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ACTIVITY OF GROWTH HORMONE SECRETAGOGUES, GHRELIN AND DES-ACYL GHRELIN ON CARDIAC AND SKELETAL MYOCYTES

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TABLE OF CONTENTS

Abstract

Ghrelin (GHR) is a circulating acyl-peptidyl hormone predominantly produced by the stomach, which, by acting on the hypothalamic/pituitary axis, induces a strong release of growth hormone (GH). This activity of ghrelin is mediated by the activation of the so-called GH secretagogue (GHS) receptor type 1a (GHSR-1a), a formerly G protein-coupled orphan receptor specific for a family of synthetic peptidyl and non-peptidyl GHSs. GHSR-1a is mainly expressed in the pituitary and hypothalamus, but is present also in other central and peripheral tissues. Indeed, apart from stimulating GH secretion, ghrelin and many synthetic GHSs stimulate food intake and adiposity; influence sleep and behavior; control gastric motility and acid secretion; affect glucose levels; modulate pancreatic exocrine and endocrine function; stimulate the secretion of prolactin and ACTH, and negatively influence the pituitary-gonadal axis at both central and peripheral level.

Ghrelin is a 28 aminoacid peptide esterified with octanoic acid on Ser 3. Acylation is an absolute requirement for ghrelin binding to GHSR-1a and its endocrine activities. Indeed, des-acyl ghrelin (D-GHR) does not bind to GHSR-1a and is devoid of any GH releasing activity. However, we have demonstrated, for the first time, that des-acyl ghrelin is biologically active, as it shares with ghrelin and GHSs some cardiovascular actions, antiapoptotic effects, and the ability to enhance skeletal myoblasts differentiation. Other groups have recently demonstrated that des-acyl ghrelin is also able to modulate cell proliferation, and has as well an adipogenic effect on bone marrow adipocytes, strengthening our hypothesis of the existence of a yet unknown receptor, distinct from GHSR-1a, through the binding to which ghrelin and des-acyl ghrelin exert their nonendocrine activities.

Abbreviations

7-TM	seven transmembrane domains
ACTH	adrenocorticotropic hormone
bFGF	basic fibroblast growth factor
BLAST	Basic Local Alignment Search Tool
CHF	chronic heart failure
CNS	central nervous system
CT-1	cardiotrophin-1
D-GHR	des-acyl ghrelin
DM	differentiation medium
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
GH	growth hormone
GHR	ghrelin
GHRH	GH-releasing hormone
GHRP	GH-releasing peptide
GHS(s)	growth hormone secretagogue(s)
GHSR	growth hormone secretagogue receptor
GM	growth medium
GPCR(s)	G protein-coupled receptor(s)
HPLC	high-performance liquid chromatography
IGF-1	insulin-like growth factor-1
LDH	lactate dehydrogenase
MAPK	mitogen-activated protein kinase
MCK	muscle creatine kinase
MHC	myosin heavy chain
MLC	myosin light chain
MRF(s)	muscle regulatory factor(s)
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide
PI-3K	phosphatidylinositol 3-kinase
RIA	radio immuno assay
TGF	transforming growth factor
TNF-α	tumor necrosis factor-α

Papers included in this thesis

1. **Filigheddu N**, Fubini A, Baldanzi G, Cutrupi S, Ghè C, Catapano F, Broglio F, Bosia A, Papotti M, Muccioli G, Ghigo E, Deghenghi R, Graziani A. Hexarelin protects H9C2 cardiomyocytes from doxorubicin-induced cell death. Endocrine 2001; 14:113–119.

2. Baldanzi G, **Filigheddu N**, Cutrupi S, Catapano F, Bonissoni S, Fubini A, Malan D, Baj G, Granata R, Broglio F, Papotti M, Surico N, Bussolino F, Isgaard J, Deghenghi R, Sinigaglia F, Prat M, Muccioli G, Ghigo E, Graziani A. Ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and PI 3-kinase/AKT. J Cell Biol. 2002; 159:1029-37.

Introduction

The Growth Hormone Secretagogues

In 1977, Bowers and colleagues published a paper describing the GH-releasing properties of peptides derived from met-enkephalins that lacked any opiate properties [1]. The first compound, called growth hormone releasing peptide (GHRP), produced a weak GH response *in vitro* and was not effective *in vivo*. Further development produced GHRP-6, the first hexapeptide to actively release GH *in vivo*, even after oral administration. Further research, aimed to select orally active molecules with better bioavailability and longer half-lives than GHRP-6, led to the synthesis of other GHRPs, among which hexarelin has probably been the most studied, as well as of non-peptidyl compounds, among which the most significant was MK0677, developed by Merck.

The primary action of the GHRPs was the stimulation of GH release via a mechanism distinct from that of GH releasing-hormone (GHRH). GHRP-6 activated the phospolipase-C pathway, resulting in a rise in inositol triphosphate and intracellular calcium, a pathway distinct from the cAMP-phosphokinase A pathway of the GHRH receptor.

The Growth Hormone Secretagogue Receptor

In 1996, the Merck group combined expression cloning and their compound MK0677, looking for changes in intracellular calcium to indicate a positive response, to identify and clone the GH secretagogue receptor (GHSR) [2].

Ghrelin receptor is a typical GPCR of 366 amino acids belonging to the rhodopsin family with seven transmembrane domains (7-TM) [2-4].

From the ghrelin receptor cDNA two different mRNAs have been isolated [2]. The first, GHSR type 1a, encodes a 7-TM GPCR with binding and functional properties consistent with its role as ghrelin receptor. The other GHSR cDNA, type 1b, is produced by an alternative splicing mechanism [2]. The GHSR gene consists of two exons; the first exon encodes TM-1 to TM-5, and the second exon encodes TM-6 to

TM-7. Type 1b is derived from only the first exon and encodes only five of the seven predicted TM domains. The type 1b receptor is thus a COOH-terminal truncated form of the type 1a receptor and is pharmacologically inactive. The GHSR has several homologs, whose endogenous ligands are gastrointestinal peptides or neuropeptides. Figure 1 shows a dendrogram alignment of the ghrelin receptor superfamily. This superfamily contains receptors for ghrelin, motilin, neuromedin U [5-8], and neurotensin [9]. All of these peptides are found in gastrointestinal organs and regulate gastrointestinal movement and other functions. The ghrelin receptor is most homologous to the motilin receptor, the human forms sharing 52% identical aminoacids [10]. Moreover, their ligands, ghrelin and motilin peptides, have similar aminoacid sequences and preliminary studies have shown that motilin can stimulate the ghrelin receptor, albeit with lower affinity. In contrast, ghrelin does not activate motilin receptor. The ghrelin receptor is well conserved across all vertebrate species examined. This strict conservation suggests that ghrelin and its receptor serve important physiological functions.

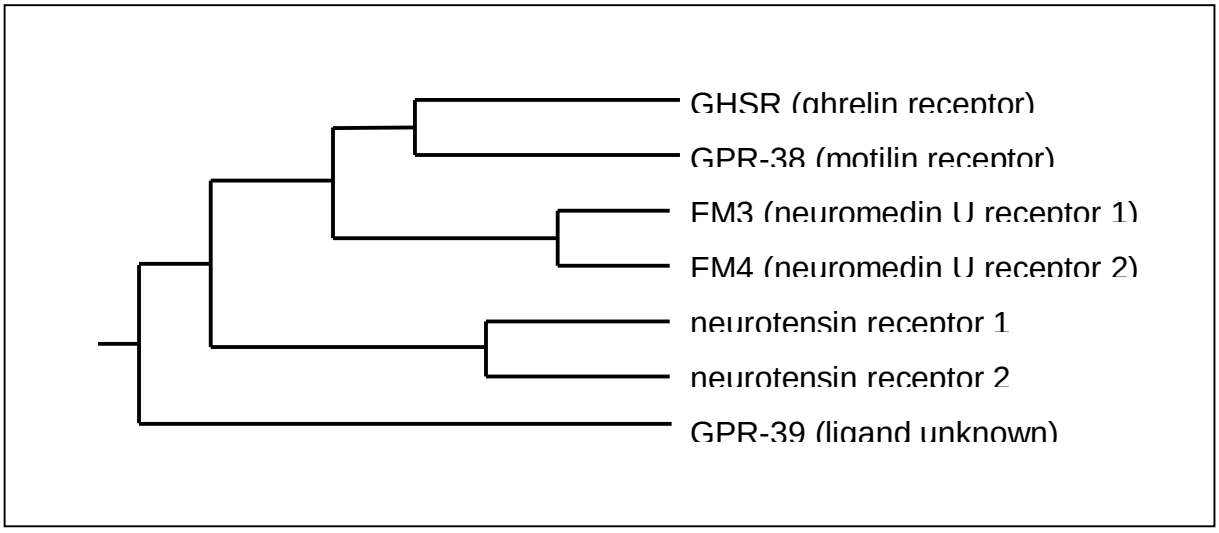

FIG. 1. **Dendrogram alignment of GHSR and other GPCRs.** The ghrelin receptor is part of a GPCR superfamily that contains the motilin, neuromedin U and neurotensin receptors, and is most homologous to the motilin receptor. Because their endogenous ligands, ghrelin and motilin, have partly homologous amino acid sequences, the ghrelin and motilin systems may have evolved from a common ancestral system. This superfamily also contains an orphan receptor, GPR39, whose endogenous ligand is expected to be a peptide.

It is suggested that a novel unidentified subtype of ghrelin receptor exists, not only because, as we have shown, both ghrelin and des-acyl ghrelin bind to H9c2 cardiomyocytes, which do not express the GHSR-1a receptor [11], but also because ghrelin binding activity has been demonstrated in 3T3-L1 cells by radiolabeled ghrelin, although RT-PCR detected no signal for GHSR-1a in these cells [12]. However, BLAST searches of the human genome using GHSR cDNA as a search sequence have not revealed any ghrelin receptor homologs. Further study is required to search for an as-yet-unidentified ghrelin receptor subtype.

Ghrelin and Des-acyl Ghrelin

The cloning of the GH secretagogue receptor provided the tool necessary to identify its natural ligand: at the end of 1999, the group led by Kojima and Kangawa published the structure of the natural ligand for the GHS receptor, designated ghrelin (derived from ghre, the proto Indo-European root of the word “grow”) [13]. They used the GHSR expressed in a cell line as a bioassay to screen tissue extracts from rats, a positive response causing a rise in intracellular calcium. They assayed extracts from several organs and, surprisingly, the strongest signal was obtained using extracts from the stomach.

The active peptide was purified by gel filtration, ion exchange, and reverse liquid chromatography and its sequence determined by Edman degradation. Initially, a synthetic peptide based on the cDNA sequence isolated from a rat stomach cDNA library was synthesized and compared to purified natural ghrelin. This comparison revealed that the synthetic peptide, unlike the purified protein, did not increase intracellular calcium, had a retention time in HPLC shorter than that of natural peptide and, intriguingly, its molecular mass was 126 atomic mass units (amu) smaller than natural peptide. Further studies demonstrated that the difference in mass was due to an esterification on serine 3 with an n–octanoic acid. This biochemical post-translational modification is the first observed in peptides isolated from natural sources and is postulated to be crucial for the biological activity of the peptide and it seems to be necessary for crossing the blood-brain barrier. The mature 28-amino acid peptide is cleaved from its precursor preproghrelin and then acylated by an n -octanoic acid residue, although other types of acylations (10 carbon fatty acid group

with and without insaturations) have been observed [13-15]. Moreover, also short fragments including the first four to five residues of ghrelin (with intact acylation on serine 3) are able to activate signal transduction of GHSR-1a.

In blood, the nonacylated form of ghrelin, des-acyl ghrelin, circulates in amounts far greater than acylated ghrelin [16]. Ghrelin in the plasma binds to high-density lipoproteins (HDLs) that contain a plasma esterase, paraoxonase, and clusterin [17]. Because a fatty acid is attached to the Ser3 of ghrelin via an ester bond, paraoxonase, a potent esterase, may be involved in deacylation of acyl-modified ghrelin. Thus des-acyl ghrelin may represent either a pre-form of acyl-modified ghrelin or the product of its deacylation. Des-acyl ghrelin does not displace radiolabeled ghrelin at the binding sites of acylated ghrelin in hypothalamus and pituitary and shows no GH-releasing and other endocrine activities in neither rats nor humans. In our laboratory, we have shown for the first time [11] a biological effect of des-acyl ghrelin and demonstrate that ghrelin and des-acyl ghrelin both recognize common high-affinity binding sites on H9c2 cardiomyocytes, which do not express the ghrelin receptor GHSR, suggesting in this way the existence of another ghrelin receptor in the cardiovascular system. Moreover, it has been reported that des-acyl ghrelin shares with acyl-modified ghrelin some nonendocrine actions, including the modulation of cell proliferation and, to a small extent, adipogenesis [18]. Further studies are required to determine to which receptor does des-acyl ghrelin bind.

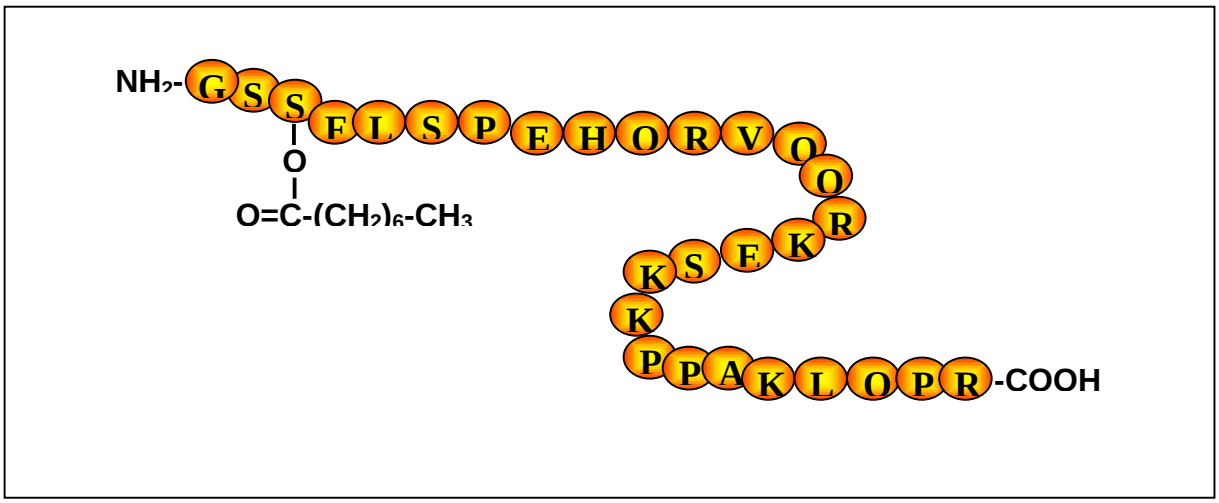

FIG. 2. **Structure of human ghrelin.** Human ghrelin is a 28-amino acid peptide, in which Ser3 is modified by a fatty acid, primarily *n*-octanoic acid. This modification is essential for ghrelin endocrine activity.

Tissue Distribution and Regulation of Ghrelin Secretion

In all the vertebrate species, ghrelin is produced prevalently in the stomach and more abundantly by the X/A-like cells within the oxyntic glands of the gastric fundus mucosa [19], although minor amounts are present elsewhere in the body. The placenta [20], testis [21], kidney [22], pituitary [23], small intestine [24], pancreas [25], lymphocytes [26], brain [27], lung [28] and ovary [29-30] also express significant levels of ghrelin. The ubiquitous expression of ghrelin in several tissues suggests the possibility of local paracrine and/or autocrine actions.

Circulating ghrelin is present at concentrations of 100±140 fmol/ml [13]; however, little is known about the regulation of ghrelin secretion from the stomach or the hypothalamus. The most important factor for the regulation of ghrelin secretion is feeding. Plasma ghrelin concentration is increased during fasting and decreased after food intake [31, 32]. However, plasma ghrelin concentration is sensitive to the composition of meals, as it is decreased by a high-fat meal [33]. Blood glucose level may also be critical: oral or intravenous administration of glucose decreases plasma ghrelin concentration [34, 35]. Plasma ghrelin concentration is low in obese people and high in lean people [36].

Biological Actions of Ghrelin

Besides the strong GH-releasing activity, ghrelin has other significant actions, including: (1) orexigenic action coupled with control of energy homeostasis, (2) control of acid secretion and gastric motility [37], (3) influences on pancreatic activity [38, 39], (4) influences on sleep [40], (5) cardiovascular and hemodynamic actions, (6) effects on proliferation on several cell lines, and (7) differentiating effects.

Despite the plethora of physiological actions exerted by ghrelin, inhere I shall focus my attention prevalently on its role on the cardiovascular system, its activity as proliferation modulation factor and its pro-differentiative action.

Cardiovascular and hemodynamic effects

The first report on the cardiovascular actions of GHS in rats appeared in 1997 [41]. These studies revealed that the peptidyl GHS hexarelin has a strong cardioprotective action, as it markedly protects against cardiovascular damage in GH-deficient rats with post-ischemic ventricular dysfunction, improves cardiac performances in rats after myocardial infarction [42], protects against diastolic dysfunctions of myocardial stunning in isolated, perfused rabbit heart [43] and enhances left ventricular contractility in pigs with dilated cardiomyopathy [44]. In humans, the acute administration of hexarelin was found able to increase left ventricular ejection fraction without any variations of mean blood pressure, heart rate and catecholamine levels [45, 46]. The same positive inotropic effect of hexarelin was found in patients with ischemic, but not in those with idiopathic dilated cardiomyopathy [47].

Recently, it has been demonstrated, both in rats and humans, that also ghrelin possesses cardiovascular activities: it has been shown that administration of ghrelin improves cardiac structure and function, and attenuates the development of cardiac cachexia in rats with heart failure [48-50], while in humans, the administration of ghrelin in normal young volunteers is followed by reduction in cardiac afterload and increase in cardiac output without any change in heart rate [51]. In humans, it has also been shown that infusion of ghrelin decreases systemic vascular resistance and increases cardiac output in patients with heart failure [52, 53].

These results suggest that ghrelin not only has cardiovascular protective effects, but also that it may regulate energy metabolism through GH-dependent and -independent mechanisms.

We have shown that *in vitro* ghrelin, as well as hexarelin, is able to prevent cell death of cultured H9C2 cardiomyocytes and endothelial cells induced by either doxorubicin, serum withdrawal or activation of FAS [54, 11]. Interestingly, the same cytoprotective effect of acylated ghrelin is shared by non-acylated ghrelin and both molecules stimulate in cultured cardiomyocytes survival intracellular signaling pathways, including tyrosine phosphorylation of intracellular proteins and activation of ERK-1/2 and protein kinase B/Akt [11]. As non-acylated ghrelin is generally unable to

activate the GHSR-1a receptor [55] and stimulate GH release [13], these data indicate that acylation of the peptide is needed for endocrine actions only and that even the non-acylated ghrelin is a biologically active peptide. This evidence would imply the existence of another cardiac GHS receptor subtype, common for acylated and non-acylated ghrelin, whose activation mediates an antiapoptic effect in the cardiovascular system.

Effects on proliferation

There are several conflicting data concerning ghrelin and GHSs effects on cell proliferation. A positive effect on proliferation has been found in osteoblasts [56, 57], primary oral keratinocytes [58], erythroleukemic cell line HEL [59], zona glomerulosa cells [60, 61], rat pituitary cell line GH3 [62], 3T3-L1 preadipocytes [63, 12], pancreatic adenocarcinoma cells [64], H9c2 cardiomyocyte cell line [65], and several prostate cancer cell lines [66]. On the other hand, it has been demonstrated that GHS and ghrelin inhibit cell growth of prostatic carcinoma cell lines [18], thyroid and follicular carcinoma cell lines [67], lung carcinoma [68], brest carcinoma cell lines [69], differentiating immature Leydig cells [70] and inhibit the proliferation of splenic T cells when these are co-stimulated by anti-CD3 [71].

Pro- and anti-differentiative activity

As one of ghrelin main action *in vivo* is the stimulation of adiposity, and GHSR is abundantly expressed in adipose tissue, the role of ghrelin in adipocyte biology has been explored by several groups, but with opposing results. Indeed, it has been reported that ghrelin induces proliferation of 3T3-L1 preadipocytes and their differentiation in mature adipocytes [72, 63], but, on the other hand, the same cells overexpressing ghrelin showed significantly attenuated differentiation, while maintaining the increased proliferation rate [12]. It has also been demonstrated from different groups that ghrelin and GHSs affect directly the process of bone formation promoting both proliferation and differentiation of osteoblastic cells [56, 57]. A different role for ghrelin in differentiation has been suggested for testis Leydig cells, where it has been shown that ghrelin inhibits proliferation, and, furthermore, during

differentiation, Leydig cells precursors acquire ghrelin expression, suggesting a self-regulatory mechanism for the inhibition of proliferation [70].

Aims

The aim of my PhD project has been the characterisation of GHSs and ghrelin/des-acyl ghrelin action on cardiac, endothelial and skeletal muscle cells. In particular, the main goals of the project were, as follow:

1. to investigate *in vitro*, at cellular level, the role of GHSs and ghrelin/des-acyl ghrelin as cardioactive factors, namely, to verify whether they are able to prevent cardiomyocytes cell death induced by cardiotoxic drugs or apoptotic agents and to evaluate the cellular pathways of their action;

2. to characterize the differentiating activity of ghrelin and des-acyl ghrelin on skeletal myoblasts and evaluate their hypertrophic activity on differentiated myocytes.

Results

Protective action of the peptidyl GHS hexarelin on cardiomyocytes and endothelial cells *in vitro* (Paper 1)

Several independent observations suggested, over the years, that the peptidyl GHS hexarelin has some cardiovascular activities. Indeed, it has been demonstrated that prolonged treatment with hexarelin protects from myocardial damage induced by ischemia-reperfusion in rats and improves cardiac contractility in rats with myocardial infarction. In addition, acute administration of hexarelin increases the left ventricular ejection fraction in normal and GH-deficient humans. Altogether these findings led to the hypothesis that peptidyl GHSs could have cardiovascular activities independently of their GH-releasing activity.

In order to verify the cellular mechanisms underlying the diverse cardiotropic actions of GHSs, in this paper we have investigated the actions of hexarelin in cultured cardiomyocyte-derived cells *in vitro*.

As cardiomyocytes cell death has been demonstrated to play a crucial role in ischemia-reperfusion myocardial damage as well as in the development of dilated cardiomyopathy, we verified the hypothesis that hexarelin may exert its cardioprotective action by preventing cardiomyocytes and endothelial cells apoptosis induced by doxorubicin, an antitumoral drug highly cytotoxic in the heart.

The results reported in this paper showed that hexarelin binds to specific high-affinity receptors on H9c2 cardiomyocytes, protects these cells from cell death triggered by doxorubicin and inhibits doxorubicin-induced apoptosis in endothelial cells.

Antiapoptotic effects of ghrelin and des-acyl ghrelin on cardiomyocytes and endothelial cells *in vitro* (Paper 2)

Since the discovery of ghrelin, the endocrine and nonendocrine activities of this gastric hormone have been widely explored, both in animals and humans. It was

soon demonstrated that ghrelin, like synthetic GHSs, has cardioactive properties, as it protects heart function from experimentally induced cardiac heart failure *in vivo* [51, 50].

Based on these observations, and on the fact that in the heart antiapoptotic factors, such as IGF-1 and cardiotrophin-1 (CT-1), play a crucial role in maintaining cardiomyocytes survival and myocardial function after ischemia- and pressure overload–induced cardiomyopathies [73, 74], we have raised the hypothesis that ghrelin may feature a direct cytoprotective activity by inhibiting cell death of cardiomyocytes and endothelial cells.

In this paper, besides showing the ability of ghrelin to inhibit apoptosis induced by doxorubicin, serum withdrawal or FAS activation, we have demonstrated that the same action is shared by des-acyl ghrelin. Both the peptides explicate the cardioprotective action activating specific signaling pathways, including stimulation of protein-tyrosine phosphorylation and activation of ERK-1/2 and PI 3-kinase/Akt, suggesting that they may act as a survival factor in cardiomyocytes and endothelial cells.

Effects of ghrelin and des-acyl ghrelin on skeletal muscle cells (Paper in preparation)

INTRODUCTION

Skeletal muscle satellite cells are quiescent mononucleated myogenic cells, located between the sarcolemma and the basal membrane of terminally-differentiated muscle fibers. These are normally quiescent in adult muscle, but act as a reserve population of cells, able to proliferate in response to injury and give rise to regenerated muscle and to more satellite cells. Regeneration of skeletal muscle tissue includes sequential processes of muscle cell proliferation and commitment, cell fusion, muscle fiber differentiation, and communication between cells of various tissues of origin. To form new fibers in a muscle damaged by disease or direct injury, satellite cells must be activated to proliferate, and subsequently fuse into an elongated multinucleated cell.

The compensative mechanisms leading to muscle regeneration are poorly understood, however they appear to recapitulate the embryonic program of differentiation, although the extracellular factors regulating such process may be different. During embryogenesis, skeletal muscle development depends upon complex multifactorial processes [75]. Myogenic cell differentiation can be subdivided into temporally separable events controlled by cyclins and cyclin-dependent kinases (Cdks) complexes [76]:

1. expression of early muscle specific markers known as muscle regulatory factors (MRFs), as myogenin and MyoD, which are turned on sequentially, and indicate commitment to the differentiation pathway;
2. p21 and p27 induction, which correlates with irreversible cell cycle arrest and inhibition of apoptosis;
3. expression of myosin heavy and light chains (MHC, MLC), muscle creatine kinase (MCK) and the acetylcholine receptor, which are hallmarks of phenotypic differentiation;
4. fusion of myocytes into multinucleated myotubes, as the terminal step of muscle differentiation, one typical marker of which is the expression of caveolin-3 [77].

Myoblasts differentiation *in vitro* is negatively regulated by high levels of serum or by specific growth factors present in the medium as fibroblast growth factor (FGF) or transforming growth factor (TGF), while insulin and insulin-like growth factors (IGFs) are potent stimulators of myogenesis [78]. Insulin and IGF-1 stimulate both proliferation and differentiation, and this contradictory behaviour has been ascribed to a biphasic action of these factors.

The growing interest on skeletal muscle regeneration is associated to the opening of new therapeutic strategies for several muscular degenerative pathologies such dystrophies, muscular atrophy and cachexia, that is the loss of muscle mass and function, associated to aging, cancer, chronic heart failure and AIDS, as well as the treatments of skeletal muscle injury after trauma.

Inhere we investigate the hypothesis that ghrelin and des-acyl ghrelin may stimulate differentiation and fusion of skeletal myoblasts, contributing thus to their trophism and regeneration. Such hypothesis arises from the following observations:

i) skeletal muscle contains high levels of high affinity peptidyl GHSs binding sites [79];

ii) increase of total circulating ghrelin concentration tightly correlates with cachexia in chronic heart failure (CHF) patients [80].

iii) ghrelin strongly prevents skeletal muscle cachexia induced by heart failure in rats, however we should note that prevention of heart failure-induced cachexia may depend also on its GH-mediated anabolic activity, as well as on its GH-independent increase of appetite, inhibition of fat utilization and increase of carbohydrate utilization [50].

Moreover, it has been suggested that GHSs may have a beneficial effect on immobility-induced muscle wasting and in the subsequent rehabilitation [81, 82].

To investigate the action of ghrelin and des-acyl ghrelin on skeletal muscle, we have used the well established murine C2C12 skeletal myoblast cell line, as well as primary satellite cells extracted from mice muscles, both able to differentiate, upon suitable conditions, in multinucleated myotubes.

MATERIALS AND METHODS

Reagents - Synthetic ghrelin and des-acyl ghrelin were provided by Neosystem. The anti-MHC (MF-20) and anti-myogenin antibodies were a kind gift of Prof. Maria Prat and Dr. Mara Brancaccio, anti-phospho-Erk1/2, anti-Erk1/2 antibodies, were purchased from Cell Signaling. Reagents were obtained from Sigma, unless otherwise indicated.

Isolation of primary satellite cells – Muscles from lower limbs and diaphragm were excised from 18-24 days-old mice, washed in PBS and digested in a 0,1% solution of collagenase B (Roche) in dispase II (Roche) at 37 ℃ for at least 40 minutes. The digestion was stopped by the addiction of FCS and the suspension was pre-plated in growth medium (see below) in plastic culture dishes over night in order to remove fibroblasts. Afterwards, the supernatant, containing the satellite cells, was re-plated on gelatin- or collagen coated dishes and cultured at low confluence to avoid spontaneous differentiation.

Cell cultures – C2C12 myoblasts were grown at low density in a proliferative medium (growth medium, GM) consisting in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS, Gibco), penicillin (100 u/ml), streptomycin (100 μg/ml) and antimycotic (0,25 μg/ml). To induce differentiation, cells

were allowed to become confluent and the medium was changed to differentiation medium (DM) consisting in DMEM supplemented with 2% horse serum, penicillin, streptomycin and antimycotic as above. Primary satellite cells were maintained in a proliferation medium composed of HAM F10 supplemented with 20% FCS, 25 ng/ml bFGF, 3% chicken embryo extract, penicillin, streptomycin and antimycotic as above. To induce differentiation, cells were seeded at high density and the medium was switched to DMEM supplemented with 5% HS, antibiotics and antimycotic as above.

Phase contrast microscopy – C2C12 and satellite cells were grown in plastic tissue culture dishes and photographed using an inverted microscope.

Western Blot Analysis – At the end of indicated treatments, cells were washed in ice-cold phosphate buffered saline and solubilized with a buffer containing Hepes (pH 8) 25 mM, NaCl 135 mM, EDTA 5 mM, EGTA 1 mM, $ZnCl_2$ 1 mM, NaF 50 mM, NP 40 1%, glycerol 10%, leupeptin 0.05 mg/ml, pepstatin 0.005 mg/ml, PMSF 200 µM, Na_3VO_4 1 mM. The lysates were shakerated at 4°C for 15 min and centrifuged at 13,000 x g for 15 min at 4 °C. Protein concentrations were determined with Bio-Rad Protein Assay and equal amounts of supernatants (20-50 µg protein/lane) were separated by 5-10% SDS-PAGE. Proteins were then electronically transferred to polyvinylidene difluoride filters (Hybond-P, Amersham Life Science). To reduce nonspecific binding, the filters were bathed in methanol and exsiccated for 30 min at room temperature. The filters were incubated with the primary antibodies for 1 h at room temperature, washed with TBS-Tween 0.1% and then incubated whit the appropriate secondary antibody (Amersham) for 45 min at RT. The results of immunoblotting were visualized with Renaissance Western Blot Chemiluminescence (NEN, Life Science Products).
Equal protein loading was further controlled by Ponceau red staining.

Immunofluorescence – Cells were plated on 24 wells-plates and treated as indicated. At the end of the treatments cells were fixed in 4% paraformhaldeyde, permeabilized with 0.5% Triton X-100, decorated with anti-MHC for 1 hour at room temperature or over night at 4°C, incubated with the secondary antibody for 30' and with DAPI at room temperature and visualized with a fluorescence microscopy.

Images were acquired (10 fields/well) and analysed to determine differentiation and fusion indexes.

Differentiation index, fusion index – To quantify the differentiation and fusion of cells after treatments, we used the differentiation index and the fusion index defined as the percentage of MHC-positive cells above total cells and the average number of nuclei in MHC-positive cells with at least 3 nuclei above total number of cells, respectively.

Cell proliferation and hypertrophy induction – Cells were starved over night in 0,2% FCS and then cultivated for 24 hours with or without ghrelin and des-acyl ghrelin both in 10% FCS medium (to evaluate inhibition of proliferation) or in 0% FCS medium (to evaluate stimulation of proliferation). At the end of treatments, cells were incubated with [^{3}H]-thymidine (2 μCi/ml) for 3 hours, washed with PBS, treated with 5% trichloroacetic acid (TCA) for 30 minutes at 4 °C to precipitate proteins and then lysed by the addiction of NaOH 0.5 M and 0.5% SDS. To evaluate the induction of hypertrophy, together with treatments, [^{3}H]-leucine (1 μCi/ml) was added to the medium, and after 24 hours, cells were processed for the proliferation quantification as above. Positive controls for proliferation and hypertrophy were 20% FCS medium and angiotensin II 100 nM, respectively. The amount of incorporated thymidine and leucine was evaluated by β-counter (GS-250 Molecular Imager Biorad) analysis. To evaluate the hypertrophy, the ratio [^{3}H]-leucine/[^{3}H]-thymidine was calculated and data normalised to the value obtained for not-treated cells.

GHSR-1a expression - Total RNA from cultured cells was extracted by Nucleospin RNA II (Macherey-Nagel) following the manufacturer's instructions, while RNA from mouse brain mechanically triturated in liquid nitrogen was extracted by Trizol (Gibco). The RNA obtained was retrotranscribed with SuperScript reverse transcriptase (Invitrogen) and then 1 μl of each cDNA was used to perform the PCR of GHSR-1a, adding the optimised buffer for the reaction, dATP, dCTP, dGTP, dTTP (10 mM), the DNAzyme EXT polymerase (Finnzymes) and the following primers:

GHSR-1a exon 1-for	5'-AGTATCGGCCCTGGAACTT-3'
GHSR-1a exon 1-rev	5'-ACGCTCGACACCCATACCAT-3'

GHSR-1a exon 2-for	5’-TGGTGTTTGCTTTCATCCTC-3’
GHSR-1a exon 2-rev	5’-CGGGAACTCTCATCCTTCAGA-3’
GHSR-1a complete-for	5’-AAGGTGGTGGTCACCAAGG-3’
GHSR-1a complete-rev	5’-CGGTACTTCTTGGACATGATG-3’.

Real time PCR – Each Real Time RT-PCR reaction to quantify myogenin expression in our samples was performed using myogenin primers (Assay on Demand Mm00446194_m1 Applied Biosystems) following the instructions of Applied Biosystems. As active reference 18s RNA (mammalian 18s PDAR, Applied Biosystems) was used.

To quantify caveolin-3 expression we performed a Sybr Green assay; for this purpose we used Platinum Sybr Green qPCR SuperMix UDG (Invitrogen) and we designed the following primers: Cav-3 forward 5’-GCGACCCCAAGAACATCAATG-3’ and Cav-3 reverse 5’-CCTTCCATACACCGTCGAAGC-3’. In this case as active reference we used GAPDH, amplified with primers suitable for Sybr Green assay: GAPDH forward 5’ CTGGCCAAGGTCATCCATGA 3’ and GAPDH reverse 5’ AGGGGCCATCCACAGTCTT 3’. The instrument used for amplification detection was the 7000 Sequence Detection System model from Applied Biosystems.

Statistical analysis - Data are presented as the mean ± SEM; the statistical significance was tested using Student's T test.

RESULTS

Ghrelin and des-acyl ghrelin induce differentiation and fusion in skeletal muscle myocytes

The addiction of ghrelin and des-acyl ghrelin to the growth media of C2C12 myoblast cell line and primary satellite cells induced morphological changes to both cell types similar to those induced by the differentiation media. In Fig. 3A contrast phase microscopy shows the morphological changes induced by des-acyl ghrelin (10 nM–1 μM) on primary satellite cells after 24 and 48 hours of incubation in high serum medium. In C2C12 cells treated with both ghrelin and des-acyl ghrelin, within 24

hours the expression of the skeletal muscle-specific basic helix-loop-helix transcription factor myogenin, marking the onset of myogenesis, was increased, while within 48 hours, when polynucleated myotubes had formed, the expression of the terminal differentiation marker myosin heavy chain (MHC) was visible (Fig. 3B).

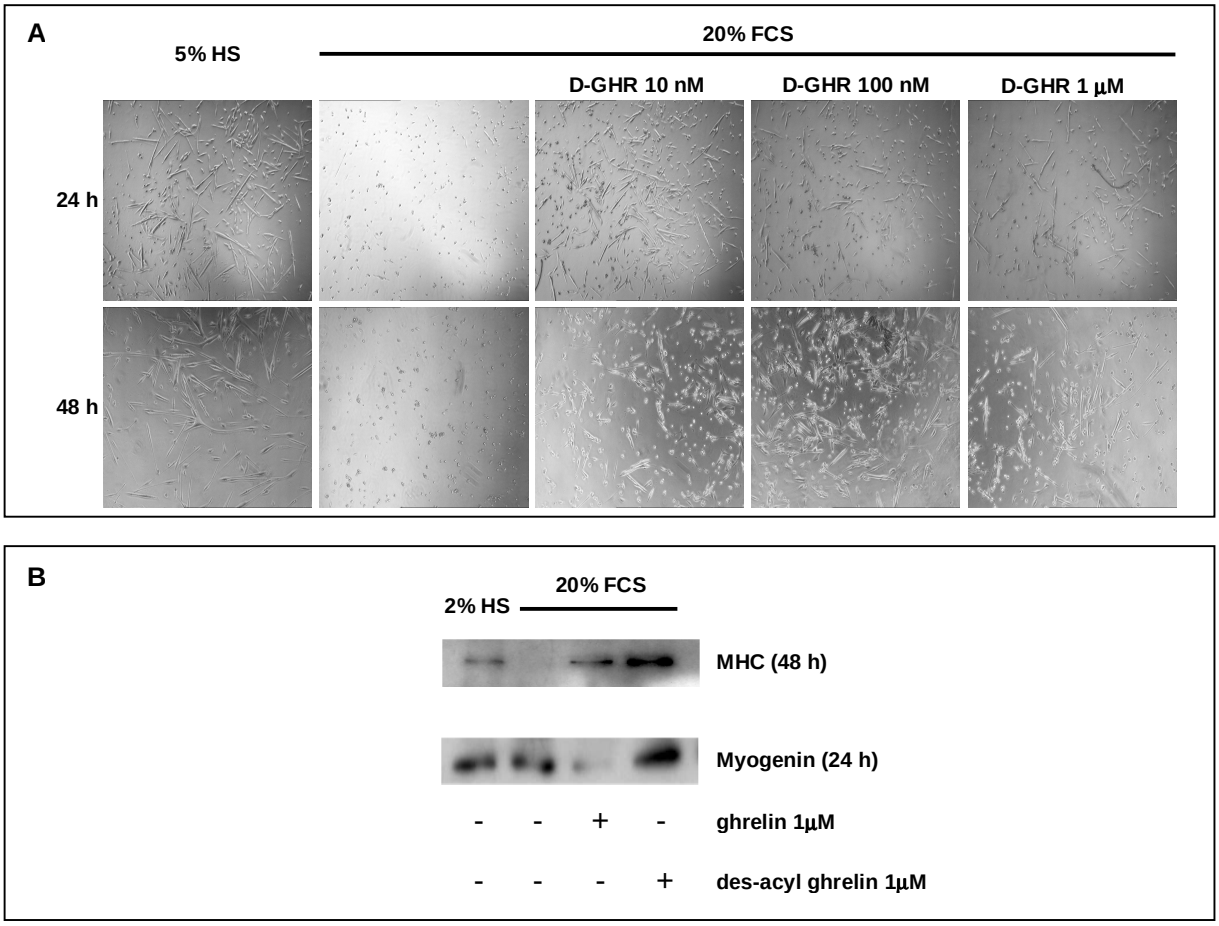

Fig. 3. **Ghrelin and des-acyl ghrelin induce differentiation of skeletal muscle myoblasts.** (A) Contrast phase microscopy of primary satellite cells treated for 24-48 hours with des-acyl ghrelin. (B) C2C12 myoblasts were assessed for differentiation by immunoblot analysis for expression of myogenin and MHC at the indicated times.

To further analyze the degree of differentiation of skeletal muscle cells, we calculated two parameters: the differentiation index, indicating how many cells compared to the totality of cells acquire the terminal differentiation marker MHC, and the fusion index, indicating the extent of the final step of differentiation, i.e. the fusion of myoblasts to form myotubes. The latter is achieved calculating the average number of nuclei contained in the myotubes compared to the total number of nuclei, taking in account only myotubes with at least three nuclei. Although it may appear a

redundancy, these two aspects of differentiation are distinct and not always correlating. For example, culturing primary satellite cells at high density in growth medium will give rise to round-shaped cells expressing differentiation markers but not fusing in polynucleated myotubes (personal observations).

Both ghrelin and des-acyl ghrelin were able to induce differentiation and fusion in C2C12 myocytes, as illustrated in Fig. 4. As it can be seen in Fig. 4A, the differentiation induced by ghrelin and des-acyl ghrelin, although significant, is lesser than that elicited by the differentiation medium. However, if we consider the fusion indexes (Fig. 4B), we may see that they are all similar. Together, these data suggest that ghrelin and des-acyl ghrelin are more fusogenic: in the samples treated with ghrelin and des-acyl ghrelin the number of MHC-positive cells per field will be lower, compared with the samples in differentiation medium, but every MHC-positive cell will have more nuclei.

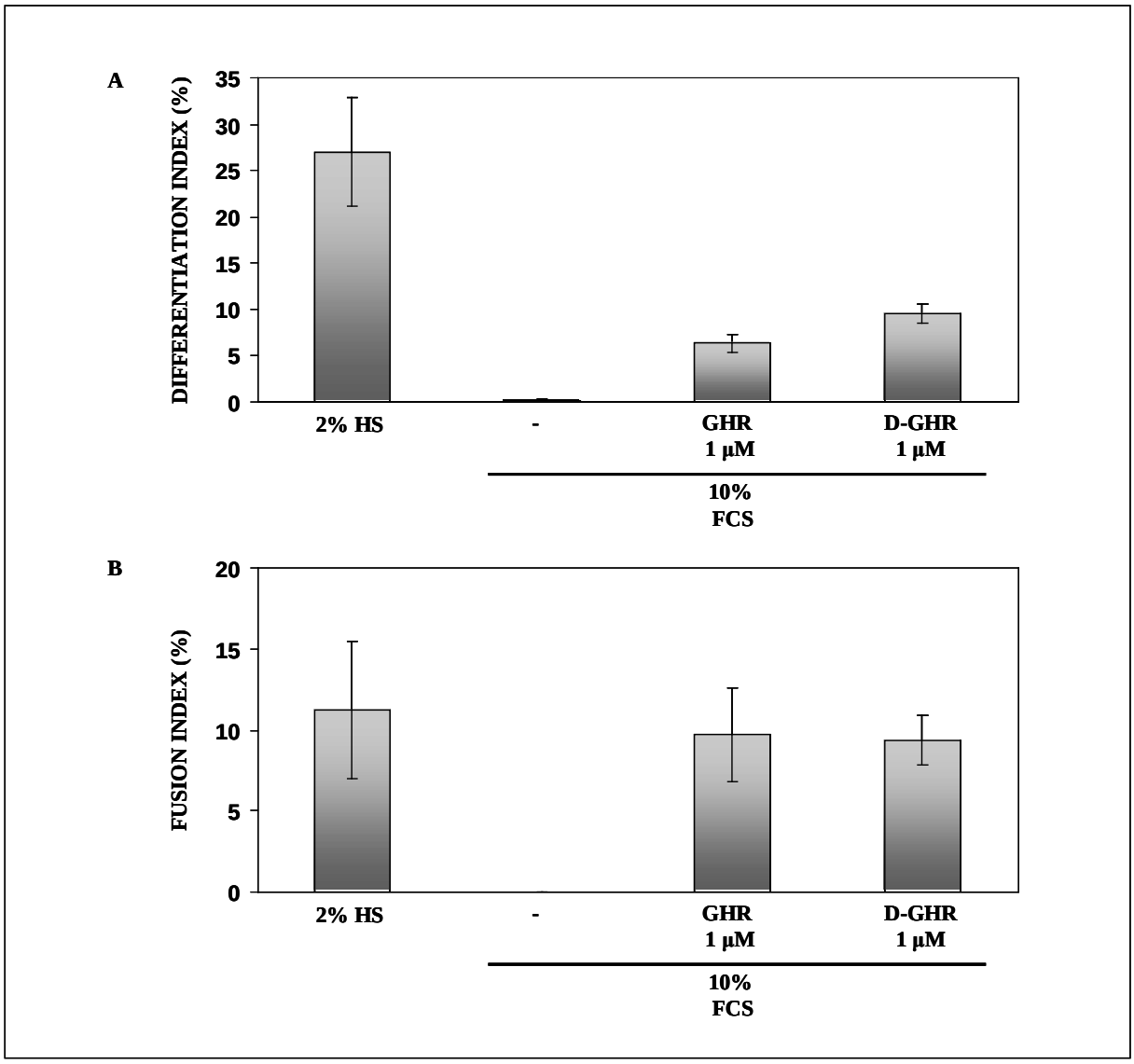

Fig. 4. **Ghrelin and des-acyl ghrelin induce differentiation and fusion of C2C12 myoblasts.** Cells were treated in GM for 48 hours, fixed and assayed for the expression of MHC by immunofluorescence. Ten fields for each treatment were analysed and the amount of MHC-positive cells compared to the total number of cells (A) and the average of nuclei in MHC-positive cells with at least three nuclei (B) were evaluated. $p<0.001$ for each treatment.

The effect on myoblasts fusion was further investigated treating C2C12 with different concentrations of des-acyl ghrelin in growth medium for 48 or 72 hours and determining the levels of caveolin-3, a fusion specific marker, by real-time RT-PCR. As showed in Fig. 5, after 72 hours of treatment, the level of caveolin-3 was increased up to three fold compared to the untreated control. It can be noted, as well, that the effects of des-acyl ghrelin are not dose-dependent.

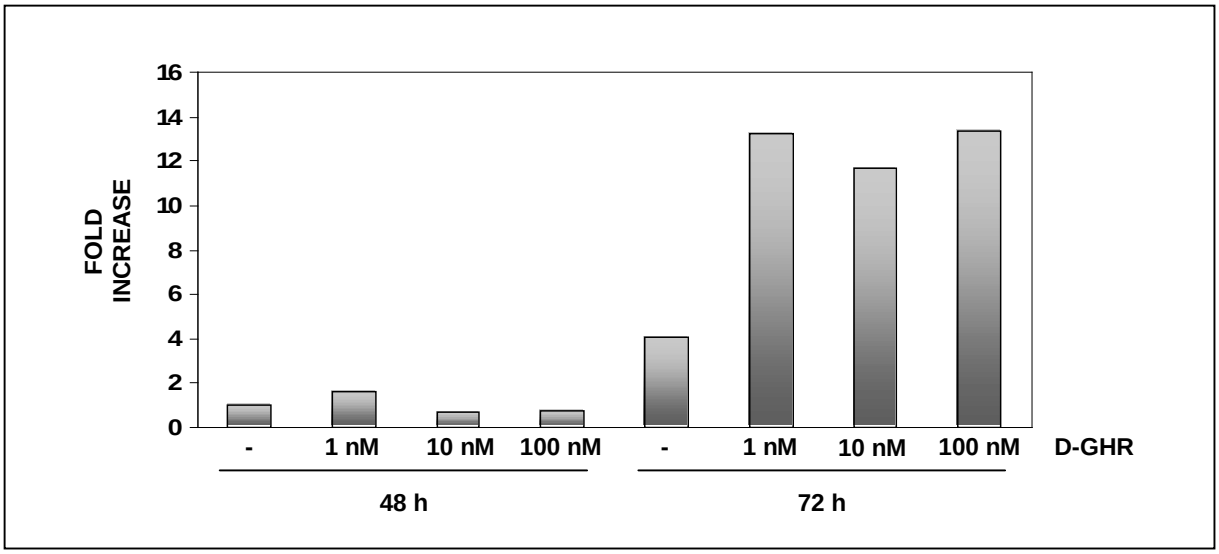

Fig. 5. **Des-acyl ghrelin-induced expression of caveolin-3.** C2C12 myoblasts were incubated with des-acyl ghrelin at the indicated concentrations in GM for 48 or 72 hours and then lysed. The RNAs extracted and retrotranscribed were assayed by real-time PCR for the expression of caveolin-3.

Based on these results, we raised the hypothesis that ghrelin and des-acyl ghrelin may have a synergic action on differentiation induced by differentiation medium. For this purpose, we investigated the expression on the early differentiation marker myogenin in real-time RT-PCR of C2C12 compelled to differentiate in differentiation medium in presence or absence of ghrelin or des-acyl ghrelin. As showed in Fig. 6, the expression of myogenin is increased by the simultaneous administration of ghrelin and des-acyl ghrelin in the differentiation medium. Also the fusion was enhanced by the addiction of ghrelin or des-acyl ghrelin, as showed by the fusion index in Fig. 7.

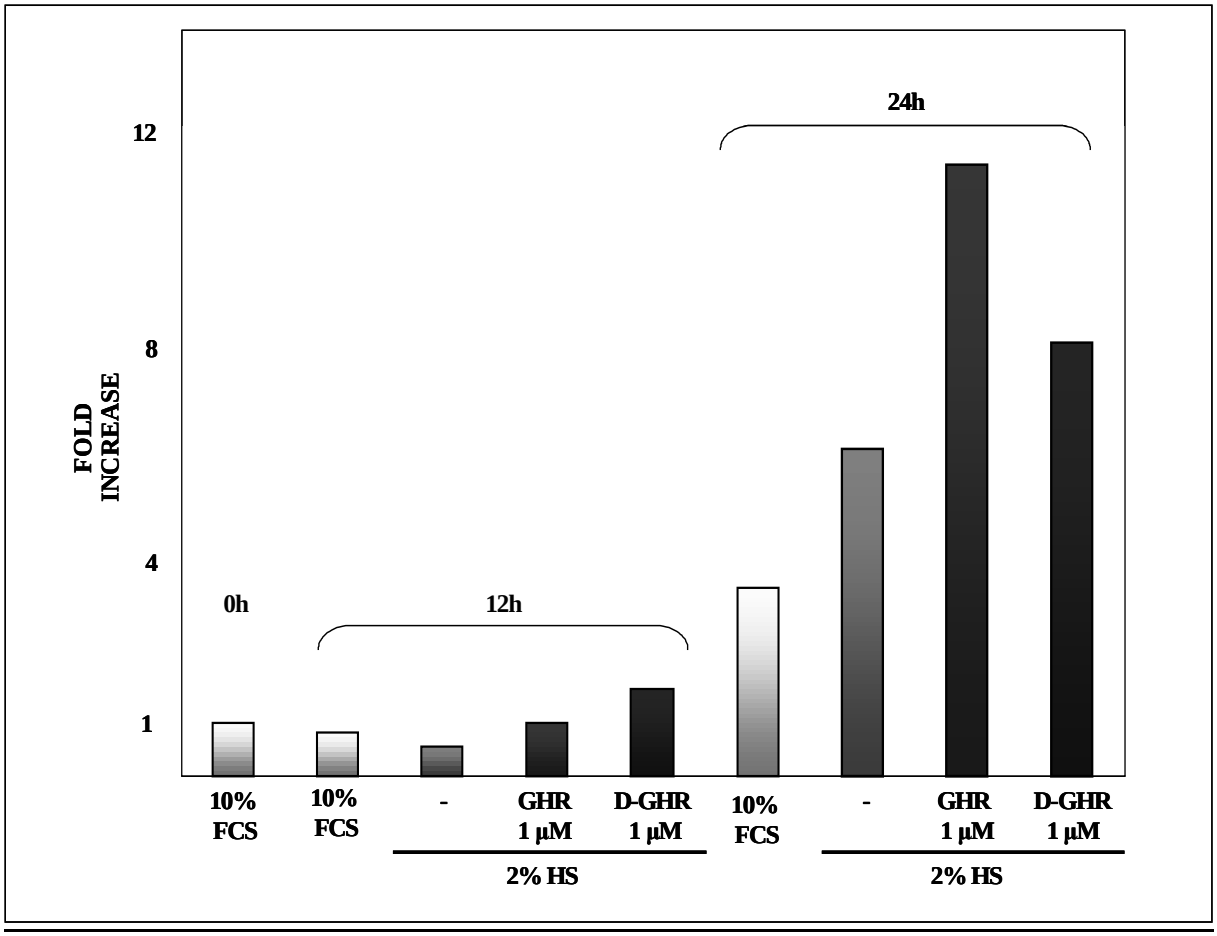

Fig. 6. **Ghrelin and des-acyl-ghrelin enhance myogenin expression in differentiating C2C12 myoblasts.** C2C12 myoblasts were incubated in differentiation medium with or without ghrelin and des-acyl ghrelin 1 µM and lysed after 24 or 48 hours of treatment, the RNA was extracted, retrotranscribed and the real-time PCR for the determination of the amount of myogenin performed on cDNAs.

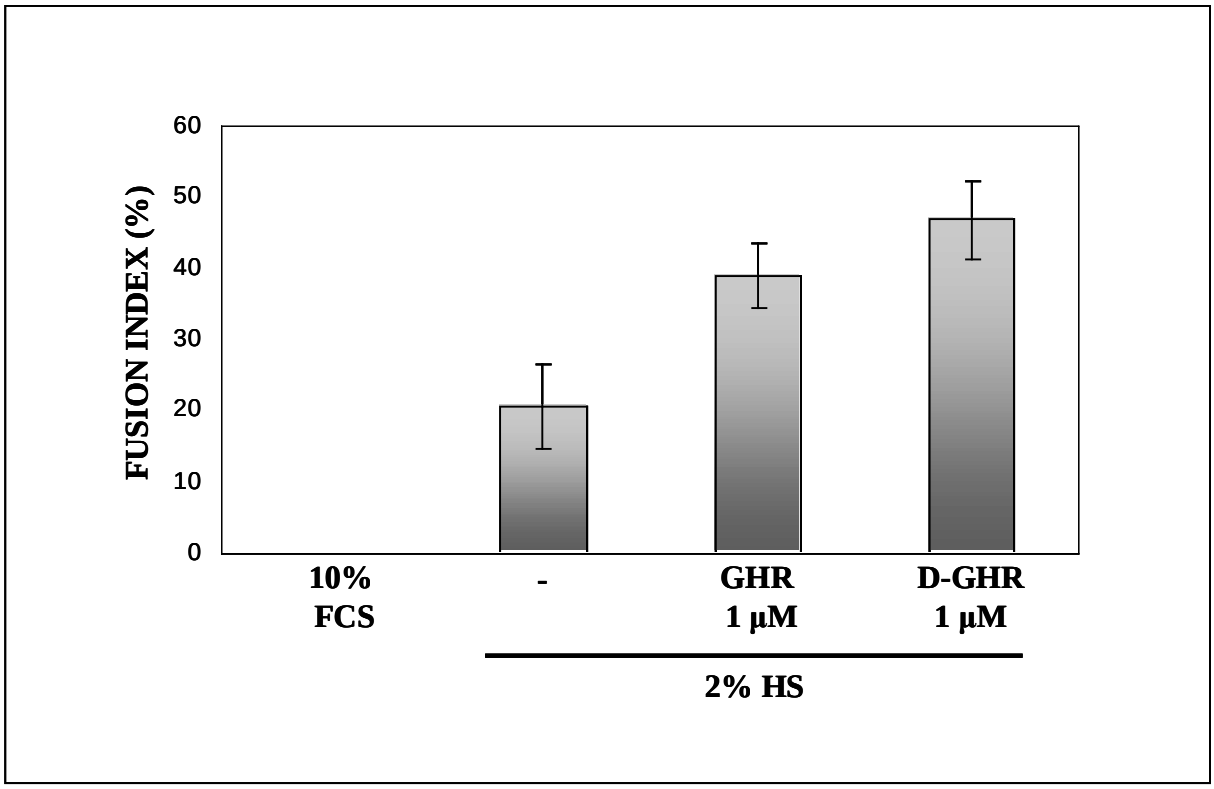

Fig. 7. **Ghrelin and des-acyl ghrelin enhance fusion of C2C12 myoblasts in differentiation medium.** C2C12 myoblasts treated with ghrelin and des-acyl ghrelin 1 µM in differentiation medium were fixed after three days of treatment and the differentiation index was calculated as above. $p<0.05$ for both treatments compared to cells in DM.

As we have seen that the effects of ghrelin and des-acyl ghrelin on C2C12 myoblasts are equivalent, moving on a system of primary cell culture, as that of satellite cells, we decide to analyse prevalently the effects elicited by des-acyl ghrelin. As showed in Fig. 8, after 24 hours of treatment in growth medium, des-acyl ghrelin does not seem able to induce differentiation of satellite cell, at least at low concentrations, (Fig. 8A), while the effect on fusion is comparable with that elicited by the differentiation medium (Fig. 8B). However, for longer periods of treatments, the effect of des-acyl ghrelin on differentiation of satellite cells in growth medium becomes more evident (Fig. 9A), while the effect on fusion is far stronger than that induced by the differentiation medium (Fig. 9B).

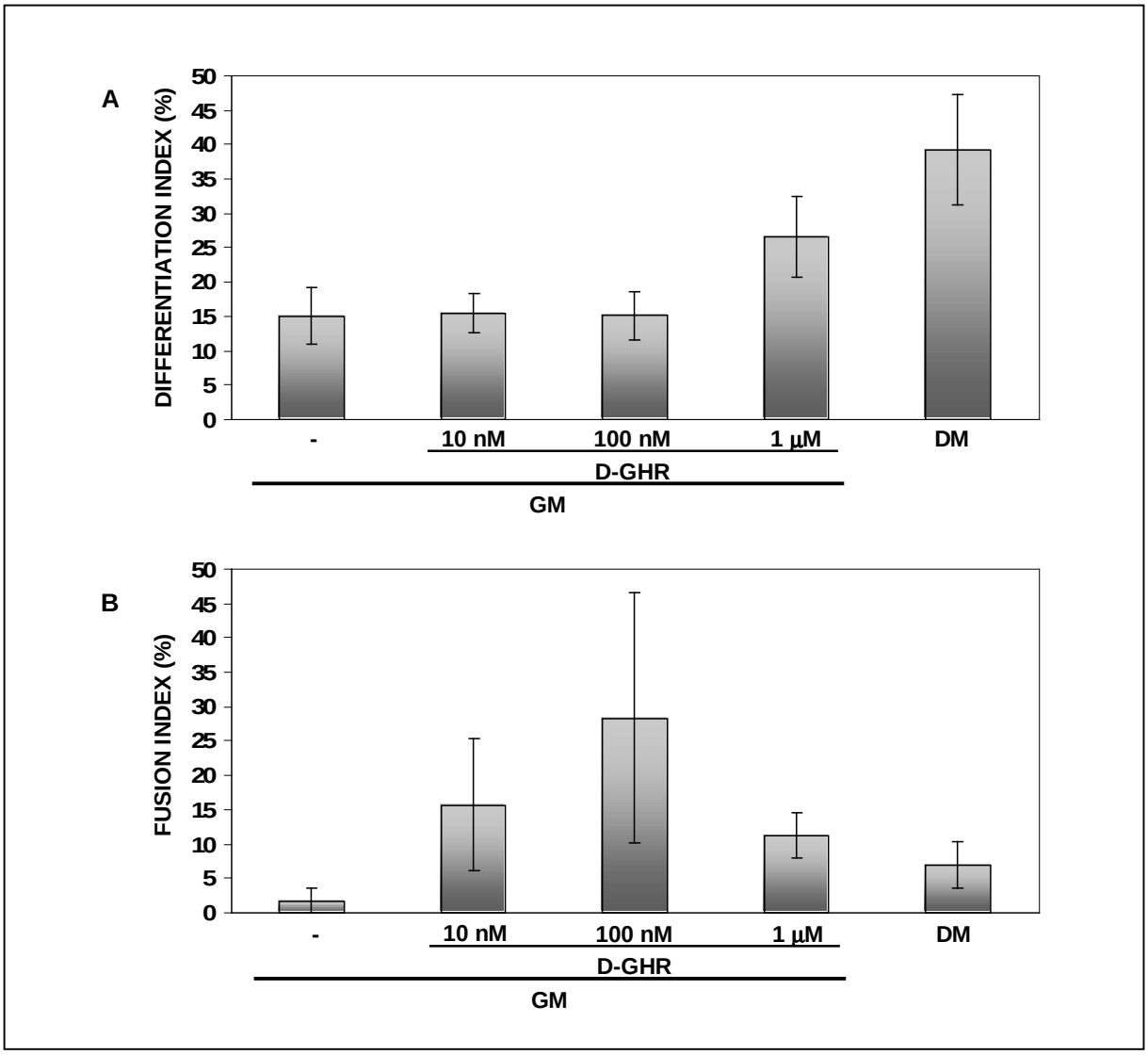

Fig. 8. **Differentiation and fusion of satellite cells in GM after 24 hours of treatment.** Satellite cells were incubated with des-acyl ghrelin at the indicated concentrations in GM for 24 hours and then fixed, immunostained and photographed for the determination of differentiation (A) and fusion (B) indexes as previously described. In (A) $p<0.05$ for treatment with D-GHR 1mM compared with cells in GM; in (B) $p<0.05$ for each treatment compared to cells in GM.

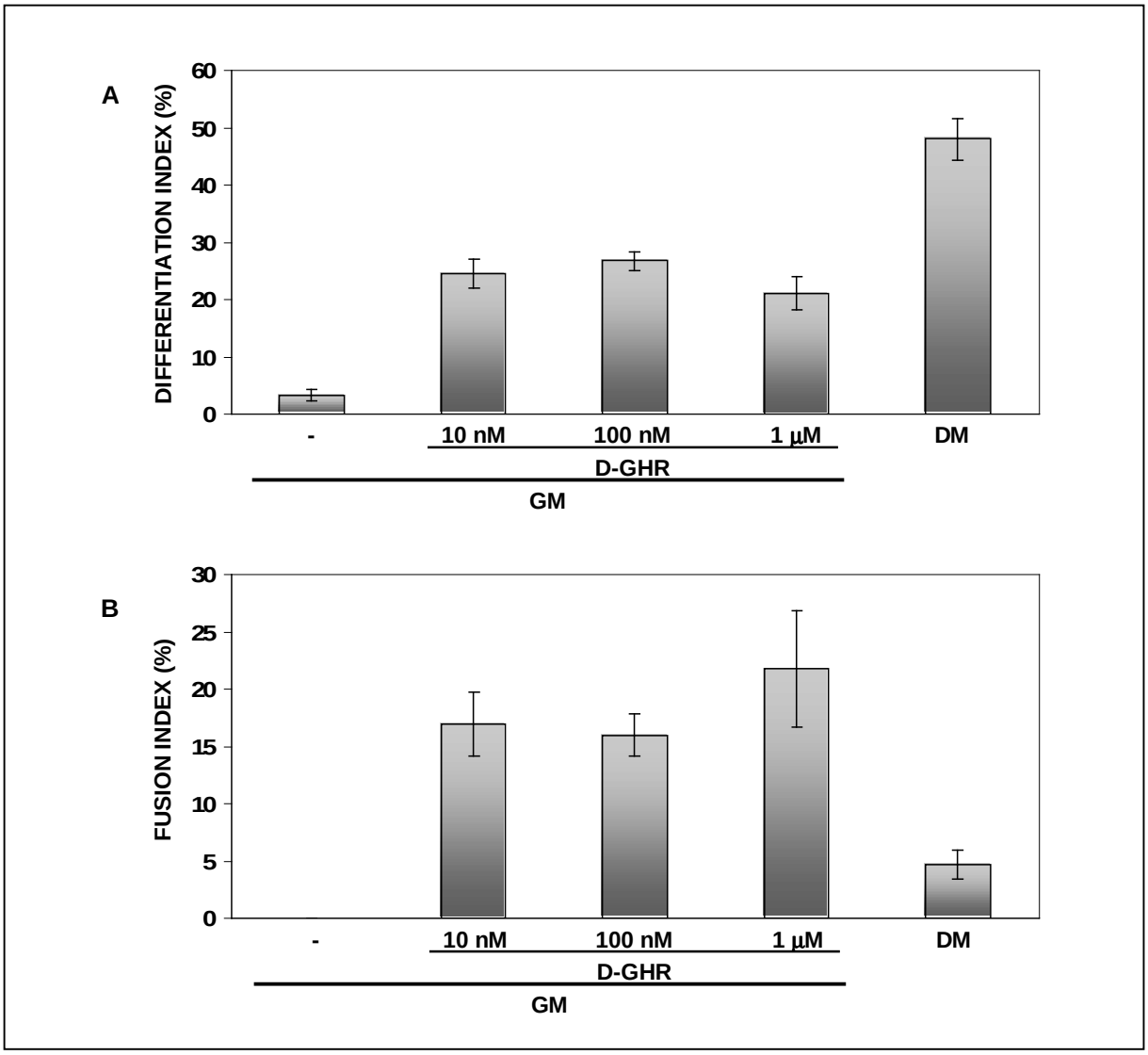

Fig. 9. **Differentiation and fusion of satellite cells in GM after 72 hours of treatment.** Satellite cells were incubated with des-acyl ghrelin at the indicated concentrations in GM for 72 hours and then fixed, immunostained and photographed for the determination of differentiation (A) and fusion (B) indexes as previously described. $p<0.001$ for each treatment compared to cells in GM

Verifying the hypothesis of a putative synergic action on differentiation induced by differentiation medium also on primary satellite cells, we could not appreciate any effect on differentiation (Fig. 10A), while the myoblasts fusion was enhanced by the addiction of des-acyl ghrelin (Fig. 10B).

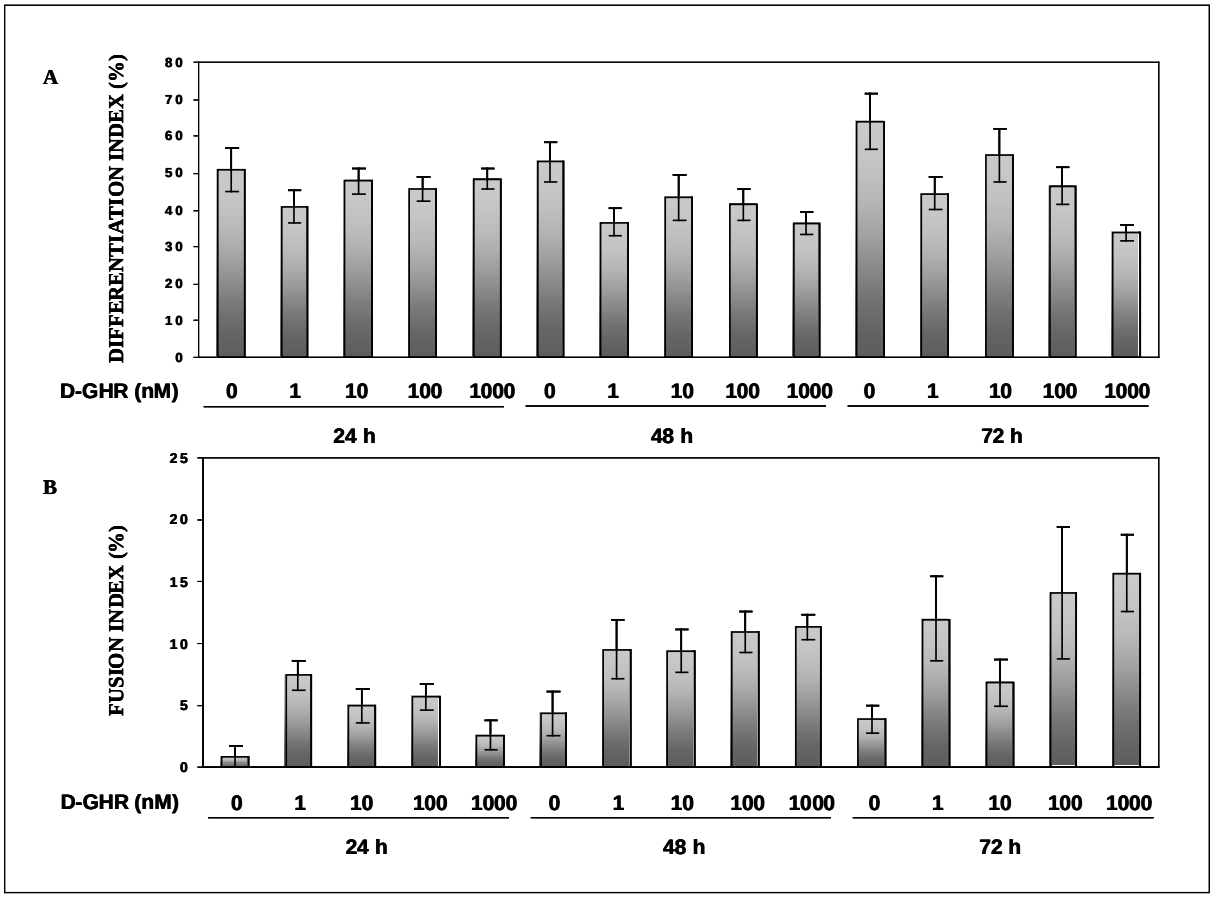

Fig. 10. **Des-acyl ghrelin enhance fusion of differentiating satellite cells.** Satellite cells were treated with the indicated concentrations of des-acyl ghrelin in DM for 24, 48 and 72 h, then fixed, immunostained and photographed for the determination of differentiation (A) and fusion (B) indexes as previously described. In (B), $p<0.05$ for each treatment except D-GHR 10 nM at 72 h compared to untreated cells.

<u>Ghrelin and des-acyl ghrelin bind to a common receptor, distinct from GHSR-1a, and activate a common biochemical and biological response in C2C12 myocytes</u>

We have assayed the expression of GHSR-1a in C2C12 myoblasts and differentiated myotubes using cDNA from the whole mouse brain as a positive control. Expression was measured by RT-PCR of GHSR-1a first exon, second exon and full length using intron-spanning primers to distinguish the eventual presence of GHSR-1b or genomic DNA. No expression was detected in both undifferentiated and differentiated C2C12 myocytes (Fig. 11), suggesting that ghrelin activity in skeletal muscle is not mediated by GHSR-1a. These findings support our previous hypothesis of the existence of a novel ghrelin receptor, distinct from GHSR-1a, through which ghrelin and des-acyl ghrelin exert several of their biological actions.

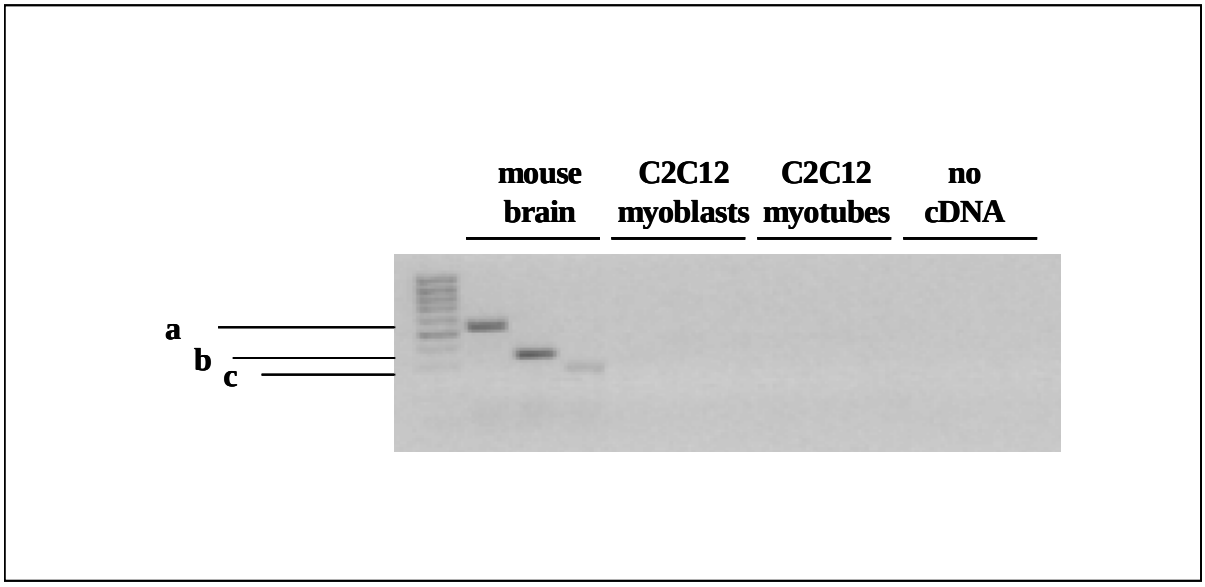

Fig. 11. **GHSR-1a is not expressed in C2C12 myoblasts and myotubes.** RT-PCR was performed on total RNA extracted from whole mouse brain, C2C12 myoblasts and differentiated myotubes with primers specific for the full-length receptor (a), exon 1 (b) and exon 2 (c).

In an attempt to further characterise the action of ghrelin on skeletal muscle, we have also investigated the possible pathways activated by ghrelin and des-acyl ghrelin on differentiated C2C12 myotubes. Our data show that on these myotubes both ghrelin and des-acyl ghrelin activate ERK-1/2 (Fig. 12), while we were not able to detect activation of PI3K/Akt pathway (data not shown).

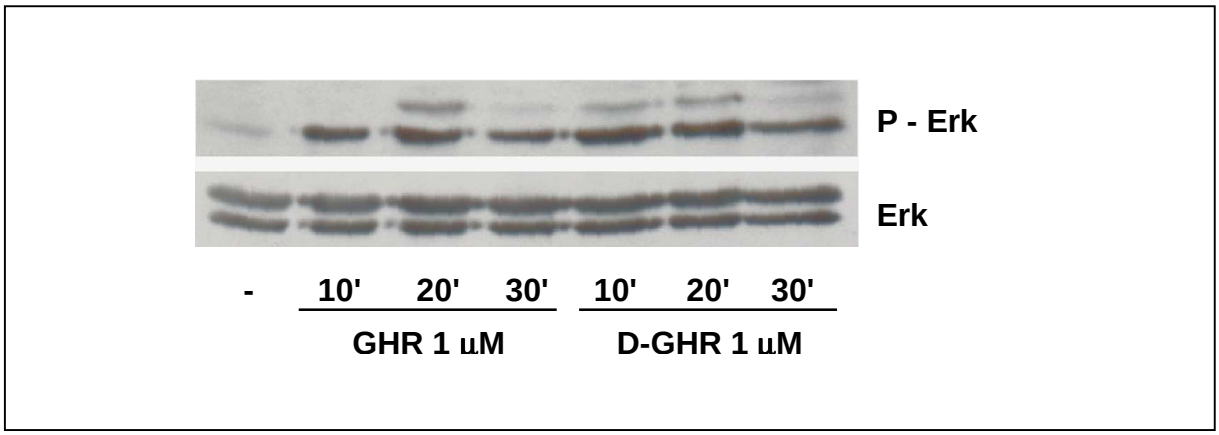

Fig. 12. **Ghrelin and des-acyl ghrelin induce activation of ERK-1/2 in C2C12 myotubes.** C2C12 myoblasts were differentiated in DM for 3 days and then treated with ghrelin and des-acyl ghrelin 1 μM for the indicated times. Total lysates were analyzed by Western blot with specific anti-phosphoERK-1/2 antibodies (top) and anti-ERK-1/2 antibodies (bottom).

The activation of ERK-1/2 on differentiated myocytes has been suggested to correlate with the onset of muscular hypertrophy [83]. On this base, we have investigated the ability of ghrelin and des-acyl ghrelin to induce skeletal muscle hypertrophy analysing the incorporation of [^{3}H]-thymidine and [^{3}H]-leucine in myoblasts after treatment with the peptides. Satellite cells starved over night, were treated with ghrelin, des-acyl ghrelin and angiotensin II as positive control of hypertrophy and incubated for 24 hours with [^{3}H]-leucine and for 3 hours with [^{3}H]-thymidine. The results showed, as expected for factors inducing differentiation, an inhibition of proliferation reflected by the reduced incorporation of [^{3}H]-thymidine (Fig. 13A), while the incorporation of [^{3}H]-leucine, indicating an increase in cell protein content, was not affected by the treatments (Fig. 13B).

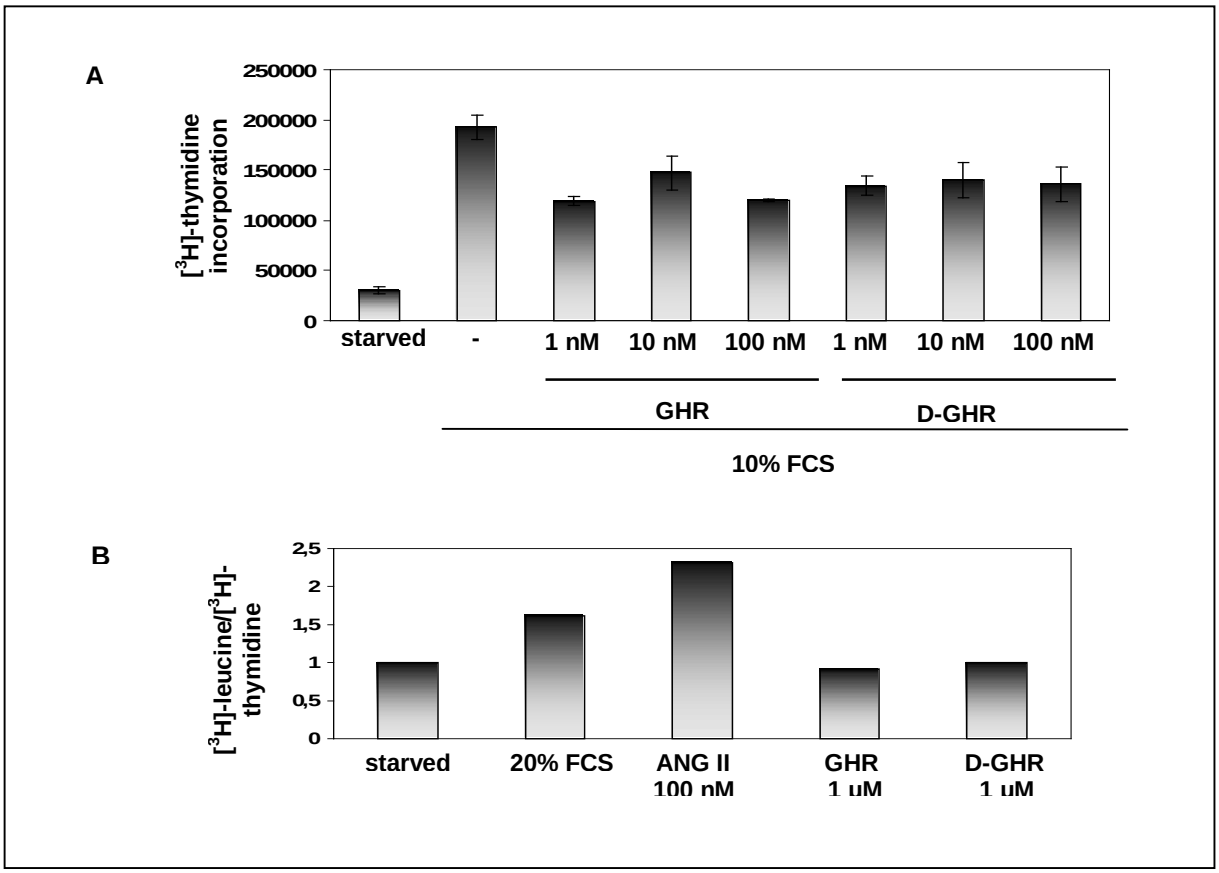

Fig. 13. **Ghrelin and des-acyl ghrelin do not induce skeletal muscle hypertrophy.** Satellite cells starved over night were treated with ghrelin, des-acyl ghrelin, high serum and angiotensin II at the indicated doses for 24 hours. [^{3}H]-leucine was administrated at the same time of treatments, while [^{3}H]-thymidine at the end of treatments for 3 hours with. (A): incorporation of [^{3}H]-thymidine after 24 hours of treatments with ghrelin or des-acyl ghrelin. (B): the ratio between the amounts of the

incorporated [^{3}H]-leucine and the incorporated [^{3}H]-thymidine was taken as index of hypertrophy. In (A), $p<0.05$ for each treatment compare to untreated cells.

Conclusions

Ghrelin is a 28 amino acid peptidic hormone secreted into the circulation from the stomach but also synthesized in a number of tissues suggesting both endocrine and paracrine effects. The unique acyl modification of the peptide with octanoic acid on its third serine residue is necessary to some of its biological effects, such as GH release, but not for other effects, including antiapoptotic effects, modulation of cell proliferation and differentiation in several cell types.

We have recently showed that both ghrelin and des-acyl ghrelin inhibit apoptosis of cardiomyocytes and endothelial cells [11], through binding to common high affinity binding sites, distinct from GHSR-1a, the only ghrelin receptor identified so far.

The finding that ghrelin up-regulation is specifically associated to cachexia [80], and that its administration strongly prevents CHF-associated cachexia [53], and that the skeletal muscle feature high binding sites for synthetic GHS [79], lead us to speculate that it may act also on skeletal muscle. As ghrelin and des-acyl ghrelin circulating level are up-regulated by starvation and strongly down-regulated by food uptake [84], we may speculate that ghrelin/des acyl -ghrelin may have been evolved as hormones regulating a response to starvation which coordinates an integrated response in the heart, in the pituitary and which would maintain the integrity of skeletal muscle.

Little is known about the importance of circulating ghrelin and des-acyl ghrelin in the regulation of physiological processes, as ghrelin knockout animals are apparently completely normal [85], but the redundancy of other factors could mask relevant effects of this peptide. However, ghrelin knockout animals were studied up to 20 weeks of age and theoretically it is possible that some of the effects of lack of ghrelin will become manifest during aging. Analogously, the complete GHSR knockout mice show little differences compared to the wild-types: although they are slightly smaller and have a modestly reduced IGF-I level, their appetite, food intake, and body composition as well as ghrelin, insulin, and leptin levels in response to fasting are similar to wild-type littermates. As expected, they do not respond to ghrelin injection with GH release or appetite induction suggesting that these effects are certainly relayed by the GHSR-1a isoform [86].

Although not essential for embryo development, from our data ghrelin seems to be one of the factors involved in the complex process of myogenesis in the adulthood, i.e. in regenerative processes of skeletal muscle. We may hypothesize that a muscular injury, that is the demolition of muscle proteins, can elicit in the muscle an autocrine/paracrine loop leading to activate skeletal myocytes turnover. According to this hypothesis, ghrelin would contribute to skeletal muscle plasticity, promoting the differentiation and fusion of myoblasts in the damaged muscles. Another hypothesis is that ghrelin could contribute in maintaining the muscular mass integrity in conditions, like fasting, promoting the degradation of skeletal proteins to supply energy. If this hypothesis would be proved, the activation of the receptor mediating ghrelin/des-acyl ghrelin differentiative activity, as well as the over-expression of the hormone, may provide novel therapeutic strategies for the reduction or retardation of several skeletal muscle pathologies, including dystrophies, atrophies and cachexia.

Based on these considerations, to further investigate the role of ghrelin in promoting skeletal muscle regeneration, we are currently developing two novel tools: the generation of transgenic mice specifically expressing GHR in the skeletal muscle and the generation of a ghrelin-expressing lentiviral vector.

For the generation of the transgenic mouse expressing ghrelin in rapid contracting skeletal muscle, ghrelin cDNA has been cloned in a plasmidic vector under the control of the skeletal muscle-specific myosin light chain 1 (MLC1) promoter, kindly donated by Dr. Rosenthal (EMBL- Rome). If ghrelin play a role in promoting muscle regeneration, as well as in inhibiting muscle atrophy, we expect that ghrelin transgenic mice will be protected form skeletal muscle damage and atrophy, either denervation-induced or aging-induced.

For the lentiviral construct generation, we have cloned ghrelin in the lentiviral vector MA1, kindly donated by Prof. Naldini (HSR-Tiget, Milan), containing a synthetic bidirectional promoter that simultaneously promotes the transcription of two divergent mRNA sequences, one of which is encoding for a green fluorescent protein (EGFP). The generated construct has been transfected in satellite cells to verify *in vitro* the ability of this MA1/GHR vector to afford the expression of the ghrelin gene. Our preliminary data showed that both ghrelin and GFP are expressed by the MA1 vector, as seen by RT-PCR. Moreover, des-acyl ghrelin is secreted in culture medium, as assayed by RIA.

Main Findings

- Our paper published on *Endocrine* was the first report showing a specific cellular function for a GHS outside the endocrine system. Moreover, our data concerning the binding sites of hexarelin in the heart suggested for the first time the hypothesis of a receptor, expressed in the heart, different from GHSR-1a as featuring a selective specificity for peptidyl GHSs and not for nonpeptidyl GHSs.

- With the paper on *J Cell Biol* we provide for the first time the evidence of a biological action of des-acyl ghrelin. Indeed, des-acyl ghrelin, which is devoid of any GH-releasing activity even *in vitro*, is as effective as ghrelin in inhibiting apoptosis and in activating intracellular signaling pathways. Moreover our work consolidate our hypothesis that ghrelin (and GHSs) antiapoptotic activity is not mediated by GHSR-1a, on the following basis: 1) both ghrelin and des-acyl ghrelin recognize a common high affinity binding site, although only ghrelin and not des-acyl ghrelin binds to and activates GHSR-1a, 2) no expression of GHSR-1a was detected in H9c2 cardiomyocytes, 3) the affinity constant of ghrelin binding sites on H9c2 cardiomyocytes are 10-fold higher than affinity constant of GHSR-1a as measured on pituitary and hypothalamus membranes.

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