In view of the conflicting results, we investigated the effects of GHREL and OBS on the proliferative activity of rat adrenocortical cells in a model of bilateral enucleation-induced adrenocortical regeneration in the rat. Rats were sacrificed 5 or 8 days after surgery. Twenty-four hours before being sacrificed, the appropriate groups were infused with 3 nmol GHREL or OBS/100 g. The mitotic index was assessed using the stachmokinetic method with vincristine. In comparison with intact rats, expression levels of ppGHREL, BAX, JUN-B and JUN-C genes were notably higher in regenerating adrenals, and neither GHREL nor OBS infusion affected these levels. Expression levels of the GHS-R, GPR39v2 and FOS genes were affected neither by adrenal enucleation nor GHREL or OBS infusion. Expression of only two studied genes, GPR39v1 and EGR1, was regulated by OBS. In the regenerating adrenal glands, GPR39v1 and EGR1 mRNA levels were higher than the levels in intact animals. GHREL infusion had no effect while OBS infusion notably stimulated GPR39v1 mRNA levels in the regenerating adrenal gland and evoked an opposite effect on EGR1 mRNA. OBS administration resulted in a potent decrease in the mitotic index of the studied cells, an effect found at both days 5 and 8 of the experiment. GHREL exerted a similar effect only at day 5 of adrenocortical regeneration. Neither GHREL nor OBS had an effect on blood aldosterone concentrations. GHREL infusion lowered plasma corticosterone concentration at day 5 but not 8 of the experiment, while OBS administration was ineffective. Thus, this study is the first to demonstrate that, *in vivo,* both GHREL and OBS inhibit the growth of the regenerating adrenal cortex. Moreover, the data suggest that the effect of OBS might be, at least in part, mediated by the EGR1 pathway known to be critical in cell proliferation.

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Introduction

Intracellular processing of prepro-ghrelin (ppGHREL) generates two biologically active peptides: the well-studied ghrelin (GHREL) and the obestatin (OBS), also named ghrelin-associated peptide (GAP). The potent GH-secretagogue and orexigenic effects of GHREL are well recognized, while the role of OBS is still unclear (reviewed in refs. 1-8).

GHREL is an endogenous ligand of the growth hormone secretagogue receptor which in human has two variants: GHS-R1a and GHS-R1b (9). OBS was initially reported to be the endogenous ligand of GPR39 (6); however, recent data do not support this suggestion.

The prepro-ghrelin gene as well as ppGHREL-derived peptide receptor genes are widely expressed in the body suggesting that ppGHREL-originating peptides may regulate target cells (1,3,7,8). This system is also involved in regulating the hypothalamo-pituitary-adrenal (HPA) axis (1,10-12). In this regard, the stimulating (either the direct or indirect) effect of GHREL on CRH, ACTH and glucocorticoid hormone secretion is well documented, while the role of OBS is still disputable. Only limited data indicate that neither intravenous nor intracerebroventricular administration of OBS affects the secretion of ACTH and corticosterone in rats (13,14).

The role of GHREL in regulating the proliferative activity of various cell types is frequently discussed in the literature. The reported studies have obtained variable results depending primarily on the experimental model applied. In cultured human and rat zona glomerulosa cells, GHREL, via MAPK-mediated pathway(s), was found to increase the proliferative activity and decrease the apoptotic deletion rate of adrenocortical cells (15-17). On the other hand, either stimulating or antiproliferative effects of GHREL were found in the human NCI-H295 and SW13 cell lines (18,19). Recently, we found that neither GHREL nor OBS affected the proliferative activity of 4-day cultured rat adrenocortical cells (12). In view of these conflicting results, we investigated the effects of GHREL and OBS on the proliferative activity

of rat adrenocortical cells *in vivo*. Since the ppGHREL gene is highly expressed during intrauterine development, we applied an experimental model in which cells resemble embryonic ones (20). Importantly, in a model of enucleation-induced adrenocortical regeneration, we were able to demonstrate the potent inhibitory effect of GHREL and OBS infusion on the proliferative activity of the studied cells.

Materials and methods

Reagents. Ghrelin (rat) and obestatin (rat) were purchased from Bachem AG (Bubendorf, Switzerland). If not otherwise stated, all other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) or from POCh (Gliwice, Poland).

Animals. Male Wistar rats from the Laboratory Animal Breeding Center, Department of Toxicology, Poznan University of Medical Sciences were used. Their age and weight are mentioned in appropriate sections of the text. Animals were maintained under standardized conditions of light (14/10-h light/dark cycle, illumination onset 06.00 a.m.) at 23˚C with free access to standard pellets and tap water.

Experimental design. The Local Ethics Committee for Animal Studies approved the study protocol. In rats weighing ~140 g, under ether anaesthesia and via dorsal approach, both adrenal glands were enucleated according to the standard method (21). Rats were sacrificed by decapitation 5 or 8 days after surgery, and their adrenals were promptly removed. Twenty-four hours before sacrifice, the appropriate rat groups were infused (ALZET mini-osmotic pump, model 2001, Durect Corp., Cupertino, CA, USA) with 3 nmol GHREL/100 g, 3 nmol OBS/100 g or with physiologic saline solution (control groups). Three hours earlier rats subjected to metaphase assay (n=6 per group) were administered an intraperitoneal injection of 0.1 mg/100 g body weight of vincristine (Gedeon-Richter, Budapest, Hungary). Animals were decapitated at 11.00 a.m., and their trunk blood was collected in the presence of 1 mg/ml EDTA. Plasma was separated and stored at -36˚C for the hormone assay. Regenerating adrenals were processed for light microscopy for the metaphase-index assay. Furthermore, gene expression studies (QPCR) were performed on groups of enucleated rats not injected with vincristine.

QPCR. The applied methods were previously described (12,22-27). After decapitation, adrenals were promptly removed, freed of adherent fat and processed for the studies. From fragments of the glands, total RNA was extracted using Tri reagent (Sigma) and purified on columns (RNeasy Mini Kit, Qiagen) (12,24,26,28). mRNA was extracted from the total RNA using the PolyATract® mRNA Isolation System III (Promega, Madison, WI). The amount of total mRNA was determined by measuring optical density at 260 nm, and the purity was estimated using a 260/280-nm absorption ratio, which was consistently higher than 1.8 (NanoDrop spectrophotometer, Thermo Scientific). RT was performed using AMV reverse transcriptase (Promega) with Oligo dT (PE Biosystems, Warrington, UK) as primers. The reaction was performed at 42˚C for 60 min (Thermocycler Uno II, Biometra). The primers used were designed by Primer 3 software (Whitehead Institute for Biomedical Research, Cambridge, MA), and the reaction amplified fragments spanning introns (Table I). The primers were purchased from the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw.

QPCR was performed by means of the Lightcycler 2.0 instrument (Roche) with software version 4.05. The SYBR Green detection system was used with the above-mentioned primers. Every 20-μl reaction mixture contained 4 μl template cDNA (or standards, or control), 0.5 μM each gene-specific primer and a previously determined optimal $MgCl_2$ concentration (3.5 μM for one reaction). LightCycler FastStart DNA Master SYBR Green I mix (Roche) was used. The real-time PCR program included a 10-min denaturation step to activate the Taq DNA polymerase followed by a three-step amplification program: denaturation at 95˚C for 10 sec, annealing at 56˚C for 5 sec and extension at 72˚C for 10 sec. Specificity of the reaction products was checked by determination of the melting points (0.1˚C/sec transition rate).

PCR efficiency was assessed by a serial dilution method. Products of the traditional RT-PCR reaction were separated in 2% agarose gel, and specific bands were extracted by the DNA gel extraction kit (Millipore). The amount of extracted DNA was estimated spectrophotometrically. The extracted DNA was diluted (10-fold serial dilutions) in order to obtain the standard curve for the efficiency calculation. The applied version of LightCycler software (4.05) allowed for the evaluation of the amplification efficiency plots.

Metaphase index. Adrenals were fixed in Bouin's solution for 24 h and embedded in paraffin. Sections (6-μm) were stained with haematoxylin and eosin (H&E), and the metaphase index (number of vincristine-arrested metaphase cells per 1,000 cells) was calculated at a magnification of x400 by counting 5,000 cells in the outer cortex of each regenerating adrenal parenchyma (29).

Hormone assay. Aldosterone and corticosterone were extracted from plasma and measured by RIA as previously described (12,24,30-34). Aldosterone RIA: sensitivity, 5 pg/ml; intra- and interassay CVs, 5 and 7%, respectively. Corticosterone RIA: sensitivity, 50 pg/ml; intra- and interassay CVs, 7 and 9%, respectively.

Statistics. Data were expressed as means ± SEM, and the statistical significance of the differences between experimental groups was estimated using the Students' t-test.

Results

By means of QPCR, we studied the gene expression of ppGHREL (prepro-GHREL), GHS-R (growth hormone secretagogue receptor), GPR39v1 and GPR39v2 (orphan G protein-coupled receptor 39, variants 1 and 2) in the adrenals of rats at days 2, 28, 45 and 90 of postnatal development. As demonstrated in Fig. 1, the highest expression of all of the studied genes was found at day 2 and was followed by a

Table I. Conventional RT-PCR and QPCR analyses of the gene expression of prepro-ghrelin (ppGHREL), growth hormone secretagogue receptor (GHS-R), orphan G protein-coupled receptor 39, variants 1 and 2 (GPR39v1 and GPR39v2), early growth response 1 (EGR1), Bcl-2-associated_X_protein (BAX), oncogene FOS (FOS), proto-oncogene b-jun (JUN-B) and proto-oncogene c-jun (JUN-C).

cDNA	Genbank accession no.	Primer	Primer sequence (5'-3')	Position	PCR product size (bp)
ppGHREL	NM_021669	S	CCAGCAGAGAAAGGAATCCAAG	135-156	142
		A	AACATCGAAGGGAGCATTGAAC	255-276	
GHS-R	NM_032075	S	AGATGCTTGCTGTGGTGGTG	782-801	182
		A	GTACAGAATGGGGTTGATGG	944-963	
GPR39v1	NM_001114392	S	GAAGAGCAAGCGGGGTACT	950-968	127
		A	CAGTGTCACCACGATCAGTCTC	1055-1076	
GPR39v2	NM_001100943	S	CTGTGGCTTTCATGTGTTGG	710-729	160
		A	GACCCAGACTCACTCAGGA	850-869	
EGR1	NM_012551	S	GAGCCGAGCGAACAACCCTA	187-296	82
		A	CCACCAGCGCCTTCTCGTTA	249-268	
BAX	NM_017059	S	TGCAGAGGATGATTGCTGAC	227-246	227
		A	GGAGGAAGTCCAGTGTCCAG	414-433	
FOS	NM_022197	S	TTTCAACGCGGACTACGAG	167-185	164
		A	AGTTGGCACTAGAGACGGACA	310-330	
JUN-B	NM_021836	S	AATGGAACAGCCTTTCTATCAC	282-303	99
		A	GGTTTCAGGAGTTTGTAGTCG	360-380	
JUN-C	NM_021835	S	GCCACCGAGACCGTAAAGA	318-336	61
		A	CCTGTGCGAGCTGGTATGAGTA	357-378	
HPRT	NM_012583	S	CAGTCAACGGGGGACATAAAAG	391-412	146
		A	ATTTTGGGGCTGTACTGCTTGA	515-536	

Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as the reference gene. Oligonucleotide sequences for sense (S) and antisense (A) primers are shown.

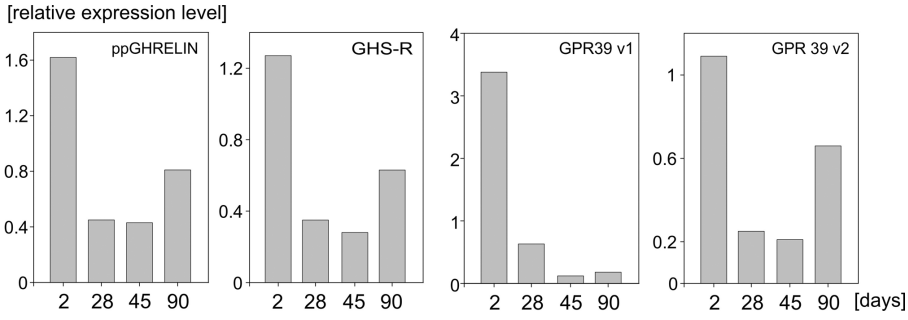

Figure 1. QPCR analyses of the gene expression of prepro-ghrelin (ppGHREL), growth hormone secretagogue receptor (GHS-R1), orphan G protein-coupled receptor 39, variants 1 and 2 (GPR39v1 and GPR39v2) in the adrenals of male rats at days 2, 28, 45 and 90 of postnatal development. Bars indicate the relative expression of the studied genes in relation to expression of the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene; means from 3 independent analyses are shown.

notable drop in subsequent days. However, in 90-day-old rats, the expression levels of the ppGHREL, GHS-R1a and GPR39v2 genes increased notably, achieving values ~50% of those noted at day 2. Therefore, the subsequent studies were performed on rats older than 90 days.

In the next step, in the regenerating adrenal glands of rats infused with GHREL, OBS or physiological saline, we tested expression of the above-mentioned genes, as well as genes involved in differentiation and mitogenesis (Fig. 2). In comparison with intact rats, the expression levels of ppGHREL, BAX, JUN-B and JUN-C genes were notably higher in the regenerating adrenals, and neither GHREL nor OBS infusion affected these levels. Expression levels of GHS-R, GPR39v2 and FOS gene, on the other hand, were affected neither by adrenal enucleation nor GHREL or OBS infusion. Expression of only two studied genes, GPR39v1 and EGR1, was

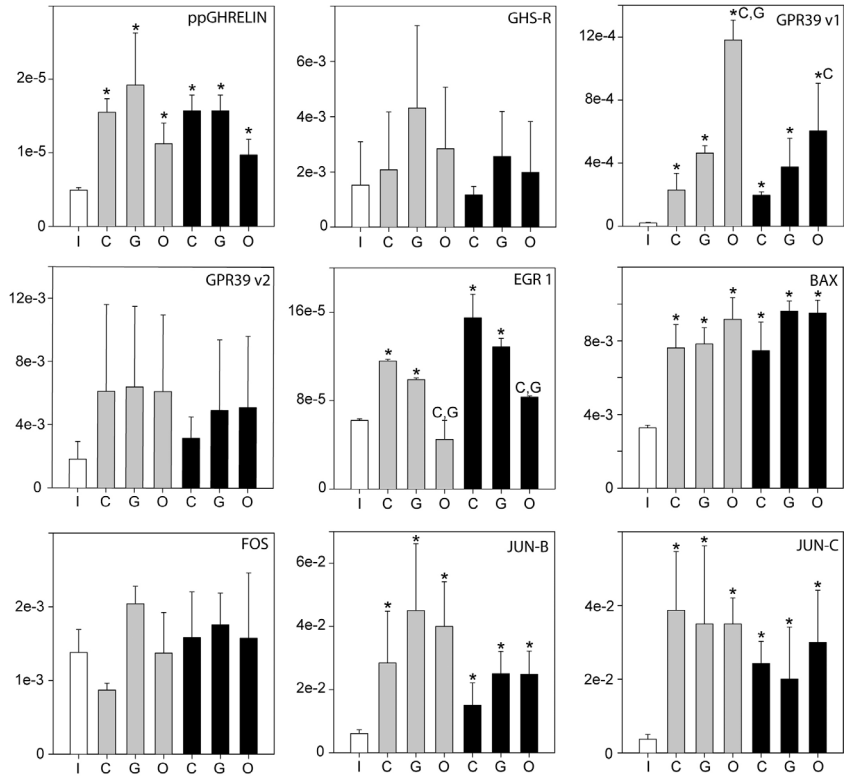

Figure 2. Effects of ghrelin or obestatin infusion (3 nmol/100 g/24 h) on expression of different genes in the enucleation-induced regenerating rat adrenal glands at days 5 and 8 after surgery. By means of QPCR, the expression levels of the following genes were determined: prepro-ghrelin (ppGHREL), growth hormone secretagogue receptor (GHS-R1a), orphan G protein-coupled receptor 39, variants 1 and 2 (GPR39v1 and GPR39v2), early growth response 1 (EGR1), Bcl-2-associated_X_protein (BAX), oncogene FOS (FOS), proto-oncogene b-jun (JUN-B) and proto-oncogene c-jun (JUN-C). Bars indicate relative expression of the studied genes in relation to gene expression of HPRT (hypoxanthine-guanine phosphoribosyltransferase). Means from 3 independent analyses and SD are shown. White bars, adrenals of intact rats; shaded bars, day 5 of experiment; black bars, day 8 of experiment. I, intact; C, control rats (0.9% NaCl solution); G, GHREL- and O, OBS-infused rats. Statistical comparison of differences (Student's t-test): *differs from intact rats ($p<0.05$); C and/or G above the bar indicates a difference from the control (C) or GHREL (G) group(s) on the same day ($p<0.05$).

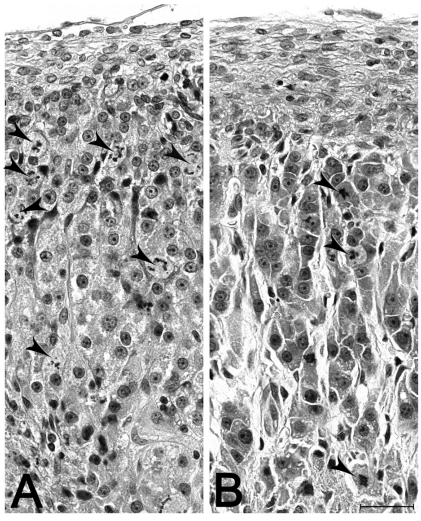

Figure 3. Metaphases in the enucleation-induced regenerating adrenal gland at day 5 of the experiment. Stachmokinetic method with vincristine. (A) Control rat, (B) OBS-infused rat. Arrowheads, metaphases. H&E staining. Scale bar, 50 μm.

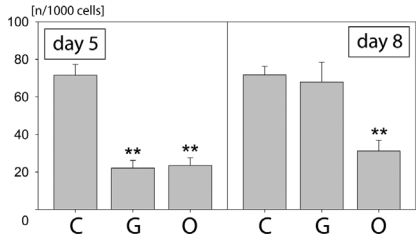

Figure 4. Effects of ghrelin or obestatin infusion (3 nmol/100 g/24 h) on the mitotic index (n/1000) of the regenerating adrenal glands at days 5 and 8 of the experiment. Stachmokinetic method with vincristine. Results are expressed as the means ± SE. In each group, n=6. C, control rats (0.9% NaCl solution); G, GHREL- and O, OBS-infused rats. Statistical comparison of differences (Student's t-test): **$p<0.001$ compared to the control group on the same day.

regulated by OBS. In the regenerating adrenal glands, GPR39v1 and EGR1 mRNA levels were higher than in intact animals. GHREL infusion did not affect the expression level of either of the genes. OBS infusion, on the other hand, notably stimulated GPR39v1 mRNA levels in the regenerating adrenal gland and evoked an opposite effect in the case of the EGR1 gene.

To study the effects of GHREL and OBS on the proliferative activity of adrenocortical cells in the regenerating adrenal glands, we applied the metaphase-arrest technique (Fig. 3). As demonstrated in Fig. 4, at days 5 and 8 of enucleation-induced adrenocortical regeneration, the proliferative activity of the studied cells was extremely high (~70 metaphase arrested cells/1000 cells). Infusion of rats with

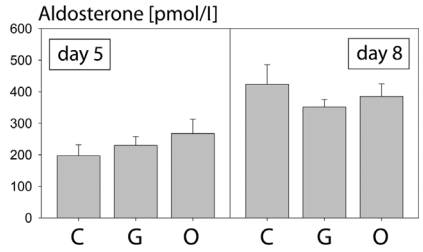

Figure 5. Effects of ghrelin or obestatin infusion (3 nmol/100 g/24 h) on plasma aldosterone concentrations (pmol/l) at days 5 and 8 of the experiment. Results are expressed as the means ± SE. In each group, n=8. C, control rats (0.9% NaCl solution); G, GHREL- and O, OBS-infused rats. No statistically significant differences (within the same group) were found (Student's t-test).

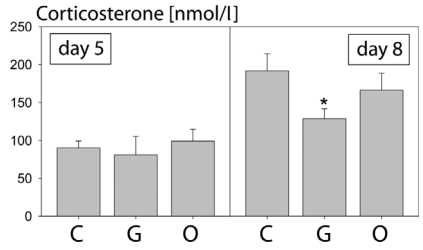

Figure 6. Effects of ghrelin or obestatin infusion (3 nmol/100 g/24 h) on plasma corticosterone concentrations (nmol/l) at days 5 and 8 of the experiment. Results are expressed as the means ± SE. In each group, n=8. C, control rats (0.9% NaCl solution); G, GHREL- and O, OBS-infused rats. Statistical comparison of differences (Student's t-test): *p<0.05 compared to the control group on the same day.

OBS (3 nmol/24 h/100 g) resulted in a potent decrease in the mitotic index of the studied cells, an effect found at both days 5 and 8 of the experiment. GHREL, on the other hand, exerted a similar effect at day 5, but not at day 8 of adrenocortical regeneration.

Both at days 5 and 8, infusion of either GHREL or OBS had no effect on blood aldosterone concentrations of rats with enucleation-induced regenerating adrenal glands (Fig. 5). GHREL infusion, on the other hand, lowered the plasma corticosterone concentration at day 5 but not 8 of the experiment, while OBS administration was ineffective (Fig. 6).

Discussion

In recent literature, the role of OBS is frequently discussed. Since the initial report of Zhang *et al* (6), it has been suggested that OBS exerts biological effects opposed to that evoked by GHREL (reviewed in refs. 3,7). Actual data indicate, however, that OBS exerts no effects on food intake, body weight gain or energy homeostasis (4,13,36).

Regarding the HPA axis, GHREL affects all of its components. The stimulating (either direct or indirect) effect of GHREL on CRH, ACTH and glucocorticoid hormone secretion is well documented, while the role of OBS is still disputable (1,10-12). In the rat neither i.v. nor i.c.v. administration of OBS affected ACTH and corticosterone secretion (13,14). Our studies also demonstrated that OBS infusion into rats with a regenerating adrenal cortex does not change plasma aldosterone and corticosterone concentrations. Furthermore, we previously demonstrated that OBS has no effect on basal or ACTH-stimulated corticosterone secretion by rat adrenocortical cells in primary culture (12). Regarding other steroid secreting cells, OBS promoted the secretion of progesterone, but not testosterone or estradiol in cultured porcine ovarian granulosa cells (36). These findings suggest that OBS is not involved in regulating HPA axis function in the rat.

Expression of ppGHREL and related receptors in the rat adrenal gland is well documented (12,15,23,37,38). ppGHREL, GHS-R, GPR39v1 and GPR39v2 (putative OBS receptor) mRNAs are present in the cortex. This part of the gland is also provided with GOAT4 mRNA, which encodes the GHREL O-acyl transferase that specifically octanoylates serine-3 of the GHREL peptide (39). As applied in the present study, enucleation-induced adrenal regeneration is a classic model of rapid adrenocortical growth (20). Using this model, in the present study, the expression levels of the ppGHREL and GPR39v1 genes were notably higher than levels in intact rats while expression of GHS-R and GPR39v2 remained unchanged. Neither GHREL nor OBS infusion affected the ppGHREL mRNA levels in the regenerating adrenals. This finding suggests that, in regenerating adrenals, expression levels of the ppGHREL gene are not regulated by circulating GHREL. On the contrary, elevated levels of GPR39v1 mRNA in regenerating adrenals underwent a further increase in the OBS-infused rats. This up-regulation of the putative OBS receptor by OBS infusion is an imortant finding. However the physiologic relevance of this control remains to be clarified. It should be emphasized that, in cultured rat adrenocortical cells, OBS did not alter the expression levels of either the GPR39v1 or GPR39v2 gene (12). These findings suggest that the OBS-infusion-evoked up-regulation of adrenal GPR39v1 gene expression in enucleated glands is an indirect one and is possibly mediated by factor(s) other than OBS.

As mentioned above, in the regenerating rat adrenal gland, GHS-R and GPR39v2 mRNA levels were higher than in intact glands and remained unaltered in the GHREL- and OBS-infused rats. This finding suggests that *in vivo* the expression level of the GHS-R gene is not regulated by GHREL. In contrast, previous studies on the homologous regulation of GHS-R1a mRNAs in rat adrenal cultured cells, adrenal slices and human adrenocortical cell lines demonstrated an inhibitory effect of GHREL on expression of the GHS-R1a gene (12,18,37).

In regenerating rat adrenal glands, we also studied the expression levels of several genes involved in differentiation and mitogenesis. Of these, the expression levels of the BAX, JUN-B and JUN-C genes were notably higher in the regenerating adrenals, and neither GHREL nor OBS infusion affected these levels. On the other hand, the expression level of the FOS gene, which encodes protein associated with cell proliferation and differentiation, was similar in intact and regenerating adrenals and was unaffected by GHREL or OBS infusion.

In the experimental model applied in this study, the most significant changes were found in the expression levels of the EGR1 gene. The EGR1 gene encodes protein that activates the transcription of target genes whose products are required

for mitogenesis and differentiation. EGR1 mRNA levels were notably higher in the regenerating glands than in intact adrenals and were unaffected by GHREL infusion. OBS infusion, on the other hand, notably lowered the expression levels of EGR1; the effect was paralleled by a decrease in the metaphase index of the adrenocortical cells.

A current survey of the literature on direct OBS involvement in regulating proliferative activity of various cells provides conflicting results. As reported, in primary culture of hRPE (human retinal epithelial) cells, OBS induced proliferation in a dose-dependent manner, an effect which is dependent on MEK/ERK 1/2 phosphorylation (40). Furthermore, the stimulating effects of OBS on BrdU incorporation into HIT-T15 (hamster insulinoma tumor cells) and rat insulinoma-derived INS-1 cells were also reported (41). Likewise, in cultured porcine ovarian granulosa cells, OBS stimulated the expression of PCNA, cyclin B1 and MAPK (36). The stimulating effect of OBS on cell proliferation was also reported for a primary culture of piglet hepatocytes and adipose cells (7). In contrast, in 3T3-L1 preadipocytes (a cell line derived from the Swiss 3T3 mouse fibroblast cell line), OBS inhibited the proliferative activity as assessed by the MTT assay (42). Based on the same assay, the antiproliferative effect of OBS on the TT (human medullary thyroid carcinoma cells) and BON-1 (carcinoid cells) cell lines was also reported (43). In contrast, OBS did not modify the cell cycle in the murine cardiomyocyte cell line HL-1 (44). Recently, we found that, in serum-deprived medium, neither GHREL nor OBS affected BrdU incorporation into the primary culture of rat adrenocortical cells (12). It should be emphasized that, in cultured rat adrenocortical cells, GPR39v1 and GPR39v2 (orphan G protein-coupled receptor 39, variants 1 and 2) mRNAs are present, and it remains to be ascertained whether these putative OBS receptor isoforms are also expressed at the protein level. In view of these findings, a potent inhibitory effect of OBS on the proliferative activity of regenerating adrenal cortex was unexpected. Moreover, this effect was accompanied by a notable decrease in the expression level of the EGR1 gene. The above presented findings suggest that the inhibitory effect of OBS on adrenocortical regeneration in the rat is an indirect one and is exerted probably at the levels of the hypothalamus and/or pituitary gland. Regarding our finding, it should be mentioned that OBS injections (i.p.) did not affect ^{3}H-thymidine incorporation into the rat pancreas (45).

It should be emphasized that, in the present experimental model, GHREL inhibited the mitotic activity of regenerating adrenocortical cells only at day 5, but not day 8. In this context it is worth noting that, in GHREL-infused rats, the expression levels of EGR1 remained unchanged. This raises an intriguing possibility that the inhibitory effect of GHREL and OBS on adrenocortical cell proliferation *in vivo* may be mediated by different pathways.

As mentioned earlier, adrenal regeneration is a classic model of rapid adrenocortical growth, which is modulated, apart from ACTH, by several other regulatory peptides (20,46-50). This group of peptides also includes those involved in the regulation of energy homeostasis, among them leptin (51,52), orexin (31,53,54), cholecystokinin (55), pentagastrin (56), galanin (57,58), neuromedin U (59,60) and beacon (61,62). The results of our study suggest that both GHREL and OBS may be included into this expanding group of peptides.

In conclusion, this study is the first to demonstrate that, *in vivo,* GHREL and OBS inhibit the growth of regenerating adrenal cortex. Moreover, the data suggest that the effect of OBS might be, at least in part, mediated by the EGR1 pathway known to be critical in cell proliferation.

Acknowledgements

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