# Ghrelin and growth hormone secretagogue receptor are expressed in the rat adrenal cortex: evidence that ghrelin stimulates the growth, but not the secretory activity of adrenal cells

Paola G. Andreis[a], Ludwik K. Malendowicz[b], Marcin Trejter[b], Giuliano Neri[a], Raffaella Spinazzi[a], Gian Paolo Rossi[c], Gastone G. Nussdorfer[a,*]

[a] *Department of Human Anatomy and Physiology, Section of Anatomy, School of Medicine, University of Padua, Via Gabelli 65, I-35121 Padua, Italy*
[b] *Department of Histology and Embryology, School of Medicine, Karol Marcinkowski University of Medical Sciences, PL-60781 Poznan, Poland*
[c] *Department of Clinical and Experimental Medicine, School of Medicine, University of Padua, I-35121 Padua, Italy*

Received 19 November 2002; revised 11 January 2003; accepted 11 January 2003

First published online 22 January 2003

Edited by Jacques Hanoune

**Abstract** **Ghrelin is an endogenous ligand of the growth hormone secretagogue receptor (GHS-R), which has been originally isolated from rat stomach. Evidence has been previously provided that adrenal gland possesses abundant ghrelin-displaceable GHS-Rs, but nothing is known about the possible role of ghrelin in the regulation of adrenocortical function. Reverse transcription-polymerase chain reaction demonstrated the expression of ghrelin and GHS-R in the rat adrenal cortex, and high adrenal concentrations of immunoreactive ghrelin were detected by radioimmune assay (RIA). Autoradiography localized abundant [$^{125}$I]ghrelin binding sites in the adrenal zona glomerulosa (ZG) and outer zona fasciculata (ZF). Ghrelin (from $10^{-10}$ to $10^{-8}$ M) did not affect either basal steroid hormone (pregnenolone, progesterone, 11-deoxycorticosterone, corticosterone, 18-hydroxycorticosterone and aldosterone) secretion from dispersed ZG and zona fasciculata/reticularis (ZF/R) cells (as evaluated by quantitative high pressure liquid chromatography), or basal and agonist-stimulated aldosterone and corticosterone production from cultured ZG and ZF/R cells, respectively (as measured by RIA). Ghrelin ($10^{-8}$ and $10^{-6}$ M) raised basal, but not agonist-stimulated, proliferation rate of cultured ZG cells (percent of cells able to incorporate 5-bromo-2′-deoxyuridine), without affecting apoptotic deletion rate (percent of cells able to incorporate biotinylated nucleosides into apoptotic DNA fragments). The tyrosine kinase (TK) inhibitor tyrphostin-23 and the p42/p44 mitogen-activated protein kinase (MAPK) inhibitor PD-98059 abolished the proliferogenic effect of $10^{-8}$ M ghrelin, while the protein kinase A and C inhibitors H-89 and calphostin-C were ineffective. Ghrelin ($10^{-8}$ M) stimulated TK and MAPK activity of dispersed ZG cells, and the effect was abolished by preincubation with tyrphostin-23 and PD-98059, respectively. Tyrphostin-23 annulled ghrelin-induced activation of MAPK activity. Taken together, the present findings indicate that (i) ghrelin and GHS-R are both expressed in the rat adrenal cortex, ghrelin binding sites being very abundant in the ZG; (ii) ghrelin does not affect the secretory activity of rat adrenocortical cells, but significantly enhances the proliferation rate of cultured ZG cells, without affecting apoptotic deletion rate; and (iii) the ZG proliferogenic action of ghrelin involves the TK-dependent activation of the p42/p44 MAPK cascade.**



*Key words:* Ghrelin; Growth hormone secretagogue receptor; Adrenocortical cell; Steroid hormone secretion; Cell proliferation; Cell apoptosis

## 1. Introduction

Ghrelin is a 28 amino acid peptide, originally isolated from rat stomach, which acts as an endogenous ligand of the growth hormone (GH) secretagogue receptor (GHS-R) [1], two subtypes of which have been identified: the fully functional GHS-R1a and the biologically inactive GHS-R1b [2,3]. Ghrelin and GHS-Rs are expressed in the hypothalamus [4], abundant ghrelin binding sites are present in the pituitary gland [5], and the main biological effects of ghrelin known to date are exerted on the hypothalamo-pituitary complex. Ghrelin, in addition to eliciting pituitary GH release [1,6,7], exerts an orexinogenic action [8–10] and is possibly involved in the neuroendocrine and behavioral responses to stresses via its stimulating effect on the hypothalamic adrenocorticotropic hormone (ACTH) secretagogues CRH and vasopressin [11–13].

Evidence has been provided that ghrelin is expressed in some endocrine tissues, like human and rat pancreatic islets and rat Leydig cells [14,15], where it stimulates insulin release and inhibits agonist-enhanced testosterone secretion, respectively. Human adrenal glands were found to possess abundant ghrelin-displaceable GHS-Rs [16], and to express both ghrelin and GHS-R1a mRNAs [17]. Recent findings indicate that rat adrenals, although not expressing ghrelin mRNA, are provided with GHS-R1a. However, ghrelin was unable to exert any appreciable effect on basal and ACTH-stimulated corticosterone secretion from quarters of the entire gland [18]. This last observation does not rule out the possibility that ghrelin affects the secretion of hormones other than corticosterone, e.g. aldosterone produced by zona glomerulosa (ZG). Moreover, it could be possible that GHS-Rs may be involved in the control of rat adrenal growth. Thus, the present study was undertaken to investigate (i) the ghrelin and GHS-R expression in the rat adrenal cortex, (ii) the zonal localization of

*Corresponding author. Fax: (39)-049-827 2319.
*E-mail address:* gastone.nusdorfer@unipd.it (G.G. Nussdorfer).

0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00051-6

ghrelin binding sites, and (iii) the ghrelin effects on secretion and growth of rat adrenocortical cells cultured in vitro.

## 2. Materials and methods

### 2.1. Animals and reagents

Adult male Sprague–Dawley rats (260 ± 30 g body weight) were purchased from Charles-River (Como, Italy), and the protocol of the experiment was approved by the local Ethics Committee for Animal Studies. Rats were decapitated, and their adrenal glands were promptly removed and cleaned from adherent adipose tissue. Hypothalamus and testis were also removed from some animals.

Rat ghrelin was purchased from Neosystem Laboratoires (Strasbourg, France), [$^{125}$I]ghrelin from Phoenix Pharmaceuticals (Belmont, CA, USA), and His-D-2-methyl-Trp-Ala-Trp-D-Phe-Lis-$NH_2$ (hexarelin) from Peninsula Laboratories (St. Helens, UK). Anti-p42/p44 antibody was provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA), Dulbecco's modified Eagle's minimum essential medium (DMEM) by Gibco (Paisley, UK), Medium 199 by Difco (Detroit, MI, USA), and cyanoketone (WIN-24540) by Sterling-Winthrop (Guilford, UK). H-89, calphostin-C, tyrphostin-23 and PD-98059 were obtained from Biomol (Milan, Italy). Endothelin-1[1–31] (ET-1[1–31]) was purchased from Peptide Institute (Osaka, Japan). ACTH, angiotensin-II (Ang-II), 5′-bromo-2′-deoxyuridine (BrdU), bovine serum albumin (BSA), human serum albumin (HSA), fetal calf serum (FCS) and all other chemicals and laboratory reagents were obtained from Sigma Chemical Company (St. Louis, MO, USA).

### 2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted from the adrenal cortex (enucleated adrenal gland), hypothalamus and testis of six rats with the guanidium isothiocyanate method, and total RNA was reverse transcribed to cDNA [15]. PCR procedures followed those described earlier [19], and the sense and antisense primers for ghrelin and GHS-R were selected according to Tene-Sempere et al. [15]. GHS-R primers were flanking a coding area common to both GHS-R1a and GHS-R1b splice variants [18]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as housekeeping gene. In a thermal cycler (Delphi 100; Oracle Biosystems, MJ Research, Waterston, MA, USA), after an initial step at 97°C for 5 min, we used a denaturation step at 96°C for 90 s, an annealing step for 90 s, and an extension step at 72°C for 3 min for a total of 36 cycles. An additional extension step at 72°C for 10 min was then carried out. Primer sequences, annealing temperature and the predicted size of PCR products are shown in Table 1. To rule out the possibility of amplifying genomic DNA, one PCR was carried out without prior RT of the RNA. Detection of the PCR amplification products was first performed by size fractionation on 2% agarose gel electrophoresis. Then, after purification using the QIA quick PCR purification kit (Qiagen, Hilden, Germany), amplicons were identified by sequencing (Alf Sequencer; Pharmacia Biotech, Freiburg, Germany).

### 2.3. Ghrelin radioimmune assay (RIA)

Adrenal cortex, hypothalamus and testis of six rats were homogenized in phosphate-buffered saline (PBS), and their protein concentration was determined by the Lowry method, using BSA as a standard. Ghrelin was separated and purified by chromatography, using a Sep-Pak C18 cartridge (Waters, Milford, MA, USA), and its concentration in eluates was determined using the ghrelin (rat) RIA kit of Phoenix Pharmaceuticals (sensitivity: $IC_{50}$, 7.2 fmol/tube; intra- and interassay CVs: 6.3 and 8.5%, respectively).

### 2.4. Autoradiography

Adrenal glands of three rats were immediately frozen at −30°C by immersion in isopentane, and stored at −80°C. Frozen sections (10–15 μm thick) were cut in a cryostat (Leitz 1720 Digital) at −20°C, and processed for autoradiography as previously detailed [20]. They were labeled in vitro by incubation for 120 min at 37°C with $10^{-8}$ M [$^{125}$I]ghrelin, and the selectivity of binding was checked by addition of $10^{-6}$ M cold ghrelin or $10^{-7}$ M hexarelin. The reaction was stopped by washing the samples three times in 50 mM Tris–HCl buffer. After rinsing, the sections were rapidly dried, fixed in paraformaldehyde vapors at 80°C for 120 min, and coated with NTB2 nuclear emulsion (Eastman Kodak, Rochester, NJ, USA). Autoradiographs were exposed for 2 weeks at 4°C, and then developed with undiluted Kodak D19 developer. They were stained with hematoxylin-eosin, and observed and photographed with a camera-connected Leitz Laborlux microscope. For each adrenal gland, three unstained autoradiograms were analyzed by computer-assisted densitometry, as previously detailed [20]. In each autoradiogram, 10 areas of the ZG, outer ZF, inner ZF and ZR, and adrenal medulla (about 36 000 pixels each) were analyzed. The density value of the capsule was taken as the background value.

### 2.5. Adrenocortical cell cultures

Adrenal glands were gently decapsulated to separate ZG from the inner zones, then halved and enucleated to eliminate adrenal medulla. Dispersed ZG and zona fasciculata/reticularis (ZF/R) cells were obtained by sequential collagenase digestion and mechanical disaggregation [20]. Dispersed ZG cells were suspended in DMEM, containing 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin, and plated in 35 mm tissue culture dishes. Cells were seeded at a density of $5\times10^5$ cells/dish to obtain confluency after 24 h of culture (secretion experiments) or at a density of $3\times10^4$ cells/dish (growth experiments). Cells were cultured at 37°C in a humidified atmosphere of 95% air/5% $CO_2$, and employed after 24 h of culture [21].

### 2.6. Adrenocortical cell secretion

Dispersed ZG and ZF/R cells were put in Medium 199 and Krebs–Ringer bicarbonate buffer with 2% glucose, containing 5 mg/ml HSA. They were incubated ($3\times10^5$ cells/ml) with ghrelin (from $10^{-10}$ to $10^{-6}$ M), in the presence or absence of $10^{-5}$ M cyanoketone to prevent further metabolism of pregnenolone [22]. The incubation was carried out in a shaking bath at 37°C for 90 min in an atmosphere of 95% air/5% $CO_2$. Medium was collected, and steroid hormone concentrations were measured by high pressure liquid chromatography (HPLC), as previously described [22]. Pregnenolone was detected by ultraviolet (UV) absorbance at 290 nm, and other steroid hormones (see Table 2) at 240 nm wavelength, and identified by comparison of their retention times with those of the standards. Quantification of steroid hormones was based on peak area measurement, the sensitivity of our assay being 1 pmol/ml. Intra- and interassay CVs were 6.9 and 7.8%, respectively.

Confluent ZG cell cultures were incubated for 120 min at 37°C in fresh DMEM, containing ghrelin (from $10^{-10}$ to $10^{-6}$ M) alone and in the presence of $10^{-9}$ M ACTH or Ang-II. Other cultures were incubated in DMEM without any peptide (baseline value). The medium was collected, and aldosterone and corticosterone concentrations were measured, without previous extraction and purification, using commercial RIA kits: ALDO CTK2 (IRE-Sorin, Vercelli, Italy; sensitivity: 14 fmol/ml; intra- and interassay CVs: 7.1 and 8.4%, respectively); and CTRX-RIA (Eurogeneticx, Milan, Italy; sensitivity: 73 fmol/ml; intra- and interassay CVs: 7.6 and 9.1%, respectively).

Table 1
RT-PCR primers and PCR products

| Primers | Sequence | Annealing temperature | Product size |
|---|---|---|---|
| Ghrelin sense | 5′-TTGAGCCCAGAGCACCAGAAA-3′ | | |
| Ghrelin antisense | 5′-AGTTGCAGAGGAGGCAGAAGCT-3′ | 55°C | 347 bp |
| GHS-R sense | 5′-AGGCAACCTGCTCACTATGCTG-3′ | | |
| GHS-R antisense | 5′-GACAAGGATGACCAGCTTCACG-3′ | 60°C | 321 bp |
| GAPDH sense | 5′-CCCTTCATTGACCTCAACTA-3′ | | |
| GAPDH antisense | 5′-GCCAGTGAGCTTCCCGTTCA-3′ | 57°C | 585 bp |

*2.7. Adrenocortical cell proliferation*

Non-confluent ZG cell cultures were incubated for 24 h at 37°C in fresh DMEM (supplemented with 10% FCS), containing (i) ghrelin (from $10^{-10}$ to $10^{-8}$ M) alone and in the presence of $10^{-8}$ M ET-1-[1–31]; (ii) ghrelin ($10^{-8}$ M) alone and in the presence of $10^{-5}$ M H-89, $10^{-5}$ M calphostin-C, $10^{-5}$ M tyrphostin-23 or $10^{-4}$ M PD-98059; and (iii) DMEM alone and with H-89, calphostin-C, tyrphostin-23 or PD-98059. The concentrations of ET-1[1–31] and the signaling cascade inhibitors were chosen according to Mazzocchi et al. [21]. During the last 12 h of incubation BrdU was added to the culture medium to a final concentration of 20 mg/ml [21]. Cultures were fixed in 4% paraformaldehyde for 30 min, and BrdU-positive (S-phase) cells were detected using the cell proliferation kit of Amersham Pharmacia (Aylesbury, UK). The percentage of positive cells (proliferation index) was evaluated by counting 3000 cells per dish, and six dishes for each experimental point were employed.

*2.8. Adrenocortical cell apoptosis*

Non-confluent ZG cell cultures were incubated for 24 h at 37°C in fresh DMEM (supplemented or not with 10% FCS), containing ghrelin (from $10^{-10}$ to $10^{-6}$ M). Other cultures were incubated in DMEM without ghrelin (baseline value). Cultures were fixed in 4% paraformaldehyde for 30 min, and apoptotic cells were detected using the TACS Klenow in situ apoptosis detection kit of Sigma. The percentage of positive cells (apoptotic index) was evaluated by counting 3000 cells per dish, and six dishes for each experimental point were employed.

*2.9. Tyrosine kinase (TK) and mitogen-activated protein kinase (MAPK) activity*

Dispersed ZG cells were put in Medium 199 and Krebs–Ringer bicarbonate buffer with 2% glucose, containing 5 mg/ml BSA. They were incubated with ghrelin (from $10^{-10}$ to $10^{-6}$ M) or without ghrelin (baseline value). Other preparations were preincubated for 30 min with $10^{-5}$ M tyrphostin-23 or $10^{-4}$ M PD-98059, and then exposed or not to ghrelin ($10^{-8}$ M). The incubation was carried out in a shaking bath at 37°C for 15 min, and was stopped by two quick washes with ice-cold PBS. Dispersed cells were lysed and homogenized, as previously described [23]. Homogenates were centrifuged at 4°C at $800\times g$ for 10 min, and then at $12\,000\times g$ for 15 min. Supernatants were removed, the protein concentration was determined by the Lowry method, and then stored at −80°C. TK activity was assayed, using poly($Glu^4$,$Tyr^1$) as substrate [24]. MAPK activity was measured using anti-MAPK p42/p44 polyclonal antibody and myelinic basic protein as substrate [25].

*2.10. Statistics*

Data were expressed as means ± S.E.M. of six separate experiments. Their statistical comparison was done by analysis of variance (ANOVA), followed by Duncan's multiple range test.

## 3. Results and discussion

RT-PCR demonstrated sizeable expression of ghrelin and GHS-R mRNAs in the rat adrenal cortex (Fig. 1), as well as in the hypothalamus and testis (data not shown). RIA detected high concentrations of ghrelin-IR in the adrenal cortex and hypothalamus and low concentrations in the testis (Fig. 2). Autoradiography evidenced abundant [$^{125}$I]ghrelin binding sites in the adrenal gland, which were located in the outer portion of the cortex, and to a lesser extent in the adrenal medulla (Fig. 3A). The binding was completely displaced not only by cold ghrelin (Fig. 3B), but also by the selective GHS-R ligand hexarelin [26] (data not shown). The quantitative densitometric analysis of autoradiograms confirmed these qualitative observations (Fig. 4), thereby indicating that [$^{125}$I]ghrelin binding sites are GHS-Rs. Taken together these findings, in addition to confirming the presence of ghrelin in the rat hypothalamus and testis [4,15], provide evidence that ghrelin is expressed as mRNA and protein in the rat adrenal cortex. A previous investigation did not demonstrate ghrelin mRNA expression in the rat adrenals [18]. At present, we are unable to provide a reasonable explanation of this discrepancy, although we wish to stress that different strains of rats (Sprague–Dawley versus Wistar rats) were used. Moreover, our study confirms that rat adrenals, like human glands [16,17], possess GHS-Rs [18], and provides the first evidence that ghrelin binding sites are mainly located in the ZG and outer ZF.

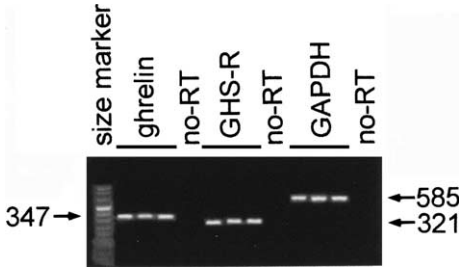


Fig. 1. Ethidium bromide-stained 2% agarose gel showing cDNA amplified with rat specific primers from RNA of three exemplary rat adrenal cortices. Lane 1 was loaded with 200 ng of a size marker (marker VIII; Roche Molecular Biochemicals, Mannheim, Germany). The amplified fragments were of the expected sizes: 347 bp for ghrelin, 321 bp for GHS-R, and 585 bp for GAPDH. No amplification of PCR mixture without prior RT of the mRNAs is shown as negative control.

The HPLC assay (Table 2) demonstrated that ghrelin did not evoke any significant change in the basal secretion of pregnenolone, progesterone, 11-deoxycorticosterone, cortico-

Table 2
Lack of effect of ghrelin on basal steroid hormone secretion from dispersed rat adrenocortical cells

| | Baseline | Ghrelin $10^{-10}$ M | Ghrelin $10^{-8}$ M | Ghrelin $10^{-6}$ M |
|---|---|---|---|---|
| ZG cells | | | | |
| Pregnenolone | 589.2 ± 160.7 | 685.1 ± 248.5 | 554.2 ± 191.6 | 515.8 ± 185.2 |
| Progesterone | 128.8 ± 36.8 | 101.8 ± 36.9 | 140.5 ± 51.2 | 139.4 ± 45.7 |
| 11-Deoxycorticosterone | 8.9 ± 4.3 | 10.7 ± 3.8 | 9.8 ± 3.7 | 8.1 ± 2.9 |
| Corticosterone | 99.0 ± 23.1 | 88.5 ± 22.6 | 104.1 ± 30.9 | 105.7 ± 39.4 |
| 18-Hydroxycorticosterone | 25.7 ± 6.3 | 20.4 ± 7.1 | 33.2 ± 11.5 | 28.1 ± 9.4 |
| Aldosterone | 49.5 ± 19.9 | 55.6 ± 15.8 | 41.8 ± 14.5 | 50.8 ± 18.7 |
| ZF/R cells | | | | |
| Pregnenolone | 609.1 ± 156.7 | 559.5 ± 175.2 | 670.8 ± 199.6 | 635.4 ± 207.5 |
| Progesterone | 121.5 ± 33.7 | 148.7 ± 49.0 | 135.6 ± 41.2 | 118.7 ± 30.8 |
| 11-Deoxycorticosterone | 81.4 ± 10.9 | 70.5 ± 15.6 | 75.7 ± 12.9 | 85.1 ± 19.5 |
| Corticosterone | 286.2 ± 94.3 | 315.4 ± 111.5 | 254.8 ± 100.1 | 306.4 ± 89.7 |
| 18-Hydroxycorticosterone | 4.4 ± 3.8 | ND | 8.2 ± 6.7 | ND |
| Aldosterone | ND | ND | ND | ND |

Values are expressed as pmol/$10^6$ cells·h, and are means ± S.D. ($n = 6$). ND, not detectable. Differences among groups are not significant.

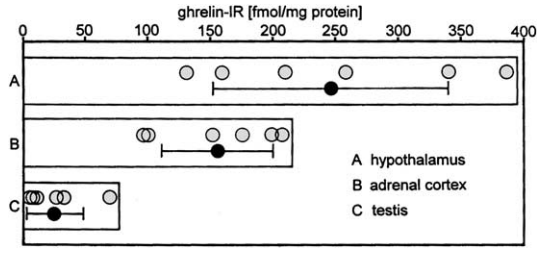


Fig. 2. Ghrelin-IR concentration in rat hypothalamus, adrenal cortex and testis. Bars enclose values of the six samples (gray circles) and their means ± S.D. (black circles).

sterone, 18-hydroxycorticosterone and aldosterone from dispersed rat ZG and ZF/R cells. As expected, ZG cell cultures released high amounts of aldosterone and displayed a secretory response to both ACTH and Ang-II, while ZF/R cell cultures mainly produced corticosterone and were responsive only to ACTH. Again, RIA showed that ghrelin did not significantly alter either basal or agonist-stimulated secretion of aldosterone and corticosterone from cultured cells (Fig. 5). Hence, ghrelin, within the range of concentrations used, affects neither basal early and late steps of steroid synthesis nor agonist-stimulated late steps. This observation is in keeping with the reported lack of effect of ghrelin on rat adrenal corticosterone production [18], and indicates that adrenal ghrelin receptors behave differently from those of rat Leydig cells, where ghrelin has been found to decrease human chorionic gonadotropin- and cyclic AMP-stimulated testosterone secretion through a mechanism probably involving down-regulation of steroid acute regulatory protein, P450 cholesterol side chain cleavage and 3β-hydroxysteroid dehydrogenase (i.e. by inhibiting agonist-stimulated early steps of steroid synthesis) [15].

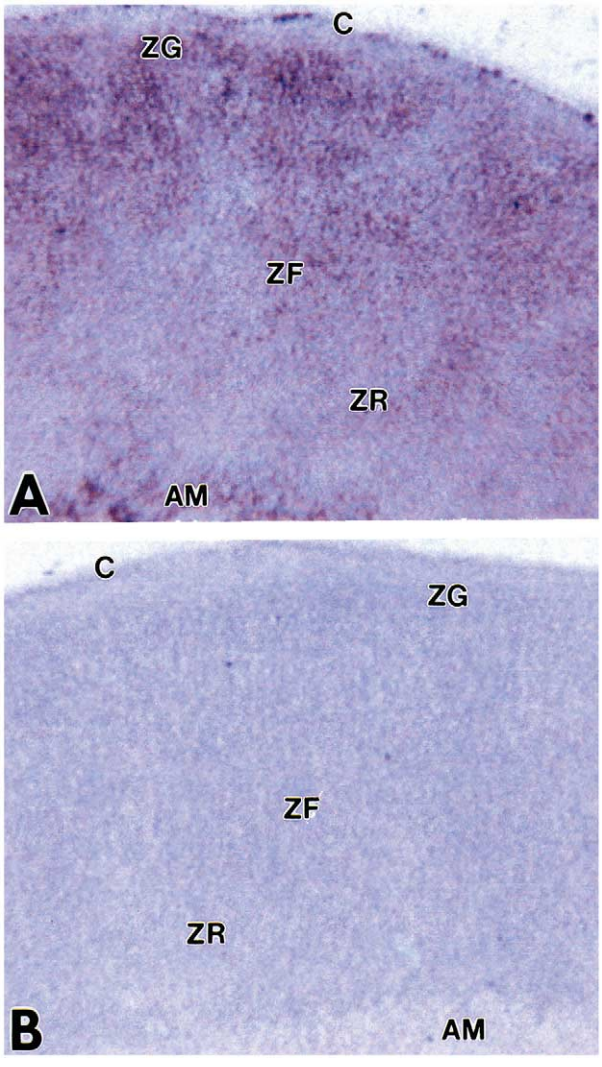


Fig. 3. Autoradiograms of hematoxylin-eosin-stained frozen sections of rat adrenal gland incubated with $10^{-8}$ M [$^{125}$I]ghrelin. Binding is present in both outer cortex and medulla of the gland (A), and is completely displaced by $10^{-6}$ M cold ghrelin (B). c, Gland capsule; AM, adrenal medulla; ZF, zona fasciculata; ZG, zona glomerulosa; ZR, zona reticularis. 70×.

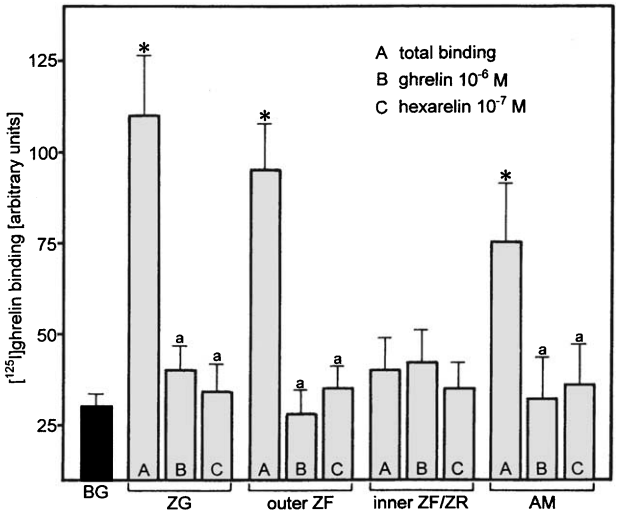


Fig. 4. Evaluation by quantitative densitometry of [$^{125}$I]ghrelin binding in the rat adrenal cortex and medulla (total binding, TB), and its displacement by cold ghrelin and hexarelin. AM, adrenal medulla; ZF, zona fasciculata; ZG, zona glomerulosa; ZR, zona reticularis. Data are means ± S.E.M. ($n = 3$). *$P < 0.01$ versus the background (BG) value; [a]$P < 0.01$ versus the respective TB value.

As discussed above, the ghrelin receptors are mainly present in the ZG, which is the layer involved in the maintenance of the adrenal growth [27]. Thus we examined the effects of ghrelin on the growth of cultured ZG cells. Ghrelin ($10^{-8}$ and $10^{-6}$ M) markedly raised basal, but not ET-1[1–31]-enhanced, proliferation index of ZG cell cultures. Conversely, ghrelin did not modify apoptotic index of ZG cells either cultured in FCS-supplemented or FCS-free medium (Fig. 6). It is to be noted that the observed proliferogenic action of ghrelin appears to conflict with the results of a previous study, which showed that this peptide causes a marked inhibition of the proliferative activity of three different human breast carcinoma cell lines [28]. However, they are in accord with the reported stimulating action of ghrelin on cardiomyocyte H9c2 and prostate cancer PC3 cell lines [29,30]. The possibility that the proliferogenic action of ghrelin on cultured ZG may be mediated by a novel receptor other than GHS-R is unlikely in light of the findings that the selective GHS-R agonist hexarelin (i) completely eliminates [$^{125}$I]ghrelin binding in the ZG (see above), and (ii) mimics the effect of ghrelin on ZG cell proliferation index (preliminary data not shown).

The $10^{-8}$ M ghrelin-induced rise in ZG cell proliferation index was not affected by either $10^{-5}$ M H-89 or $10^{-5}$ M

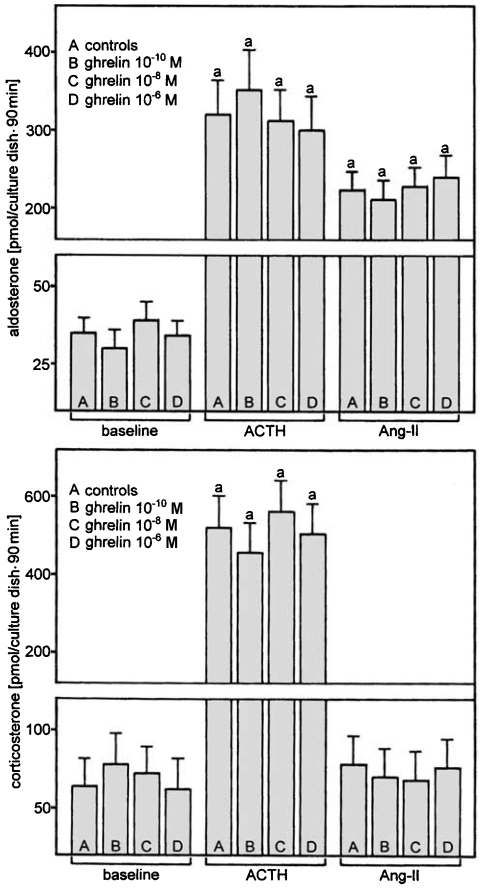


Fig. 5. Lack of effect of ghrelin on basal and agonist-stimulated aldosterone and corticosterone secretion from rat ZG cell (upper panel) and ZF/R cell cultures (lower panel), respectively. Bars are means ± S.E.M. ($n = 6$). [a]$P < 0.01$ versus the respective baseline value.

calphostin-C, but was abolished by both $10^{-5}$ M tyrphostin-23 and $10^{-4}$ M PD-98059 (Fig. 7). Ghrelin ($10^{-8}$ and $10^{-6}$ M) significantly enhanced TK and MAPK p42/p44 activities (by about 2.5- and 3.1-fold, respectively) (Fig. 8, upper panel). Preincubation with $10^{-5}$ M tyrphostin-23, but not $10^{-4}$ M PD-98059, blocked the ghrelin ($10^{-8}$ M)-induced stimulation

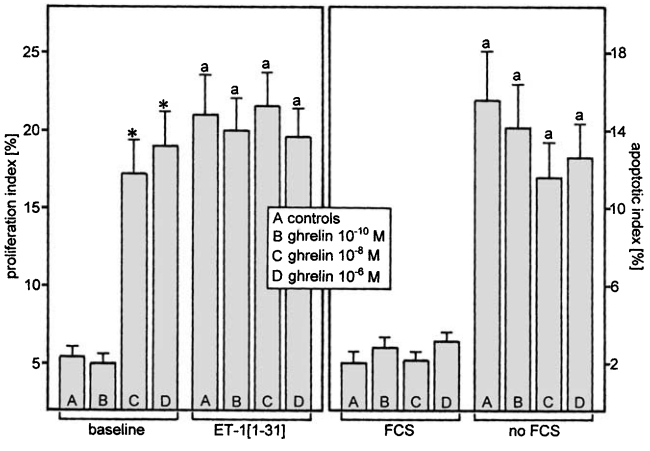


Fig. 6. Effect of ghrelin on basal and stimulated proliferation (left panel) and apoptotic rate (right panel) of cultured rat ZG cells. Bars are means ± S.E.M. ($n = 6$). *$P < 0.01$ versus the respective control value; [a]$P < 0.01$ versus the respective baseline value.

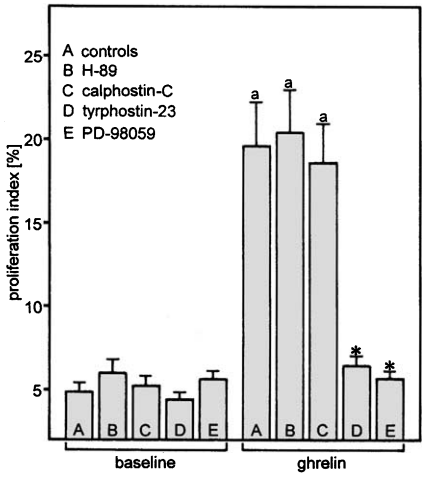


Fig. 7. Effects of H-89 ($10^{-5}$ M), calphostin-C ($10^{-5}$ M), tyrphostin-23 ($10^{-5}$ M) and PD-98059 ($10^{-4}$ M) on basal and ghrelin ($10^{-8}$ M)-stimulated proliferation rate of cultured rat ZG cells. Bars are means ± S.E.M. ($n = 6$). *$P < 0.01$ versus the respective control value; [a]$P < 0.01$ versus the respective baseline value.

of TK activity. In contrast, the ghrelin-evoked stimulation of MAPK activity was annulled by both tyrphostin-23 and PD-98059 (Fig. 8, lower panel).

These findings demonstrate that ghrelin, like ET-1[1–31] [21], exerts its growth-promoting action on cultured rat ZG cells by activating the TK–MAPK signaling pathway (Fig. 9), and this is in keeping with the lack of additivity between

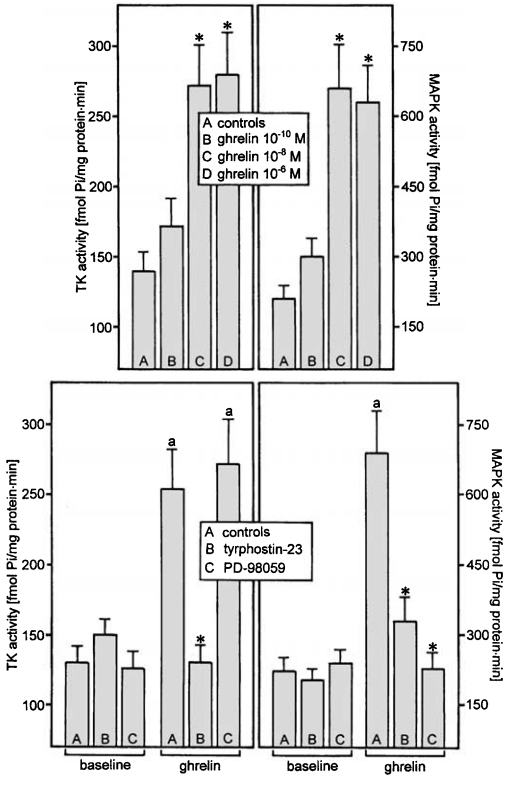


Fig. 8. Effects of ghrelin on basal TK and MAPK p42/p44 activities of dispersed rat ZG cells (upper panels), and of the preincubation with tyrphostin-23 ($10^{-5}$ M) or PD-98059 ($10^{-4}$ M) on basal and ghrelin ($10^{-8}$ M)-stimulated activities (lower panels). Bars are means ± S.E.M. ($n = 6$). *$P < 0.01$ versus the respective control value; [a]$P < 0.01$ versus the respective baseline value.

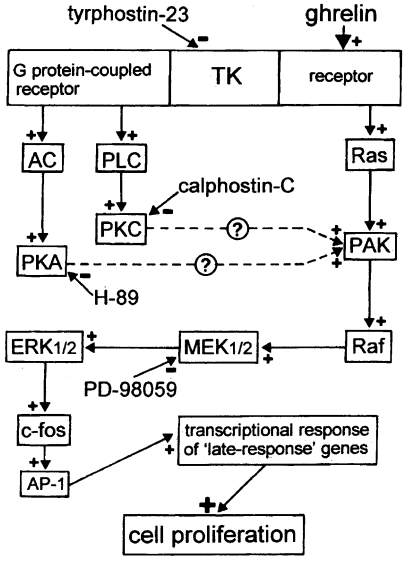


Fig. 9. Simplified scheme illustrating the main pathways inducing cell proliferation, the possible locus of action of ghrelin and the sites of action of inhibitors. Briefly, tyrosine kinase (TK), by binding to its agonists, activates Ras, a peptide belonging to a family of GTP binding proteins. Ras in turn triggers a cascade leading to the sequential activation of p21-activated kinase (PAK), Raf, MEK1/2 and extracellular (signal) regulated kinases (ERK1/2). ERK1/2 induce the expression, among others, of the *c-fos* gene, the products of which associate with other related protein to form the transcriptional factor AP-1. TK may also behave as a G protein-coupled receptor, which, through the activation of adenylate cyclase (AC) and phospholipase-C (PLC), leads to the production of protein kinases (PK)A and C. PKA and PKC can activate MAPK cascade via a not yet known Ras-independent mechanism. + and − symbols near the arrow heads indicate activation and inhibition, respectively.

ghrelin and ET-1[1–31] proliferogenic effects. This contention is based on the following pieces of evidence: (i) the TK inhibitor tyrphostin-23 [31] and the MEK1 inhibitor PD-98059 [32] block the ghrelin-induced rise in the number of BrdU-positive ZG cells; (ii) these inhibitors per se do not elicit any apparent effect on basal DNA synthesis rate, thereby ruling out the possibility that these results were due to a non-specific toxic effect on cultured ZG cells; (iii) ghrelin stimulates both TK and MAPK activity, and the effect is reversed by tyrphostin-23 and PD-98059 at the concentrations annulling the ZG proliferogenic action of the peptide; and (iv) tyrphostin-23 blocks the ghrelin-induced stimulation of MAPK activity.

The TK receptor is well known to be involved in the activation of p42/p44 MAPKs [33–35]. However, many lines of evidence indicate that TK, behaving as a G protein-coupled receptor, can activate MAPK cascade via both protein kinase (PK) C- [35–37] and PKA-dependent pathways [37,38]. Accordingly, cyclic AMP and Ang-II were found to activate MAPK and to enhance proliferation of ZG cells via a PKA- and a PKC-dependent pathway, respectively [39,40]. Likewise, ET-1[1–31] has been reported to exert its proliferogenic action on cultured ZG cells via two independent pathways involving both TK-dependent and PKC-dependent MAPK cascades [21]. In contrast, our present results make it unlikely that either PKA or PKC cascades play a relevant role in the growth effect of ghrelin on adrenals, in as much as neither the PKA inhibitor H-89 [41] nor the PKC inhibitor calphostin-C [42] are able to blunt the proliferogenic action of this peptide on cultured ZG cells (Fig. 9). It is to be noted that this finding is reminiscent of that obtained with adrenomedullin, a regulatory peptide which has been found to enhance rat ZG cell proliferation via a MAPK signaling mechanism exclusively dependent on TK activation [23,43].

In summary, the present study indicates that (i) ghrelin and GHS-R are both expressed in the rat adrenal cortex; (ii) ghrelin binding sites are abundant in the outer cortex; (iii) ghrelin does not affect the secretory activity of rat adrenocortical cells; (iv) ghrelin stimulates proliferation of cultured ZG cells, without significantly affecting apoptotic deletion rate; and (v) the ZG proliferogenic action of ghrelin involves the TK-dependent activation of the p42/p44 MAPK cascade. In conclusion, our results allow us to include ghrelin in that group of peptides [44,45] involved in the autocrine–paracrine regulation of adrenal growth.

## References

[1] Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H. and Kangawa, K. (1999) Nature 402, 656–660.
[2] Howard, A.D., Feighner, S.C., Cully, D.F., Arena, J.P., Liberator, P.A., Rosenblum, C.I., Hamelin, M., Hreniuk, D.L., Palyha, O.C., Anderson, J., Paress, P.S., Diaz, C., Chou, M., Liu, K.K., McKee, K.K., Pong, S.S., Chaung, L.L., Elbrecht, A., Dashkevicz, M., Heavens, R., Rigby, M., Sirinathsinghji, D.J.S., Dean, D.C., Melillo, D.G., Patchett, A.A., Nargund, R., Griffin, P.R., De Martino, J.A., Gupta, S.K., Schaeffer, J.M., Smith, R.G. and Van der Ploeg, L.H.T. (1996) Science 273, 974–977.
[3] McKee, K.K., Palyha, O.C., Feighner, S.D., Hreniuk, D.L., Tan, C.P., Phillips, M.S., Smith, R.G., Van der Ploeg, L.H.T. and Howard, A.D. (1997) Mol. Endocrinol. 11, 415–423.
[4] Kojima, M., Hosoda, H., Matsuo, H. and Kangawa, K. (2001) Trends Endocrinol. Tab. 12, 118–122.
[5] Muccioli, G., Papotti, M., Locatelli, V., Ghigo, E. and Deghenghi, R. (2001) J. Endocrinol. Invest. 24, RC7–RC9.
[6] Takaya, K., Ariyasu, H., Kanamoto, N., Iwakura, H., Yoshimoto, A., Harada, M., Mori, K., Komatsu, Y., Usui, T., Shimatsu, A., Ogawa, Y., Hosoda, K., Akamizu, T., Kojima, M., Kangawa, K. and Nakao, K. (2000) J. Clin. Endocrinol. Metab. 85, 4908–4911.
[7] Ghigo, E., Arvat, E., Giordano, R., Broglio, F., Gianotti, L., Maccario, M., Bisi, G., Graziani, A., Papotti, M., Muccioli, G., Deghenghi, R. and Camanni, F. (2001) Endocrine 14, 87–93.
[8] Tschöp, M., Smiley, D.L. and Heiman, M.L. (2000) Nature 407, 908–913.
[9] Wren, A.M., Small, C.J., Ward, H.L., Murphy, K.J., Dakin, C.L., Taheri, S., Kennedy, A.R., Roberts, G.H., Morgan, D.G., Gathei, M.A. and Bloom, S.R. (2000) Endocrinology 141, 4325–4328.
[10] Horvath, T.L., Diano, S., Sotonyi, P., Heiman, M. and Tschöp, M. (2001) Endocrinology 142, 4163–4169.
[11] Korbonits, M., Kaltsas, G., Perry, L.A., Putignano, P., Grossman, A.B., Besser, G.M. and Trainer, P.G. (1999) J. Clin. Endocrinol. Metab. 84, 2489–2495.
[12] Asakawa, A., Inui, A., Kaga, T., Yuzuriha, H., Nagata, T., Fujimiya, M., Katsuura, G., Makino, S., Fujino, M.A. and Kasuga, M. (2001) Neuroendocrinology 74, 143–147.
[13] Ishizaki, S., Murase, T., Sugimura, Y., Kakiya, S., Yokoi, H., Takikawa, K., Arima, H., Miura, Y. and Oiso, Y. (2002) Endocrinology 143, 1589–1593.
[14] Date, Y., Nakazato, M., Hashiguchi, S., Dezaki, K., Mondal, M.S., Hosoda, H., Kojima, M., Kangawa, K., Arima, T., Matsuo, H., Yada, T. and Matsukura, S. (2002) Diabetes 51, 124–129.
[15] Tena-Sempere, M., Barreiro, M.L., Gonzàlez, L.C., Gaytán, F., Zhang, F.P., Caminos, J.E., Pinilla, L., Casanueva, F.F., Diéguez, C. and Aguilar, E. (2002) Endocrinology 143, 717–725.
[16] Papotti, M., Ghè, C., Cassoni, P., Catapano, F., Deghenghi, R., Ghigo, E. and Muccioli, G. (2000) J. Clin. Endocrinol. Metab. 85, 3803–3807.
[17] Gnanapavan, S., Kola, B., Bustin, S.A., Morris, D.G., McGee,

P., Fairclough, P., Bhattacharyya, S., Carpenter, R., Grossman, A.B. and Korbonits, M. (2002) J. Clin. Endocrinol. Metab. 87, 2988–2991.
[18] Barreiro, M.L., Pinilla, L., Aguilar, E. and Tena-Sempere, M. (2002) Eur. J. Endocrinol. 147, 677–688.
[19] Mazzocchi, G., Rossi, G.P., Neri, G., Malendowicz, L.K., Albertin, G. and Nussdorfer, G.G. (1998) FASEB J. 12, 1533–1539.
[20] Belloni, A.S., Rossi, G.P., Andreis, P.G., Neri, G., Albertin, G., Pessina, A.C. and Nussdorfer, G.G. (1996) Hypertension 27, 1153–1159.
[21] Mazzocchi, G., Rossi, G.P., Malendowicz, L.K., Champion, H.C. and Nussdorfer, G.G. (2000) FEBS Lett. 487, 194–198.
[22] Neri, G., Malendowicz, L.K., Andreis, P.G. and Nussdorfer, G.G. (1993) Endocrinology 133, 511–514.
[23] Andreis, P.G., Markowska, A., Champion, H.C., Mazzocchi, G., Malendowicz, L.K. and Nussdorfer, G.G. (2000) Endocrinology 141, 2098–2104.
[24] Nichols, M.R. and Morimoto, B.H. (1999) Arch. Biochem. Biophys. 366, 224–230.
[25] Hayashida, W., Horiuchi, M. and Dzau, V.J. (1996) J. Biol. Chem. 271, 21985–21992.
[26] Guillaume, V., Magnan, E., Cataldi, M., Dutour, A., Sauze, N., Renard, M., Razafindraibe, H., Conte-Devolx, B., Deghenghi, R., Lenaerts, V. and Oliver, C. (1994) Endocrinology 135, 1073–1076.
[27] Nussdorfer, G.G. (1986) Int. Rev. Cytol. 98, 1–405.
[28] Cassoni, P., Papotti, M., Ghé, C., Catapano, F., Sapino, A., Graziani, A., Deghenghi, R., Reissmann, T., Ghigo, E. and Muccioli, G. (2001) J. Clin. Endocrinol. Metab. 86, 1738–1745.
[29] Jeffery, P.L., Herington, A.C. and Chopin, L.K. (2002) J. Endocrinol. 172, R7–R11.
[30] Pettersson, P.L., Muccioli, G., Granata, R., Deghenghi, R., Ghigo, E., Ohlsson, C. and Isgaard, J. (2002) J. Endocrinol. 175, 201–209.
[31] Levitzi, A. and Gilon, C. (1991) Trends Pharmacol. Sci. 12, 171–174.
[32] Alessi, D.R., Cuenda, A., Cohen, P., Dudly, D.T. and Saltiel, A.R. (1995) J. Biol. Chem. 270, 27489–27494.
[33] Campbell, J.S., Seger, R., Graves, J.D., Graves, L.M., Jensen, A.M. and Krebs, E.G. (1995) Recent Progr. Horm. Res. 50, 131–159.
[34] Van Biesen, T., Luttrel, L.M., Hawes, B.E. and Lefkowitz, R.J. (1996) Endocr. Rev. 17, 698–714.
[35] Fanger, G.R. (1999) Histol. Histopathol. 14, 887–894.
[36] Hawes, B.E., Van Biesen, T., Koch, W.J., Luttrel, L.M. and Lefkowitz, R.J. (1995) J. Biol. Chem. 270, 17148–17153.
[37] Han, X.B. and Conn, P.M. (1999) Endocrinology 140, 2241–2251.
[38] Wan, Y. and Huang, X.Y. (1998) J. Biol. Chem. 273, 14533–14537.
[39] Miyamoto, N., Seo, H., Kanda, K., Hidaka, H. and Masui, N. (1992) Endocrinology 130, 3231–3236.
[40] Tian, Y., Smith, R.D., Balla, T. and Catt, K.J. (1998) Endocrinology 139, 1801–1809.
[41] Chijwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T. and Hidaka, H. (1990) J. Biol. Chem. 265, 5267–5272.
[42] Kobayashi, E., Ando, K., Nakano, H., Iida, T., Ohno, H., Morimoto, M. and Tamaoki, T. (1989) J. Antibiot. 42, 1470–1474.
[43] Semplicini, A., Ceolotto, G., Baritono, E., Malendowicz, L.K., Andreis, P.G., Sartori, M., Rossi, G.P. and Nussdorfer, G.G. (2001) J. Hypertens. 19, 599–602.
[44] Nussdorfer, G.G. (1996) Pharmacol. Rev. 48, 495–530.
[45] Delarue, C., Contesse, V., Lenglet, S., Sicard, F., Perraudin, V., Lefebvre, H., Kodjo, M., Leboulenger, F., Yon, L., Gallo-Payet, N. and Vaudry, H. (2001) Rev. Endocr. Metab. Disord. 2, 253–267.