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Ghrelin and des-acyl ghrelin both inhibit isoproterenol-induced lipolysis in rat adipocytes via a non-type 1a growth hormone secretagogue receptor

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Abstract

Besides possessing a strong growth hormone (GH)-releasing activity, the gastrointestinal octanoylated peptide ghrelin has been reported to antagonize lipolysis in rat adipocytes. It is not yet clear whether this inhibitory activity on lipolysis is also shared by the major circulating isoform, des-acyl ghrelin, that does not activate the ghrelin receptor, namely the type 1a GH secretagogue-receptor (GHS-R1a) and lacks the endocrine effects of the acylated form. Here we show that des-acyl ghrelin, like ghrelin and some synthetic GHS (hexarelin and MK0677) and carboxy-terminally ghrelin fragments such as ghrelin-(1-5) and ghrelin-(1-10), all significantly reduced, over concentrations ranging from 1 to 1000 nM, the stimulation of glycerol release caused in rat epididymal adipocytes by the nonselective β-adrenoceptor agonist isoproterenol in vitro. The order of potency on stimulated-lipolysis was: des-acyl ghrelin=ghrelin>MK0677=hexarelin>ghrelin-(1-5)=ghrelin-(1-10). This ranking was consistent with the binding experiments performed on membranes of epididymal adipose tissue or isolated adipocytes that did not express mRNA for GHS-R1a. A common high-affinity binding site was recognized in these cells by both acylated and des-acylated ghrelin and also by hexarelin, MK0677, ghrelin-(1-5) and ghrelin-(1-10). In conclusion, these findings provide the first evidence that des-acyl ghrelin, as well as ghrelin, short ghrelin fragments and synthetic GHS, may act directly as antilipolytic factors on the adipose tissue through binding to a specific receptor which is distinct from GHS-R1a.

Keywords: Des-acyl ghrelin; Ghrelin; Growth hormone secretagogue receptor; Rat adipocyte; Isoproterenol-induced lipolysis

1. Introduction

Ghrelin, a 28 amino acid octanoylated peptide, is predominantly produced by the stomach, but also expressed in several other tissues and exerts a wide spectrum of actions (Broglio et al., 2003b; Gualillo et al., 2003). Besides a potent growth hormone (GH)-releasing effect, ghrelin has emerged as an orexigenic factor probably able to trigger meal initiation and to signal the status of energy balance to the central nervous system (Muccioli et al., 2002; Woods, 2004). Circulating ghrelin levels are increased by fasting and energy restriction and decreased by meals, glucose load, insulin and somatostatin (Muccioli et al., 2002; Broglio et al., 2003c, Deghenghi et al., 2003). Ghrelin promotes food intake antagonizing leptin action through the activation of the hypothalamic orexin neurons, neuropeptide Y-Y1 receptor pathway and over-expressing the Agouti-related protein (Cowley et al., 2003; Kohno et al., 2003; Olszewski et al., 2003). Interactions with proopiomelanocortin, corticotropin-releasing hormone, orexins (hypocretins) and endocannabinoids have also been reported (Druce and Bloom, 2003; Funahashi et al., 2003; Kohno et al., 2003). In addition, ghrelin enhances weight gain and fat deposition by mechanisms which include central modulation of energy balance (Muccioli et al., 2002) and, probably, also peripheral actions, such as adipogenetic and antilipolytic

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effects (Choi et al., 2003; Thompson et al., 2004). The stimulatory effect of ghrelin on GH/insulin-like growth factor-I axis would further contribute to its anabolic action (Ghigo et al., 1998). The central and endocrine actions of ghrelin are mediated by the type 1a GH secretagogue receptor (GHS-R1a) and acylation at Serine 3 is essential for hormone's binding to this receptor (Kojima et al., 2001). On the other hand, the GHS-R1a is specific also for a family of synthetic peptidyl (hexarelin) and non-peptidyl (MK0677) (Smith et al., 1997). Des-acyl ghrelin, the major circulating form of ghrelin (Hosoda et al., 2000), is devoid of any GHS-R1a binding affinity and GH-releasing activity (Bednarek et al., 2000; Muccioli et al., 2001, Broglio et al., 2003a) and because of this it was considered to be biologically inactive. However, recent studies have established that des-acyl ghrelin exerts the same biological activities of ghrelin in rat H9c2 cardiomyocytes (Baldanzi et al., 2002), guinea pig cardiac papillary muscle (Bedendi et al., 2003) and in different human cancer cell lines (Cassoni et al., 2001, 2004) via activation of a receptor, common to both the acylated and the des-acylated ghrelin forms, that is distinct from the GHS-R1a. Recently, it has been reported that ghrelin is able to reduce the lipolytic action of the nonselective β-adrenoceptor agonist isoproterenol in rat adipocytes in vitro (Choi et al., 2003) and that des-acyl ghrelin, as well as ghrelin, promote adipogenesis in vivo (Thompson et al., 2004). We have now studied the effect of des-acyl ghrelin on the isoproterenol-induced lipolysis in adipocytes isolated from rat epididymal (i.e. visceral) adipose tissue and compared its activity with that of ghrelin and some synthetic GHS (MK0677 and hexarelin) and carboxy-terminally truncated ghrelin fragments, such as ghrelin-(1-5) and ghrelin-(1-10). In addition, we have assessed the binding activity of the above compounds to β-adrenoceptors of epididymal fat pads, as well as the expression of GHS-R1a mRNA and the presence of other specific ghrelin binding sites in this tissue or adipocytes by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis and by radioreceptor assay, respectively.

2. Materials and methods

2.1. Materials

Rat ghrelin (Gly-Ser-Ser-(O *n*-octanoyl)-Phe-Leu-Ser-Pro-Glu-His-Gln-Lys-Ala-Gln-Gln-Arg-Lys-Glu-Ser-Lys-Lys-Pro-Pro-Ala-Lys-Leu-Gln-Pro-Arg-NH_2) was purchased from Tocris Cookson (Avonmouth, Bristol, UK), whereas rat des-acyl ghrelin was from Phoenix Pharmaceuticals (Belmont, CA, USA). Rat Tyr^4-ghrelin, hexarelin (His-D-2Me-Trp-Ala-Trp-D-Phe-Lys-NH_2), MK0677 (*N*-[(1R){[1,2-dihydro-1-methanesulphonylspiro-(^{3}H-indole-3,4′ -piperidin)-1′ -yl]-2-(phenyl-methoxy)-ethyl}-2-amino-2-methylpropanamide methane sulphonate]), ghrelin-(1-5) and ghrelin-(1-10) were purchased from Neosystem (Strasbourg, France). [^{125}I]Tyr^4-ghrelin (1800–2000 Ci/mmol) was iodinated using a lactoperoxidase method and purified by reverse-phase high-performance liquid chromatography, as previously described (Muccioli et al., 2001). Radioiodinated (±)-cyanopindolol ([^{125}I]CYP) was purchased from Perkin-Elmer Life Sciences and Analytical Instruments (Monza, Italy) at a specific activity of 2200 Ci/mmol. Enzyme-linked immunosorbent assay kit for acylated ghrelin measurement was purchased from Linco Research (St. Charles, MO, USA). Free Glycerol Reagent for glycerol measurement, collagenase type I, glycerol, (±)-isoproterenol, (±)-propranolol and other chemical reagents were purchased from Sigma Aldrich (Milan, Italy).

2.2. Animals and preparation of adipocytes

Male Wistar rats (6 week old; Harlan Italy, San Pietro al Natisone, Udine, Italy), weighing 150–160 g, were used. They has free access to food and tap water in an animal room, which was maintained at 24±1 °C, 55±10% humidity with a 12-h light–dark cycle. Animals were approved for use by the Ministero della Salute (Rome, Italy) and the projects was supervised by the local ethical committee. Rats were housed and sacrificed in accordance with the European Community guidelines for care and use of animals. Approximately 2–3 g of the epididymal fat, known to be particularly sensitive to norepinephrine-stimulated lipolysis (Morimoto et al., 1997), were surgically removed from CO_2-anesthetized rats. A sample of adipose tissue, free from the connective tissue and blood vessels, was minced and suspended in Krebs–Ringer solution (120 mM NaCl, 5 mM KCl, 1.25 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.5 mM NaH_2PO_4, 0.7 mM Na_2HPO_4) containing 25 mM HEPES, 5.5 mM glucose, 1% bovine serum albumin (essentially, fatty acid-free) and 0.5 mg/ml collagenase; the buffer was adjusted to pH 7.4 after addition of bovine serum albumin. The adipose tissue suspension was incubated for 40 min at 37 °C and adipocytes were isolated as described by Zalatan et al. (2001). The quality of the adipocytes prepared was assessed by histological examination. About 30% of cells contained multilocular vesicles resembling lipid droplets typical of mature adipocytes. The presence of lipid within the intracellular vesicles was confirmed by histological staining with Sudan black and oil red O. The remaining cells were epitheloid in appearance and were regarded as preadipocytes. Cell viability, tested by means of vital dye (trypan blue), was more than 90% in all adipocyte preparations and no significant loss of cell viability was observed before or after 3 h of incubation at 37 °C. Adipocytes were immediately used to study in vitro lipolysis. The remaining fat cells preparation and a sample of the removed adipose tissue was used for RT-PCR or homogenized to obtain crude membrane preparation for β-adrenoceptor assay and ghrelin binding studies.

2.3. Incubation experiments for lipolysis and glycerol assay

Incubation experiments were performed as previously described (Zalatan et al., 2001) and the lipolysis was determined by measuring the level of glycerol released by adipocytes in the incubation medium. Briefly, lipolysis of isolated adipocytes was induced by incubating about 200,000 cells in Krebs–Ringer solution (incubation medium) containing high amount (4%) of essentially, fatty acid-free bovine serum albumin that improved glycerol release by isoproterenol. Fat cells were incubated in the presence of a submaximal concentration (40 nM) of isoproterenol, and the effect of des-acyl ghrelin, ghrelin, MK0677, hexarelin, ghrelin-(1-5) and ghrelin-(1-10) were tested by including them at different concentrations (ranging from 1 to 1000 nM) in the incubation medium, both in the absence (basal lipolysis) and in the presence of isoproterenol (stimulated lipolysis). Since the effects of some substances tested on the stimulation on glycerol release by isoproterenol could be limited by drug binding to the albumin added to the incubation medium, medium without bovine serum albumin was used in some experiments with ghrelin and its short truncated fragments. Samples were incubated at 37 °C in borosilicate tubes with gentle shaking for 3 h, a time needed to reach a steady increase in glycerol release by isoproterenol (Zalatan et al., 2001; Muccioli et al., unpublished results). Incubation was stopped by placing tubes on ice and aliquots of incubation medium were removed after centrifugation. The aliquots were incubated at 60 °C for 20 min to inactivate any residual enzymatic activity and stored at −20 °C until glycerol measurements. Glycerol levels (mean value of triplicate determinations) were measured using the Free Glycerol Reagent and expressed relative to the cellular protein content. The half-maximal effective concentration (EC_{50}) for inhibition of isoproterenol-induced lipolysis was determined for each compound under study by a GrapPad Prism 4 program (GraphPad Software, San Diego, CA, USA). In some assays, exogenous glycerol (1 mM) was added to cell samples with or without ghrelin or des-acyl ghrelin at 1000 nM concentration to test whether these substances were able to change glycerol levels in the incubation medium by other mechanisms than modulating lipolysis, for example by influencing cellular uptake and/or metabolism of glycerol.

2.4. Procedure for detection acylated ghrelin in cell incubation medium

Synthetic rat ghrelin lyophilized was dissolved in the incubation medium at concentrations ranging from 1 to 1000 nM and subjected to cell incubation conditions in the absence or in the presence of adipocytes. The media were collected at the initial and at the end (3 h) of experiments and subjected to enzyme-linked immunosorbent assay (ELISA) for acylated ghrelin measurement. This ELISA kit recognizes the octanoyl-modified portion of ghrelin, but not its des-acylated form, and is manufactured using the highly specific antibody pairs generated by Dr. Kenji Kangawa and by following his protocol.

2.5. β-Adrenoceptor assay

β-Adrenoceptors in the adipose tissue were assayed as previously described (Bojanic and Nahorsky, 1983), using the nonselective β-adrenoceptor antagonist [^{125}I]CYP as a ligand. Tissue membranes (200 μg of membrane protein from 50,000×*g* pellet, measured by the method of Lowry) were incubated with [^{125}I]CYP 100 pM at 37 °C for 60 min and appropriate concentrations of competitors in 25 mM Tris–HCl, pH 7.8, 154 mM NaCl, 1.1 mM ascorbic acid in a final incubation volume of 0.5 ml. The binding reaction was terminated following the procedure described by Bojanic and Nahorsky (1983) and the radioactivity remaining bound to the filters was measured by a Packard gamma counter A5003. Specific binding was defined as the total binding minus nonspecific binding. Nonspecific binding was determined by incubation of ligand and membranes in the presence of 200 μM (±)-isoproterenol. Specific binding constituted 60–70% of total binding. The concentration of the competitor causing 50% inhibition of specific radioligand binding (IC_{50}) was calculated by the GraphPad Prism 4 software.

2.6. RT-PCR analysis for GHS-R1a

RT-PCR was performed to detect mRNA for GHS-R1a. Total RNA extraction from rat hypothalamus and adipose tissue and complementary DNA transcription were performed as described elsewhere (Pettersson et al., 2002). The forward and reverse primer sequences for GHS-R1a were 5′ -GTCGAGCGCTACTTCGC-3′ and 5′ -GTACTGGCTGATCTGAGC-3′ , respectively (Yokote et al., 1998). The GHS-R primers were located on different exons. After an initial denaturation step (95 °C for 8 min), 32 cycles of PCR were carried out in a 50 μl volume with AmpliTaq Gold Polymerase in a GeneAmp PCR System (Perkin Elmer, Milan, Italy) under the following conditions: one cycle, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, followed by 72 °C for further 7 min for elongation. The PCR products were electrophoresed in 1.5% agarose gel and visualized by propidium iodide staining. Positive control included hypothalamic tissue, whereas a negative control was carried out by using buffer alone with no RNA. Amplification for β-actin served as internal control for the RNA samples.

2.7. Ghrelin binding assay

Ghrelin binding to membranes (30,000×*g* pellet) isolated from adipose tissue or binding to isolated adipocytes were carried out as previously described (Bedendi et al., 2003), using [^{125}I]Tyr^{4}-ghrelin as a radioligand. Tyr^{4}-ghrelin has been reported to have in vivo the same GH-

releasing potency of native ghrelin and to be a reliable probe for labelling GHS-R in tissue membranes (Muccioli et al., 2001; Bedendi et al., 2003, Cassoni et al., 2004). Receptor binding saturation studies were conducted by incubating in triplicate either whole adipose tissue or adipocyte membranes (100 μg of membrane protein, measured by the method of Lowry) at 23 °C for 2 h under constant shaking with increasing concentrations (from 0.03 to 3 nM) of radioligand in the absence and in the presence of a fixed concentration (2000 nM) of unlabelled ghrelin. Binding experiments were performed either in assay buffer containing or not bovine serum albumin. Saturation isotherms were analysed using a GraphPad Prism 4 iterative nonlinear curve fitting program and binding data were transformed by the method of Scatchard to calculate the dissociation constant (K_d) and the maximal binding capacity (B_{max}) values. To establish binding site specificity, increasing concentrations of ghrelin, des-acyl ghrelin and other substances studied in experiments for lipolysis were tested in competition assays with [^{125}I]Tyr^4-ghrelin and the IC_{50} values were calculated by the GraphPad Prism 4 software.

2.8. Statistical analysis

All results are expressed as mean values±S.E.M. Statistical analysis was carried out with one-way analysis of variance followed by Student's two-tailed *t*-test or Newman–Keuls multiple range test depending on the experiments. $P<0.05$ was chosen as level of significance. The number of experiments is indicated by n.

3. Results

3.1. Effect of ghrelin on fat cell lipolysis induced by isoproterenol in vitro and stability of ghrelin at the initial and the end of experiments

Addition of isoproterenol (40 nM) to rat adipocytes freshly isolated from epididymal fat pads significantly stimulated, after 3 h incubation, the release of glycerol (an index of lipolysis) to a level 5.6-fold greater than in the control group (Table 1). No significant effect on basal glycerol levels was observed when adipocytes were incubated in the presence of ghrelin alone at all the concentrations tested. However, when added simultaneously with isoproterenol in the incubation medium containing 4% bovine serum albumin, ghrelin significantly reduced, in a dose-dependent fashion, the isoproterenol-stimulated glycerol release (Table 1) within a range of concentrations (from 1 to 1000 nM) that have been found to be effective in stimulating GH release in vitro (Kojima et al., 1999). The calculated half-maximal effective ghrelin concentrations (EC_{50}), expressed as mean±S.E.M., n=5, were 8.9±0.9 nM. Because the ester bond is both chemically and enzymatically unstable and elimination of the octanoyl

Table 1
Effect of ghrelin on glycerol release (an index of lipolysis) stimulated by isoproterenol (40 nM) in isolated rat epididymal adipocytes

Treatment	Concentration			
	1 nM	10 nM	100 nM	1000 nM
Control	100			
Isoproterenol	563±13[a]			
Ghrelin	97±2	104±5	95±6	98±4
Isoproterenol+ghrelin	506±32	426±30[b]	366±30[c]	340±29[c]

All data are expressed as percentages of the basal rate of 100% (28±2 nmol glycerol/mg protein) in the control group. The data represent the mean±S.E.M. of five independent experiments.
[a] $P<0.001$ vs. control.
[b] $P<0.05$.
[c] $P<0.001$ vs. isoproterenol alone.

modification of ghrelin has been reported to occur during storage, handling, dissolution in the incubation medium or when ghrelin is added to cell cultures (Kanamoto et al., 2001; Hosoda et al., 2004), we measured the residual quantity of the acylated form of ghrelin exogenously added to the incubation medium at the initial and the end of experiments in the absence and in the presence of rat adipocytes. As shown in Table 2, 1, 10, 100 and 1000 nM acylated ghrelin markedly decreased to 0.108, 1.10, 9.68 and 92.1 nM, respectively when lyophilized synthetic ghrelin was reconstituted, kept frozen at −30 °C for 2 months and subjected to ELISA measurement. These results indicate (i) that the residual quantity of acylated ghrelin present in the incubation medium was ~10% of total ghrelin added and (ii) that, as also reported by Kanamoto et al. (2001) and Hosoda et al. (2004), des-acylation of ghrelin could occur during the storage, handling and/or dissolving in the medium. After 3 h of incubation at 37 °C about 73–80% of acylated ghrelin was degraded in the medium. In the presence of adipocytes, the degradation was more prominent, suggesting that fat cells themselves did augment the des-acylation process (Table 2).

3.2. Effect of des-acyl ghrelin, synthetic GHS and short ghrelin fragments on fat cell lipolysis induced by isoproterenol in vitro

Des-acyl ghrelin possessed an antilipolytic efficacy very close to that of ghrelin (see Table 1) and slightly greater than MK0677 or hexarelin (Table 3). The EC_{50} concentrations, all expressed as nM values (mean±S.E.M., n=5) were 9.1±1.7 for des-acyl ghrelin and about two times greater for both MK0677 (18.8±1.5; $P<0.001$ compared to des-acyl ghrelin) and hexarelin (22.6±1.5, $P<0.001$ compared to des-acyl ghrelin). In contrast, no inhibition of glycerol release was observed when some carboxy-terminally truncated ghrelin fragments, such as ghrelin-(1-5) and ghrelin-(1-10), were added simultaneously with isoproterenol in the incubation medium containing 4% bovine serum albumin (Table 3). Surprisingly, when ghrelin-(1-5) and ghrelin-(1-10) were incubated in medium without albumin, both

Table 2
Residual acylated ghrelin detected in the incubation medium at the initial (0 h) and at the end (3 h) of the experiment in the absence and in the presence of adipocytes

Ghrelin added (nM)	Acylated ghrelin (nM)			
	Medium only		Adipocytes	
	0 h	3 h	0 h	3 h
1	0.108±0.010	0.026±0.003[a]	0.108±0.01	0.014±0.02[b]
10	1.10±0.088	0.29±0.05[a]	1.10±0.088	0.16±0.036[b]
100	9.68±0.80	2.35±0.37[a]	9.68±0.80	1.33±0.26[b]
1000	92.1±5.12	17.9±1.72[a]	92.1±5.12	9.30±0.99[b]

[a] $P<0.001$ vs. 0 h medium only.
[b] $P<0.001$ vs. 3 h medium only.

peptides resulted effective, but much less potent than ghrelin in inhibiting glycerol release induced by isoproterenol (Table 4). The calculated EC_{50}, all expressed as nM values (mean±S.E.M., n=5), were 7.3±1.5 for ghrelin, 35.2±4.5 for ghrelin-(1-5) and 29.7±2.9 for ghrelin-(1-10). This finding suggests that the need to add high amount of bovine serum albumin to the incubation medium may involve binding of acylated ghrelin fragments to this protein and could explain the lack of inhibition of these substances on β-adrenergic lipolysis observed in the presence of bovine serum albumin. Consistent with this hypothesis are also the observations that antilipolytic prostaglandins, such as PGE_2 (Richelsen et al., 1984) that displays a strong binding to albumin (Brown and Collier, 1975), had a slight and not reproducible isoproterenol antagonism in medium containing bovine serum albumin, whereas it showed a gradual and significant dose-dependent inhibition of isoproterenol-stimulated lipolysis in medium devoid of bovine serum albumin, with the highest effect at 10 nM concentration (data not shown).

3.3. Effect of ghrelin and des-acyl ghrelin on cellular glycerol uptake

When exogenous glycerol (1 mM) was added to adipocytes in culture, ghrelin or des-acyl ghrelin, at a concentration (1000 nM) that caused the highest inhibition on glycerol release by isoproterenol, did not modify, after 3 h incubation, the glycerol levels in the incubation medium (data not shown). This result indicates that ghrelin reduced glycerol levels in the medium by inhibiting isoproterenol-induced lipolysis and not by stimulating cellular uptake and/or metabolism of glycerol.

3.4. Binding activity of ghrelin and des-acyl ghrelin to β-adrenoceptors of the adipose tissue

To assess whether the inhibitory effect of ghrelin and des-acylated ghrelin on the isoproterenol-induced lipolysis

Table 3
Effect of des-acyl ghrelin, MK0677, hexarelin and some truncated ghrelin fragments on glycerol release (an index of lipolysis) stimulated by isoproterenol (40 nM) in isolated rat epididymal adipocytes

Treatment	Concentration			
	1 nM	10 nM	100 nM	1000 nM
Control	100			
Isoproterenol	563±13[a]			
Isoproterenol+ des-acyl ghrelin	503±11	427±17[b]	372±18[d]	348±14[d]
Isoproterenol+ MK0677	536±19	473±36	404±28[c]	373±29[d]
Isoproterenol+ hexarelin	548±21	480±34	402±26[c]	377±27[d]
Isoproterenol+ ghrelin-(1-5)	554±29	560±26	534±35	556±22
Isoproterenol+ ghrelin-(1-10)	572±32	545±30	552±24	533±32

All data are expressed as percentages of the basal rate of 100% (28±2 nmol glycerol/mg protein) in the control group. The data represent the mean±S.E.M. of five independent experiments.
[a] $P<0.001$ vs. control.
[b] $P<0.05$.
[c] $P<0.01$.
[d] $P<0.001$ vs. isoproterenol alone.

Table 4
Effect of ghrelin and some truncated ghrelin fragments on glycerol release (an index of lipolysis) stimulated by isoproterenol (40 nM) in isolated rat epididymal adipocytes incubated in medium without bovine serum albumin

Treatment	Concentration			
	1 nM	10 nM	100 nM	1000 nM
Control	100			
Isoproterenol	227±16[a]			
Isoproterenol+ ghrelin	191±13	163±10[b]	139±7[d]	127±7[d]
Isoproterenol+ ghrelin-(1-5)	215±11	202±11	180±8[b]	165±8[c]
Isoproterenol+ ghrelin-(1-10)	212±11	195±6	175±8[b]	159±5[d]

All data are expressed as percentages of the basal rate of 100% (14±2 nmol glycerol/mg protein) in the control group. The data represent the mean±S.E.M. of four independent experiments.
[a] $P<0.001$ vs. control.
[b] $P<0.05$.
[c] $P<0.01$.
[d] $P<0.001$ vs. isoproterenol alone.

was related to their direct interaction with β-adrenoceptors, we have evaluated the ability of these peptides to inhibit the binding of the nonselective β-adrenoceptor antagonist [^{125}I]CYP to epididymal fat pad membranes. As shown in Fig. 1, the binding of [^{125}I]CYP was inhibited in a dose dependent manner by isoproterenol (IC_{50}=0.74±0.12 μM, mean±S.E.M., n=3) and by the nonselective β-adrenoceptor antagonist propranolol (IC_{50}=7.0±0.08 nM, mean±S.E.M. n=3), but not by ghrelin or des-acyl ghrelin (Fig. 1). This finding excludes the possibility of an interaction of ghrelin and des-acyl ghrelin with β-adrenoceptors and supports the view that the antilipolytic action of these substances could be related to a different mechanism of action.

3.5. Expression of GHS-R1a mRNA and ghrelin binding studies in the adipose tissue and adipocytes

To investigate the identity of the receptor mediating the antilipolytic action of ghrelin, des-acyl ghrelin, carboxy-terminally truncated ghrelin fragments and synthetic GHS, we have assayed the expression of GHS-R1a mRNA and the presence of other specific ghrelin binding sites in the epididymal fat or isolated adipocytes by RT-PCR analysis and by radioreceptor assay, respectively. After RT-PCR using the primer sequence described by Yokote et al. (1998) (Fig. 2), a 492 base pairs signal corresponding to rat GHS-R1a was observed (lane 2) in a classic ghrelin target tissue, such as the hypothalamus (positive control). In contrast, no signal corresponding to GHS-R1a was detected in the epididymal fat tissue (lane 3) or when buffer alone was used as the negative control (lane 1). Analysis of mRNA by means of a GHS-R1a primer reported by Choi et al. (2003)

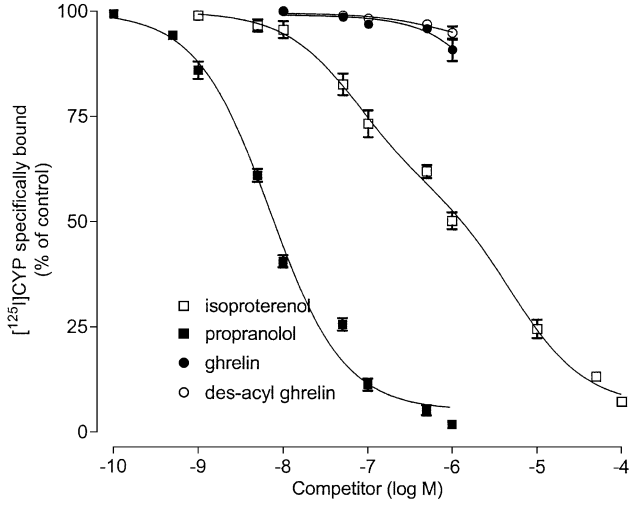

Fig. 1. Competition for [^{125}I]CYP binding to membranes of epididymal adipose tissue by isoproterenol, propranolol, ghrelin and des-acyl ghrelin. Binding assays were conducted as described in Materials and methods. The ordinate represents binding as a percentage of control (specific binding in the absence of unlabelled competitor). Each point represents the mean±S.E.M. of three independent experiments.

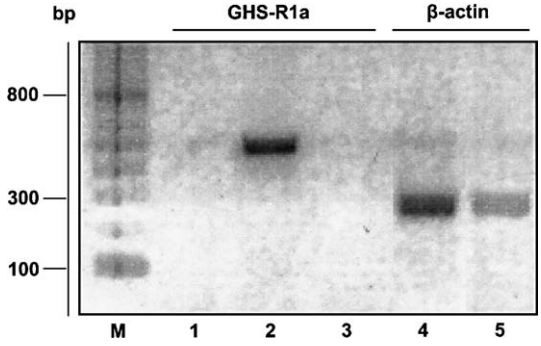

Fig. 2. RT-PCR analysis of mRNA expression for rat GHS-R1a in rat epididymal adipose tissue. The PCR products were separated by agarose gel (1.5%) electrophoresis and stained with propidium iodide. The amplified products for GHS-R1a and for β-actin were 492 base pairs (bp) and 230 bp, respectively. M, 100 bp ladder. Buffer alone was used as negative control (lane 1) and rat hypothalamus as positive control (lane 2). Lane 3: rat adipose tissue. β-Actin was used as an internal control for equivalent amounts of amplified samples (lane 4 for hypothalamus and 5 for adipose tissue). Shown is representative RT-PCR from three independent experiments on three different RNA samples.

confirmed the RT-PCR negativity in this adipose tissue (results not shown). Surprisingly, binding experiments with increasing concentrations of [^{125}I]Tyr^4-ghrelin (from 0.035 to 3 nM) revealed the existence of a saturable single class of ghrelin binding sites in the epididymal adipose tissue, with a K_d (0.48±0.06 nM) and a B_{max} (12.3±1.3 fmol/mg protein, mean±S.E.M., n=5) quite close to those found in other ghrelin target tissues, such as pituitary gland and cardiovascular tissues (Muccioli et al., 2001; Katugampola et al., 2001, Bedendi et al., 2003). The specificity of [^{125}I]Tyr^4-ghrelin binding to membranes from adipose tissue was assessed by competitive binding studies with the compounds tested on the isoproterenol-induced lipolysis. In these experiments, both unlabelled acylated and des-acylated ghrelin forms, as well as MK0677 and hexarelin competed in a dose-dependent manner with [^{125}I]Tyr^4-ghrelin for such binding sites, but MK0677 and hexarelin were significantly less potent than ghrelin or des-acyl ghrelin (Fig. 3A). The IC_{50} values calculated from competition binding studies and expressed as nM concentrations (mean±S.E.M. n=4) were 5.9±0.7 for ghrelin, 6.5±0.5 for des-acyl ghrelin, but only 10.8±0.9 for MK0677 and 14.8±2.2 for hexarelin. In contrast, ghrelin-(1-5) and ghrelin-(1-10), at maximal concentration tested, were able to displace only 26–34% of the specifically bound of radiolabelled ghrelin. However, when the truncated ghrelin fragments were tested in the assay buffer devoid of bovine serum albumin, a dose-dependent inhibition of binding was seen with both compounds (Fig. 3B). In this assay condition, ghrelin-(1-5) and ghrelin-(1-10) displaced [^{125}I]Tyr^4-ghrelin with equal efficacy, but they were significantly less potent (six to eight times) than ghrelin, a result consistent with their antilipolytic effect observed only in medium without bovine serum albumin (see Table 4). The pattern of

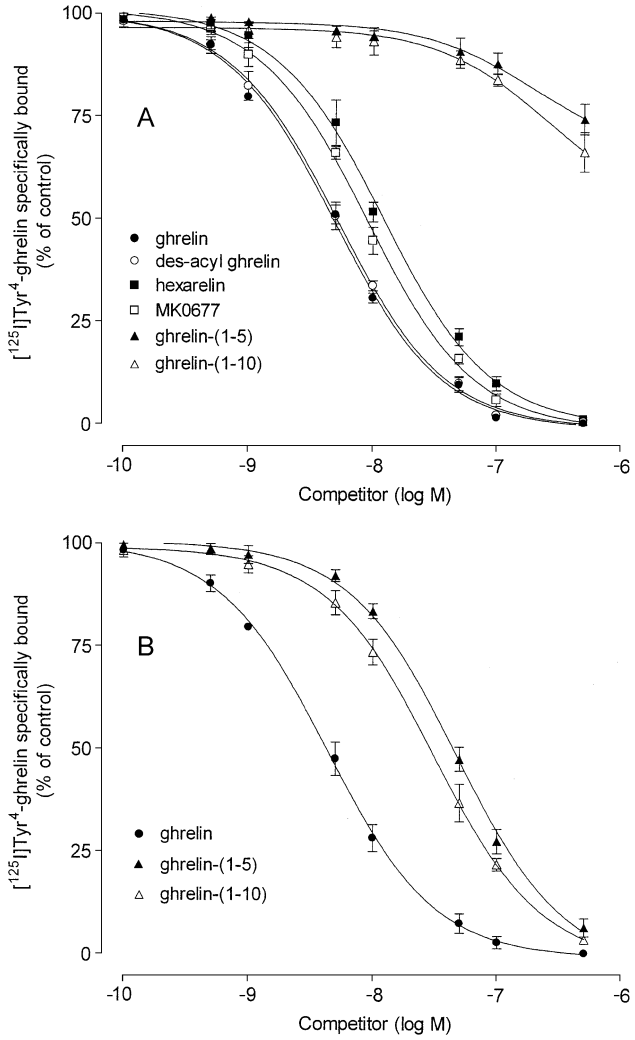

Fig. 3. Competition for [^{125}I]Tyr^4-ghrelin binding to membranes of epididymal adipose tissue by the indicated competitors. Binding assays were conducted as described in Materials and methods in assay buffer with (A) or without (B) bovine serum albumin. The ordinate represents binding as a percentage of control (specific binding in the absence of unlabelled competitor). Each point represents the mean±S.E.M. of four independent experiments.

competition binding curves in adipocytes isolated from epididymal adipose tissue resembled that reported in Fig. 3A and B (data not shown).

4. Discussion

The present study demonstrates that both des-acyl ghrelin and its acylated form and, to a lesser extent, synthetic peptidyl (hexarelin) and non-peptidyl (MK0677) GHS and some carboxy-terminally truncated ghrelin fragments, such as ghrelin-(1-5) and ghrelin-(1-10), act directly as antilipolytic factors on the adipose tissue, where they decrease the lipolytic effect of isoproterenol, a nonselective β-adrenoceptor agonist. Adipose tissue does not express the type 1a GHS/ghrelin-receptor (GHS-R1a) that has been reported to mediate the GH-releasing effect of these substances (Smith et al., 1997), but receptors able to bind ghrelin even in its des-acylated form and that are probably involved in mediating this antilipolytic action.

Des-acyl ghrelin, the major circulating form of ghrelin (Hosoda et al., 2000), is unable to bind the GHS-R1a (Bednarek et al., 2000) and lacks GH-releasing activity (Broglio et al., 2003a). Thus, it has been generally assumed that des-acyl ghrelin is an inactive peptide (Kojima et al., 2001) and although the reason why its blood concentrations are approximately four times higher than those of ghrelin has been left unexplained. Previous studies have, however, indicated that des-acyl ghrelin is not an inactive peptide, but shares the same antiproliferative effect of ghrelin on neoplastic cell lines (Cassoni et al., 2001, 2004) and, like ghrelin, prevents apoptosis induced by FAS ligand or doxorubicin in cardiomyocytes in vitro (Baldanzi et al., 2002), and reduces, even more effectively than ghrelin, the contractility of the guinea pig cardiac papillary muscle (Bedendi et al., 2003). In all these cells and tissues, a receptor has been described, which is capable of binding ghrelin in both its des-acylated and acylated forms (Baldanzi et al., 2002; Bedendi et al., 2003; Cassoni et al., 2001, 2004). This receptor, still unknown, has been assumed to be a non-type 1a GHS-R. Another non-endocrine action, more recently reported for both des-acyl ghrelin and ghrelin itself, is the promotion of rat bone marrow adipogenesis in vivo (Thompson et al., 2004). This effect was also ascribed to interaction with a non-type 1a GHS-R. As ghrelin had been reported to inhibit the lipolytic action of isoproterenol on rat adipocytes in vitro (Choi et al., 2003), we decided to clarify whether des-acylated ghrelin shares with its acylated form this antilipolytic action, as tested in rat epididymal (i.e. visceral) adipocytes. Our findings confirm the antilipolytic action of ghrelin in rat adipocytes in vitro (Choi et al., 2003) and demonstrate for the first time that des-acyl ghrelin is as active as its acylated form on these cells and, furthermore than synthetic GHS and short ghrelin fragments that are also active in inhibiting isoproterenol-induced lipolysis, though to lesser extent. Additional evidence for a specific hormonal effect was provided by the observation that des-acyl ghrelin and ghrelin could not reduce basal lipolysis or influence the cellular uptake and/or metabolism of exogenous glycerol added to the medium. The present study also showed that ghrelin reduced lipolysis in a dose-dependent manner over a range of concentrations (1–1000 nM) shown effective in stimulating GH-release in vitro (Kojima et al., 1999). Taking into account that only ~10% of total ghrelin added to the incubation medium was found in acylated form, our data suggest that ghrelin might exert its antilipolytic action not only at physiological concentrations (0.1–0.2 nM), but also at abnormally high levels (0.4–2.0 nM), as found during different physiopathological conditions, such as cachexia, anorexia nervosa, Prader-Willi syndrome and ghrelin-secreting carcinoids (Broglio et al., 2003c, Corbetta

et al., 2003; van der Lely et al., 2004). Regarding the mechanisms by which des-acyl ghrelin and ghrelin may act at the level of adipose tissue, our data show (i) that these peptides do not bind to β-adrenoceptors of the adipose tissue and (ii) that, like other des-acyl ghrelin-responsive tissues and cell lines, adipocytes display a significant high-affinity and saturable [^{125}I]Tyr^4-ghrelin specific binding that is displaced by ghrelin, truncated ghrelin fragments and synthetic GHS such as hexarelin and MK0677 showing some structural similarity with the first seven amino acids of ghrelin by molecular modelling based on nuclear magnetic resonance data (Bondensgaard et al., 2004). The most significant finding was the observations that the binding of the radioligand was also inhibited by unlabelled des-acyl ghrelin which does not interact with GHS-R1a. This displacement pattern implies that ester group, one of the principal structural features determining the binding at GHS-R1a (Bednarek et al., 2000), is not essential for activation of binding sites in the adipocytes. This finding suggests the presence of a GHS-R subtype on fat cells with a binding pocket different from that of the GHS-R1a and that could be isolated in future studies to explain where ghrelin, des-acyl ghrelin and GHS bind. Further evidence for the involvement of non-type 1a GHS-R in the antilipolytic action shared by des-acylated and acylated ghrelin is provided by the present findings (i) that rat adipocytes did not express GHS-R1a mRNA, as did the hypothalamus (Smith et al., 1997), and (ii) that des-acyl ghrelin, as well as ghrelin, truncated ghrelin analogs and synthetic GHS, all displaced [^{125}I]Tyr^4-ghrelin with IC_{50} values quite close to their half-maximal antilipolytic concentrations.

In summary, this study has shown for the first time that des-acyl ghrelin is as active as its acylated form and more than synthetic GHS and short ghrelin fragments, in negatively modulating fat cell lipolysis elicited by isoproterenol. As des-acyl ghrelin has also been shown to stimulate adipogenesis in rats (Thompson et al., 2004) and to counteract the influence of acylated ghrelin on insulin secretion and glucose metabolism in humans (Broglio et al., 2004), we conclude that des-acylated ghrelin can now be considered as a metabolically active peptide affecting glucose and lipid metabolism.

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