

Autocrine proliferative effect of ghrelin on leukemic HL-60 and THP-1 cells

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Abstract

Ghrelin is a 28 amino acid peptide hormone that is mainly produced by the stomach, but also by several tissues and tumors. Ghrelin is octanoylated on the Ser³, but is also detected as a des-acylated form. Only the acylated ghrelin activates the GH secretagogue receptor (GHS-R) type 1a to stimulate GH release, and regulate food intake and energy metabolism. For the first time, we report that ghrelin and des-acyl ghrelin are present in human promyelocytic HL-60, monocytic THP-1 and lymphoblastic SupT1 cell lines. The human leukemic cell lines did not express the functional GHS-R 1a, whereas they expressed GHS-R 1b, a truncated variant of the receptor. Leukemic cell proliferation was not modified by the addition of octanoylated or des-acyl ghrelins.

However, THP-1 and HL-60 cell proliferations were inhibited by SB801, an antibody directed against the N-terminal octanoylated portion of ghrelin, suggesting that octanoylated ghrelin stimulates cell proliferation via an autocrine pathway involving an as yet unidentified ghrelin receptor. Both octanoylated and des-acyl ghrelins did not alter the basal adenylate cyclase activity. Treatments of THP-1 and SupT1 cells by both octanoylated and des-acyl ghrelins did not modify the adenylate cyclase activity in response to vasoactive intestinal peptide, suggesting that ghrelin is unlikely to modulate the anti-inflammatory and differentiating properties of vasoactive intestinal peptide.

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Introduction

Ghrelin is a 28 amino acid peptide, purified and identified from rat stomach, and characterized by the presence of an *n*-octanoyl modification on the Ser³ residue (Kojima *et al.* 1999). Ghrelin stimulates growth hormone (GH) release from the pituitary (Peino *et al.* 2000, Takaya *et al.* 2000) and regulates food intake and energy metabolism (Tschop *et al.* 2000, Nakazato *et al.* 2001). These biological actions of ghrelin are mainly mediated by the growth hormone secretagogue receptor (GHS-R; Howard *et al.* 1996). Two GHS-R subtypes, generated by alternative splicing of a single gene, have been cloned: the GHS-R type 1a (GHS-R 1a) and the GHS-R type 1b (GHS-R 1b). The human GHS-R 1a consists of 366 amino-acids with seven transmembrane domains. Its activation leads to inositol triphosphate (IP₃) generation and Ca²⁺ release, through activation of the G-protein G₁₁. The GHS-R 1b consists of 289 amino acid residues with only the first five transmembrane domains followed by a 24 amino acid sequence encoded by an alternatively spliced intronic sequence. GHS-R 1b fails to bind GHS and ghrelin, and is not known to exhibit any GHS- or ghrelin-mediated biological activity.

Although only acylated forms of ghrelin bind to the GHS-R 1a and exert endocrine actions, the most abundant circulating form of ghrelin is the des-acyl ghrelin (Hosoda *et al.* 2000).

However, des-acyl ghrelin has been shown to provoke several effects, such as the modulation of cell proliferation in prostate carcinoma cell lines (Cassoni *et al.* 2004), the stimulation of adipogenesis (Thompson *et al.* 2004), the induction of cardiovascular effects (Bedendi *et al.* 2003), and the inhibition of apoptosis in cardiomyocytes and endothelial cells (Baldanzi *et al.* 2002). All these effects are likely mediated by a still unidentified ghrelin receptor.

Ghrelin is mainly produced by the stomach, but also by several tissues such as the intestine, pancreas, kidney, placenta, pituitary, hypothalamus, lung, testis, ovary (Gualillo *et al.* 2003), by tumors, such as pituitary adenomas (Korbonits *et al.* 2001), gastrointestinal carcinoids (Papotti *et al.* 2001), endocrine pancreatic tumors (Volante *et al.* 2002), and by cell lines, such as prostate neoplasms (Jeffery *et al.* 2002, Cassoni *et al.* 2004) and medullary thyroid carcinoma (Kanamoto *et al.* 2001). We recently showed that ghrelin was produced by the erythroleukemic HEL cell line and stimulated cell proliferation by an autocrine pathway involving an as yet unidentified ghrelin receptor (De Vriese *et al.* 2005).

The human HL-60 cell line has been established from the peripheral blood leukocytes of a patient with acute promyelocytic leukemia. These cells are predominantly promyelocytes and display distinct morphological and histochemical myeloid characteristics (Collins *et al.* 1977).

The human THP-1 cell line has been developed from the peripheral blood leukocytes of a patient with acute monocytic leukemia (Tsuchiya *et al.* 1980). The human SupT1 cell line was derived from malignant cells collected from the malignant pleural effusion of a patient with lymphoblastic leukemia. SupT1 cells express multiple T-lineage markers (Smith *et al.* 1984).

Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) are recognized by a family of G-protein-coupled receptors: PAC1 receptor which has a high affinity for PACAP and a much lower affinity for VIP, and VPAC1 and VPAC2 receptors which have a similar affinity for both VIP and PACAP. Functional VPAC1 and VPAC2 receptors are present in THP-1 cells (Delgado & Ganea 2001, Hayez *et al.* 2004), and VPAC2 are present in SupT1 cells (Gourlet *et al.* 1997) respectively. Several biochemical pathways have been shown to be modulated by VIP and PACAP in THP-1 and SupT1 cells (Delgado & Ganea 2001, Hayez *et al.* 2004).

The aims of the present paper were to study the presence of octanoylated and des-acyl ghrelin and GHS-R subtypes in the leukemic cell lines HL-60, THP-1 and SupT1, as well as to study the effects of ghrelin on leukemic cell proliferation and on adenylate cyclase activity in response to VIP.

Materials and Methods

Cell culture of leukemic cell lines

HL-60, THP-1, and SupT1 cells were grown in RPMI-1640 medium (Bio-Whittaker-Europe Verviers, Belgium) supplemented with inactivated fetal bovine serum (10% for HL-60 and THP-1, and 5% for SupT1), 100 UI/ml streptomycin-penicillin and 4 mM glutamine, and routinely passaged twice a week.

Ghrelin (and ghrelin receptors) mRNA detection by RT-PCR

HL-60, THP-1, or SupT1 cells total RNA was extracted using the SV RNA extraction kit (Promega), which includes a DNase treatment. cDNA synthesis was performed using 1 µg total RNA with the Expand Reverse Transcriptase (Roche). The PCR primers used were: human ghrelin sense 5'-AAG-GAGTCTGAAGAAGCCACCA-3' (nucleotide (nt) 148–168) and antisense 5'-GCCAGATGAGCGCTTCTAACTTA-3' (nt 416–439 in Accession no. AB029434, GenBank); human GHS-R 1a and GHS-R 1b sense 5'-TCTTCC-TTCCTGTCTTCTATC-3' (nt 662–682 in Accession no. U60179 and U60181, GenBank); human GHS-R 1a antisense 5'-AGTCTGAACACTGCCACC-3' (nt 993–1010 in Accession no. U60179, GenBank); human GHS-R 1b antisense 5'-TCAGAGAGAAGGGAGAAGG-3' (nt 852–870 in Accession no. U60181, GenBank); human β-actin sense 5'-TGACGGGGTCACCCACACTGTGCCCGTC-3' (nt 539–566) and antisense 5'-CTAGAAGCATTAGC

GGTGGACGATGGAGG-3' (nt 1171–1199 in Accession no. BC002409, GenBank). Amplification of β-actin served as a quality control for the RNA. For ghrelin cDNA amplification, 35 cycles were performed (10 s at 94 °C, 10 s at 57 °C, 1 min at 72 °C). For ghrelin receptor cDNA amplification, 40 cycles were performed (10 s at 94 °C, 20 s at 50 °C and 58 °C for respectively, GHS-R 1a and GHS-R 1b, 45 s at 72 °C). For β-actin amplification, 25 cycles were performed (1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C). Five microliters of each PCR product were submitted to electrophoresis on a 1.4% of agarose gel stained with ethidium bromide and visualized under UV light. For ghrelin, GHS-R 1a, and GHS-R 1b PCRs, positive controls included respectively a pSG5 vector containing human ghrelin cDNA (Tomasetto *et al.* 2000), a pEGFP-N1 vector containing human GHS-R 1a cDNA (Van Craenenbroeck *et al.* 2004), and human placenta cDNA.

Peptide extraction and reverse phase (RP)-HPLC separation

Peptide extraction from leukemic cells or their culture medium and RP-HPLC separation were performed as previously described (De Vriese *et al.* 2005). RP-HPLC fractions were collected, lyophilized, and submitted to RIA.

Peptide synthesis and radioiodination

All the peptides used were synthesized by solid phase methodology using the Fmoc (9-fluorenyl-methoxy-carbonyl) strategy (Gourlet *et al.* 1997) and purified as previously described (De Vriese *et al.* 2005). [Tyr²⁴]-Ghr (1–23) and [Tyr⁰]-Ghr (13–28)-OH were radioiodinated on the tyrosine by the iodogen method (Fraker & Speck 1978) and purified on a Sep-Pak C18 cartridge (Waters, Milford, MA, USA).

RIA for ghrelin

Assays were performed as previously described using radioiodinated [Tyr²⁴]-Ghr (1–23) and [Tyr⁰]-Ghr (13–28)-OH and rabbit polyclonal SB801 and SB969 antibodies, directed respectively towards the synthetic [Cys¹²]-Ghr (1–11) and [Cys⁰]-Ghr (13–28)-OH peptides (De Vriese *et al.* 2005). For RIA using SB801 and SB969 antibodies, the limit of detection for ghrelin were 3 and 7 fmol/assay; the intra-assay coefficients of variation were 3 and 1%; and the peptide recoveries were 97 and 98% respectively.

HL-60, THP-1, and SupT1 cell proliferation studies

HL-60, THP-1, and SupT1 cells were grown in 6-well plates (5 × 10⁵ cells/ml) in RPMI-1640 medium (Bio-Whittaker-Europe) containing 0% (HL-60 and THP-1) or 2% (SupT1) inactivated fetal bovine serum (FBS), 100 UI/ml streptomycin-penicillin and 4 mM glutamine, and incubated at 37 °C for various periods of time with or without octanoylated ghrelin (1 nM, 10 nM, 100 nM, and 1 µM), des-acyl ghrelin (1 nM, 10 nM, 100 nM, and 1 µM), 1%

SB801 or 1% SB969. For SupT1 cells, the addition of at least 2% FBS was necessary to obtain a linear cell growth for 72 h. Appropriate controls were performed for each condition using either water (for the ghrelins) or 1% preimmune rabbit serum (for the antibodies). The ability of the SB801 and SB969 antisera to inhibit the biological activity of ghrelin was verified by performing a dose–effect curve of ghrelin on intracellular calcium increase in Chinese hamster ovary (CHO) cells co-expressing the recombinant GHS-R1a and aequorin (Van Craenenbroeck *et al.* 2004). Under these conditions, 1% of SB801 and SB969 antisera increased 78- and 15-fold the EC₅₀ of ghrelin on intracellular calcium increase respectively. Following incubation, the cells were counted in triplicate in a Coulter multisizer III (Coulter Electronics Limited, Luton, Great Britain).

Adenylate cyclase activity

THP-1 and SupT1 cells were treated without or with 0.1 or 1 µM octanoylated ghrelin or 0.1 or 1 µM des-acyl ghrelin for 24 h. Membranes were prepared from scraped cells lysed in 1 mM NaHCO₃ by immediate freezing in liquid nitrogen. After thawing, the lysate was first centrifuged at 4 °C for 5 min at 400 g, and the supernatant was further centrifuged at 20 000 g for 15 min. The pellet was resuspended in 1 mM NaHCO₃ and used immediately. The adenylate cyclase activity was determined by the Salomon *et al.* (1974) procedure. Briefly, membrane proteins (30–60 µg) were incubated in the absence or presence of increasing concentrations of VIP (0.1 nM–1 µM) in a total volume of 60 µl containing 0.5 mM [α -³²P]ATP, 10 µM GTP, 5 mM MgCl₂, 0.5 mM EGTA, 1 mM cAMP, 1 mM theophylline, 10 mM phospho(enol)pyruvate, 30 µg/ml pyruvate kinase, and 30 mM Tris–HCl at a final pH of 7.8. The reaction was initiated by membrane addition and was terminated after a 15 min incubation at 37 °C by adding 0.5 ml of a 0.5% SDS solution containing 0.5 mM ATP, 0.5 mM cAMP, and 20 000 c.p.m. [³H]cAMP. cAMP was separated from ATP by two successive chromatographies on Dowex 50WX8 and neutral alumina.

Assay for carboxylesterase activity

HL-60, THP-1, and SupT1 cell lysates were prepared as follows: after 24-h culture, cells were sonicated in distilled water for 30 s and centrifuged at 4 °C at 1000 g for 10 min. The supernatant was collected and used for determination of carboxylesterase activity. The carboxylesterase activity was determined by measuring the hydrolysis of α -naphthylacetate (Yang & Dettbarn 1998, Duysen *et al.* 2001). One hundred microliters of cell lysate or 1.5 ml culture medium were preincubated at 37 °C during 20 min with 10 µM eserine to inhibit acetyl- and butyrylcholinesterases and 10 mM EDTA to inhibit paraoxonase, then 10 µl of 0.02 M α -naphthylacetate were added in a 100 mM phosphate buffer, pH 7.0.

The absorbance was measured at 321 nm every 10 min for up to 45 or 120 min. The enzyme activity was calculated as micromoles of the product per minute (U) using the extinction coefficient (2200 M⁻¹/cm) of the product.

Protein assay

Protein concentration was determined using Bradford's method (Bradford 1976).

Data analysis

Data are summarized as means \pm S.E.M. Results were statistically analyzed using MANOVA analysis or paired *t*-test. All the statistical values reported were obtained using GraphPad InStat version 3.02 for Windows (GraphPad Software, San Diego, CA, USA). *P* values less than 0.05 were considered significant.

Results

RT-PCR detection of GHS-R subtypes and ghrelin mRNAs in human HL-60, THP-1, and SupT1 cells

RT-PCR products of the expected size for GHS-R 1b and ghrelin (209 or 292 bp respectively) were detected in HL-60, THP-1, and SupT1 cells (Fig. 1). RT-PCR product of the expected size for GHS-R 1a (349 bp) was not detected in HL-60, THP-1, and SupT1 cells (Fig. 1). Direct sequencing of the PCR products confirmed that they corresponded to GHS-R 1a, GHS-R 1b, and ghrelin (data not shown).

A 24-h treatment of HL-60, THP-1, and SupT1 cells by 0.1 and 1 µM octanoylated ghrelin or des-acyl ghrelin did not alter the GHS-R 1a and GHS-R 1b mRNA expressions (data not shown).

Ghrelin production in HL-60, THP-1, and SupT1 cells

HL-60, THP-1, and SupT1 cells were subjected to peptide extraction and subsequent RP-HPLC analysis followed by RIA using both SB801 and SB969 antisera. In all the cell types studied, an immunoreactive ghrelin peak, detected in RIA using SB801 antiserum, eluted at a position identical to that of human octanoylated ghrelin, and two immunoreactive peaks, detected by RIA using SB969 antiserum, eluted at positions identical to human octanoylated ghrelin and human des-acyl ghrelin.

In HL-60, THP-1, and SupT1 cells, immunoreactive (IR) ghrelin after 24 h of culture eluting at the octanoylated ghrelin position was 15 \pm 10, 7 \pm 4, and 15 \pm 4 fmol/mg protein (*n* = 3) respectively, and IR ghrelin eluting at des-acyl ghrelin position was 4 \pm 1, 0.6 \pm 0.6, and 7 \pm 6 fmol/mg protein (*n* = 3) respectively (Table 1).

IR ghrelin was measured in FBS to determine whether FBS could contribute to IR ghrelin found in the culture

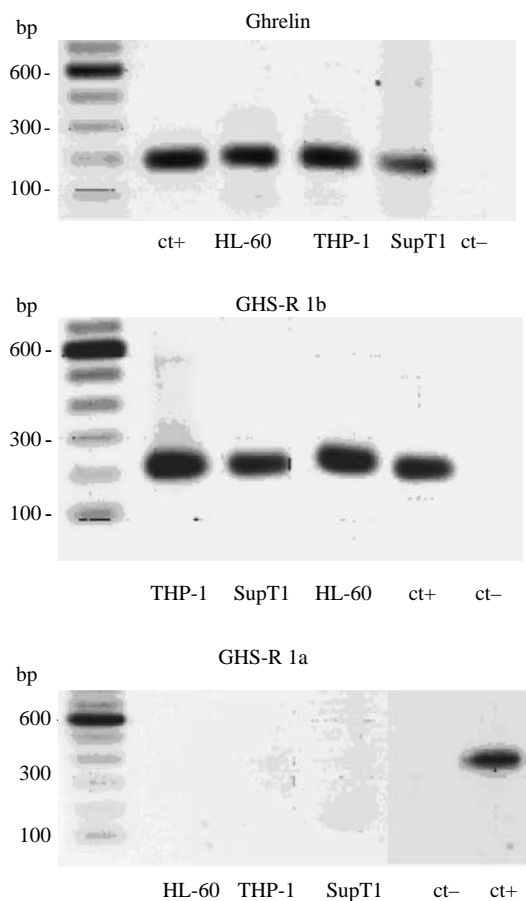


Figure 1 GHS-R subtypes and ghrelin mRNA expression in HL-60, THP-1, and SupT1 cells. RNA extraction, reverse transcription, and PCR were performed as described in Materials and Methods. Positive controls (ct+) were performed using pEGFP-N1 vector containing human GHS-R 1a cDNA diluted at 100 pg/ μ l, human placenta cDNA, or PSG5 vector containing human ghrelin diluted at 10 pg/ μ l. Negative controls (ct-) were performed by omitting cDNA in the PCR mixture.

medium of HL-60, THP-1, and SupT1 cells after 24 h. IR ghrelin assayed with the SB801 and SB969 were 0.8 and 3.7 fmol/mg protein. In HL-60, THP-1, and SupT1 culture medium, IR ghrelin after 24-h culture eluting at the

Table 1 IR ghrelin determination in HL-60, THP-1, and SupT1 cell lines. IR ghrelin was expressed as femtomoles per milligram protein. The results are the mean \pm S.E.M. of $n=3$ experiments

Cell line	Cells		Culture medium	
	Octanoylated ghrelin	Des-acyl ghrelin	Octanoylated ghrelin	Des-acyl ghrelin
HL-60	15 \pm 10	4 \pm 1	10 \pm 3	51 \pm 5
THP-1	7 \pm 4	0.6 \pm 0.6	26 \pm 4	140 \pm 38
SupT1	15 \pm 4	7 \pm 6	37 \pm 11	38 \pm 1

After Sep-Pak extraction, peptides were submitted to HPLC analysis followed by RIA using SB801 and SB969.

octanoylated ghrelin position was 10 \pm 3, 26 \pm 4, and 37 \pm 11 fmol/mg protein ($n=3$) respectively, and IR ghrelin eluting at des-acyl ghrelin position was 51 \pm 5, 140 \pm 38, and 38 \pm 1 fmol/mg protein ($n=3$) respectively (Table 1).

HL-60, THP-1, and SupT1 cell proliferation studies

The cell growth of HL-60 and THP-1 cells incubated with 0% FBS was linear for up to 72 h. For SupT1 cells, the addition of at least 2% FBS was necessary to obtain a linear cell growth for up to 72 h (data not shown).

To explore the effect of ghrelin on HL-60, THP-1, and SupT1 cell proliferations, the cells were incubated with 0% (HL-60 and THP-1) or 2% (SupT1) FBS for 24, 48, or 72 h with or without 1 nM, 10 nM, 100 nM, or 1 μ M octanoylated ghrelin, or 1 nM, 10 nM, 100 nM, or 1 μ M des-acyl ghrelin. Appropriate controls were performed for each condition using either water (for the ghrelins) or 1% preimmune rabbit serum (for the antibodies). Increasing concentrations of octanoylated ghrelin and des-acyl ghrelin had no significant effect on HL-60, THP-1, and SupT1 cell proliferation after 24-, 48-, or 72-h treatment (data not shown).

Leukemic cells were also incubated for 72 h in the presence of 1% preimmune rabbit serum (control condition), 1% SB801, or 1% SB969. The addition of preimmune rabbit serum did not significantly modify the leukemic cell proliferation. The ability of the SB801 and SB969 antisera to inhibit the biological activity of ghrelin was verified by performing a dose-effect curve of ghrelin on intracellular calcium increase in CHO cells co-expressing the recombinant GHS-R 1a and aequorin (Van Craenenbroeck *et al.* 2004). Under these conditions, 1% SB801 and SB969 antisera increased 78- and 15-fold the EC₅₀ of ghrelin on intracellular calcium increase respectively. When compared with the control, the addition of 1% SB801 significantly decreased THP-1 and HL-60 cell proliferation, but did not modify the SupT1 cell proliferation (Table 2). When compared with the control, the addition of 1% SB969 had no significant effect on THP-1, SupT1, and HL-60 cell proliferations (Table 2).

Table 2 Effect of ghrelin antibodies on cell proliferation. Results are expressed as the percentage of cell number at time 0 (mean \pm S.E.M., $n=6$)

	Control	1% SB801	1% SB969
THP-1	276 \pm 4	231 \pm 3*	275 \pm 5
SupT1	168 \pm 3	161 \pm 4	168 \pm 2
HL-60	258 \pm 5	236 \pm 4*	271 \pm 6

THP-1, SupT1, and HL-60 cells (0.5–0.7 \times 10⁶ cells/ml), grown as described in Materials and Methods, were incubated for 72 h in the absence or presence of 1% of SB801 or 1% of SB969. Appropriate controls were performed for each condition using 1% preimmune rabbit serum (for the antibodies). Data were analyzed with paired *t*-test. Data were significantly different when compared with control, * $P < 0.01$.

Effect of ghrelin and des-acyl ghrelin on adenylate cyclase activity

First of all, we determined that 0.1 and 1 μM octanoylated ghrelin and 0.1 and 1 μM des-acyl ghrelin were not able to modulate the basal adenylate cyclase activity of THP-1, SupT1 and HL-60 membranes (data not shown).

Furthermore, a 24-h treatment of THP-1 and SupT1 cells by 0.1 and 1 μM octanoylated ghrelin or des-acyl ghrelin did not statistically modify the parameters characterizing the dose-response curve of VIP on adenylate cyclase activity (basal activity, maximal effect, and log EC₅₀; Table 3). VIP was unable to induce a dose-dependent activation of adenylate cyclase on HL-60 membranes (data not shown).

Determination of carboxylesterase activity in HL-60, THP-1, and SupT1 cell lysates

Since synthetic ghrelin exogenously added to culture medium can be rapidly degraded in the presence of leukemic cells due to cellular carboxylesterase activity (De Vriese *et al.* 2005), we determined if carboxylesterase activity from HL-60, THP-1, and SupT1 cells participated in ghrelin degradation by measuring the carboxylesterase activity in cell lysates. Carboxylesterase activity of HL-60, THP-1, and SupT1 cells was 0.053, 0.184, and 0.027 U/10⁶ cells respectively.

Discussion

This study reports for the first time the expression of ghrelin, GHS-R 1b, but not GHS-R 1a mRNA transcripts in the THP-1 and SupT1 cell lines, and confirms previous data showing the presence of ghrelin mRNA transcripts in the HL-60 cell line (Hattori *et al.* 2001, Dixit *et al.* 2004, Dixit & Taub 2005). Besides, a 24-h treatment of HL-60, THP-1, and SupT1 cells by ghrelin or des-acyl ghrelin did not alter the GHS-R 1b and GHS-R 1a mRNA expression.

To quantify ghrelin production, we used two radioimmunoassays using the SB801 and SB969 antisera (De Vriese *et al.* 2005). SB801 antiserum recognizes all biologically active forms of ghrelin since C-terminal-shortened acyl ghrelins (down to Ghr (1–5)) have been shown to retain their

complete biological activity on the GHS-R 1a, without significant change in their potency (Bednarek *et al.* 2000), while SB969 antiserum allows the measurement of ghrelin, des-acyl ghrelin, as well as N- and to some extent C-terminal-shortened analogs (De Vriese *et al.* 2005). In the literature, one can often notice that no distinction is made between total ghrelin, and octanoylated and des-acyl ghrelins levels.

The human promyelocytic HL-60 and the lymphoblastic SupT1 leukemic cell lines, after 24 h of culture, produced similar quantities of total ghrelin in cells and in their culture medium. However, when compared with HL-60 and SupT1 cell lines respectively, the monocytic THP-1 cell line produced about 2.5- and 2.9-fold less total ghrelin in cells, but about 2.7- and 2.2-fold more total ghrelin in the culture medium. The proportion of octanoylated ghrelin is similar in SupT1 and HL-60 cells (69 and 79% of the total ghrelin respectively), but higher in THP-1 cells (93% of the total ghrelin). In HL-60 and THP-1 culture medium, the proportion of octanoylated ghrelin is identical (16% of the total ghrelin), while it is higher in SupT1 culture medium (49% of the total ghrelin). When compared with HL-60 and THP-1 cells, the higher proportion of octanoylated ghrelin in SupT1 culture medium could be partially explained by a lower ghrelin degradation activity. We previously showed that ghrelin exogenously added to the culture medium of HEL cells was rapidly degraded in the presence of HEL cells (~46% after 1 h) and that most of the ghrelin degradation was due to carboxylesterase activity in HEL cells (De Vriese *et al.* 2005). Since the carboxylesterase activity in SupT1 cells is 2- and 6.8-fold lower than that in HL-60 and THP-1 cells respectively, this could account for the higher proportion of octanoylated ghrelin in SupT1 culture medium. Carboxylesterase expression is strongly associated with cells of the monocytic lineage (Drexler *et al.* 1987, Uphoff *et al.* 1993, 1994) and weakly associated with cells of the lymphocytic lineage (Yourno *et al.* 1982). Our study seems to be in accordance with these data since the carboxylesterase activities of the monocytic THP-1 and promyelocytic HL-60 cell lines were higher than that of the lymphoblastic SupT1 cell line. However, although the carboxylesterase activity of the THP-1 cells is 3.5-fold higher than that of the

Table 3 Effect of ghrelin and des-acyl ghrelin treatment on the adenylate cyclase activity of THP-1 and SupT1 cell membranes. Basal activity (measured in the absence of VIP) and maximal effect (E max; measured in the presence of 1 μM VIP) were expressed as cAMP in picomoles per minute per milligram protein. Log EC₅₀ was expressed as log M. The results are the mean \pm S.E.M. ($n=3$)

	THP-1			SupT1		
	Ct	OGhr	des-OGhr	Ct	OGhr	des-OGhr
Basal activity	98.96 \pm 6.03	87.56 \pm 4.91	85.61 \pm 3.32	9.18 \pm 0.98	9.70 \pm 0.72	9.94 \pm 1.26
E max	153.20 \pm 7.07	136.40 \pm 9.18	129.90 \pm 8.78	29.39 \pm 1.05	26.65 \pm 0.78	34.76 \pm 1.27
Log EC ₅₀	-7.82 \pm 0.32	-7.30 \pm 0.36	-6.99 \pm 0.32	-7.93 \pm 0.13	-7.91 \pm 0.12	-8.00 \pm 0.13

THP-1 and SupT1 cells were treated for 24 h in the absence (Ct) or presence of 0.1 μM octanoylated ghrelin (OGhr) or 0.1 μM des-acyl ghrelin (des-OGhr). Then, the adenylate cyclase activity was determined on THP-1 and SupT1 cell membranes as described in Materials and Methods in the absence (basal activity) or presence of increasing concentrations of VIP (0.1 nM–1 μM).

HL-60 cells, the proportion of octanoylated ghrelin in THP-1 culture medium is identical, and not lower, to the one of HL-60 culture medium (16% of the total ghrelin). Since the total ghrelin in the culture medium of the THP-1 cells is 2.7-fold higher than that in the HL-60 cells, this suggests that ghrelin production could counteract the degradation activity. When considering the rapid degradation of added ghrelin in the culture medium, the amounts of octanoylated and des-acyl ghrelins in FBS would be negligible when compared with those measured in the culture medium of HL-60, THP-1, and SupT1 cells after 24 h.

We showed that HL-60, THP-1, and SupT1 express the ghrelin mRNA transcript and produce some ghrelin. Because ghrelin is known to modulate the proliferation of several cell types, such as prostatic cells, adipocytes, cardiomyocytes (Jeffery *et al.* 2002, Cassoni *et al.* 2004, Thompson *et al.* 2004, De Vriese *et al.* 2005), the HL-60, THP-1, and SupT1 cell proliferations were evaluated by adding exogenous ghrelin, des-acyl ghrelin, or SB801 and SB969 antisera used as ghrelin antagonists. HL-60, THP-1, and SupT1 cell proliferations were not significantly modified by the addition of exogenous ghrelin or des-acyl ghrelin. However, addition of SB801 antiserum significantly decreased THP-1 and HL-60 cell proliferation at 72 h, suggesting that in these cell lines ghrelin stimulates cell proliferation by an autocrine pathway involving an as yet unidentified ghrelin receptor, distinct from GHS-R 1a. Indeed, THP-1, HL-60, and SupT1 express the GHS-R 1b mRNA transcript but not GHS-R 1a mRNA transcript. An autocrine-proliferative effect of ghrelin has also been shown in the erythroleukemic HEL cells (De Vriese *et al.* 2005). In SupT1 cells, ghrelin does not seem to be involved in the control of cell proliferation.

VIP has been identified as being a potent anti-inflammatory factor since it directly inhibits the production of pro-inflammatory cytokines, upregulates interleukin-10 production (a potent anti-inflammatory cytokine), or inhibits the expression and release of pro-inflammatory chemokines (Gonzalez-Rey & Delgado 2005). VIP also acts as a negative regulatory signal in the differentiation of leukemic cells (Sherman *et al.* 1988). VIP/PACAP receptors have been identified in cells of hematopoietic origin, including THP-1 cells, expressing functional VPAC1 and VPAC2 receptors (Delgado & Ganea 2001, Hayez *et al.* 2004), and SupT1 cells, expressing VPAC2 receptors (Gourlet *et al.* 1997). The occupation of VIP/PACAP receptors leads mainly to the activation of adenylate cyclase and cAMP production. In addition to stimulating phospholipase C, ghrelin has been reported to stimulate adenylate cyclase in somatotropes (Malagon *et al.* 2003). Both octanoylated ghrelin and des-acyl ghrelin were unable to stimulate the adenylate cyclase activity on THP-1 and SupT1 membranes (data not shown). Since an increase in the cAMP level can result from a rise in the intracellular calcium concentration, we also determined the effect of octanoylated ghrelin and des-acyl ghrelin on cAMP levels, but neither peptide had any effect (data not shown). Besides, a 24-h treatment of THP-1 and SupT1 cells by

octanoylated ghrelin or des-acyl ghrelin did not significantly modify the dose-response curve of VIP on either adenylate cyclase activity (Table 3) or cAMP levels (data not shown). Therefore, neither octanoylated nor des-acyl ghrelin cannot modulate the adenylate cyclase pathway (adenylate cyclase, cAMP) activated by VIP in THP-1 and SupT1 cells. These data suggest that both octanoylated and des-acyl ghrelins are very unlikely to modulate the anti-inflammatory and differentiating properties of VIP.

In summary, ghrelin and GHS-R 1b mRNAs were detected by RT-PCR in the human HL-60, THP-1, and SupT1 cell lines. GHS-R 1a mRNA was not detected in the human leukemic cell lines. The expression of GHS-R 1a and GHS-R 1b mRNA was not modified by a 24-h treatment with ghrelin or des-acyl ghrelin. Ghrelin was produced in low quantities in HL-60, THP-1, and SupT1 cells, and in the culture medium. Ghrelin exerted an autocrine-proliferative effect on THP-1 and HL-60 cells via an as yet unidentified ghrelin receptor. Ghrelin did not modulate the adenylate cyclase/cAMP intracellular pathway, and was unable to modify the VIP-induced adenylate cyclase activity, suggesting that ghrelin is unlikely to modulate the anti-inflammatory and differentiating properties of VIP.

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