10. Introduction to

"Ghrelin and Des-Acyl Ghrelin Promote Differentiation and Fusion of C2C12 Skeletal Muscle Cells"

by

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Molecular Biology of the Cell (2007) 18: 986-994

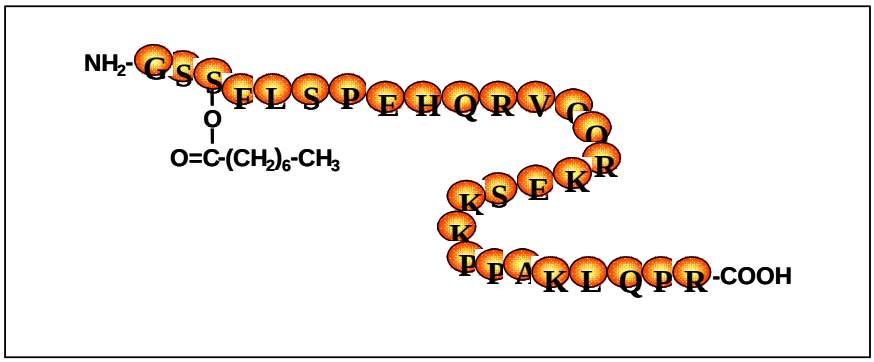
10.1. From GH secretagogue peptides to Ghrelin, through GHSR

The Growth Hormone Secretagogues (GHS) are synthetic peptides derived from met-enkephalins which are able to induce release of Growth Hormone (GH) via a mechanism which is distinct from that of the hypothalamic GH-releasing hormone (GHRH) and is mediated by PLC-induced rise in IP_3 and intracellular calcium.

Combining expression cloning and the GHS compound MK0677 (Merck), looking for changes in intracellular calcium to indicate a positive response, the GH secretagogue receptor (GHSR) was finally identified and cloned [52]. This receptor is a typical GPCR of 366 amino acids belonging to the rhodopsin family with seven transmembrane domains (7-TM) [52,53,54], with two splicing variants presently identified, called GHSR type 1a and 1b. Type 1b derives from a mRNA encoding only five of the seven predicted TM domains, is 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 [55,56].

The cloning of the GH secretagogue receptor provided the tool necessary to identify its natural ligand: the natural ligand for the GHS receptor was designated ghrelin [57]. The mature 28-amino acid peptide is produced mainly by the stomach, cleaved from its precursor preproghrelin and then acylated on serine 3 by an *n*-octanoic acid residue, although other types of acylations (10 carbon fatty acid group with and without insaturations) have been observed [57,58,59]. 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.

Once secreted in blood, the non-acylated form of ghrelin, des-acyl ghrelin, circulates in amounts far greater than acylated ghrelin [60]. Ghrelin in the plasma binds to high-density lipoproteins that contain a plasma esterase, paraoxonase, and clusterin [61]. Paraoxonase, a potent esterase, may be involved in deacylation of acyl-modified ghrelin, so that 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.



Structure of mature acylated ghrelin.

10.2. Expression and functions of ghrelin

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 [62], although minor amounts are present elsewhere in the body, namely in placenta [63], testis [64], kidney [65], pituitary [66], small intestine [67], pancreas [68], lymphocytes [69], brain [70], lung [71] and ovary [72,73] . Circulating ghrelin is present at concentrations of 100±140 fmol/ml [57] and 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 [74,75].

Besides the strong GH-releasing activity, ghrelin has other significant actions, including orexigenic action coupled with control of energy homeostasis, control of acid secretion and gastric motility [76], influences on pancreatic activity [77,78], influences on sleep [79], cardiovascular and hemodynamic actions, effects on proliferation on several cell lines, and differentiating effects.

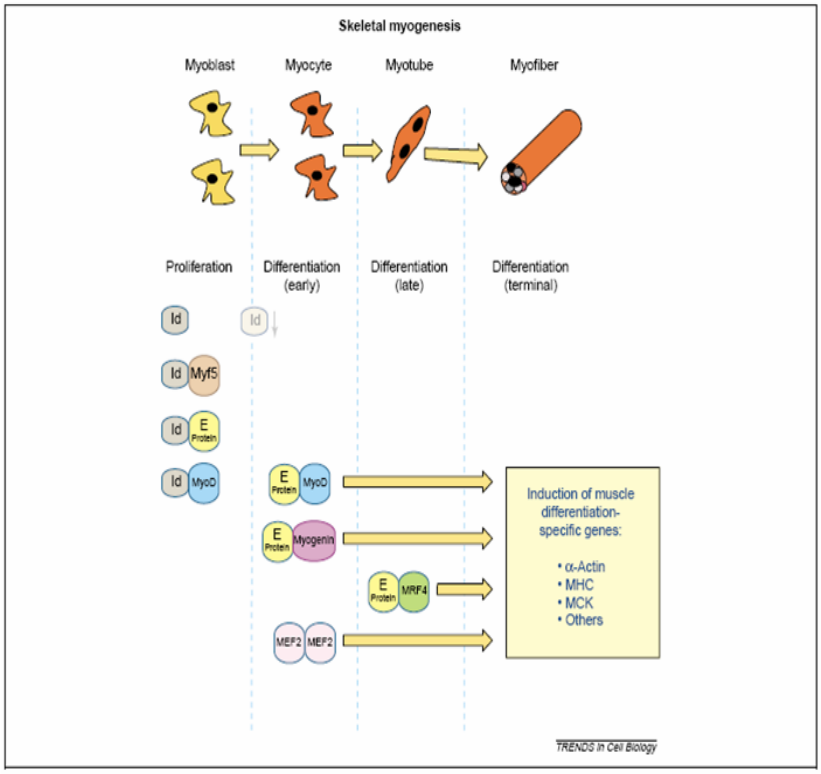
Ghrelin possesses cardiovascular activities: it has been shown that administration of ghrelin improves cardiac structure and function, and attenuates the development of cardiac cachexia in rats with heart failure [80,81,82], while in humans, the administration of ghrelin in normal young volunteers is followed by reduction in cardiac afterload and increase in cardiac output without any change in heart rate [83]. In humans, it has also been shown that infusion of ghrelin decreases systemic vascular resistance and increases cardiac output in patients with heart failure [84,85]. 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.

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 [86,87]. 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 Akt [87]. As non-acylated ghrelin is generally unable to activate the GHSR-1a receptor [88] and stimulate GH release [57], 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.

Several conflicting data concerne ghrelin and GHSs effects on cell proliferation. A positive effect on proliferation has been found in osteoblasts [89,90], primary oral keratinocytes [91], erythroleukemic cell line HEL [92], zona glomerulosa cells [93,94], rat pituitary cell line GH3 [95], 3T3-L1 preadipocytes [96,97], pancreatic adenocarcinoma cells [98], H9C2 cardiomyocyte cell line [99], and several prostate cancer cell lines [100]. On the other hand, it has been demonstrated that GHS and ghrelin inhibit cell growth of prostatic carcinoma cell lines [101], thyroid and follicular carcinoma cell lines [102], lung carcinoma [103], brest carcinoma cell lines [104], differentiating immature Leydig cells [105] and inhibit the proliferation of splenic T cells when these are co-stimulated by anti-CD3 [106].

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 [106, 96], but, on the other hand, the same cells overexpressing ghrelin showed significantly attenuated differentiation, while maintaining the increased proliferation rate [97]. It has also been demonstrated that ghrelin and GHSs affect directly the process of bone formation promoting both proliferation and differentiation of osteoblastic cells [89, 90]. 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 [105].

10.3. Skeletal muscle differentiation



Schematic representation of skeletal muscle differentiation from myoblasts to myofibres. From Lluis *et al.*, TICB 2006 (Ref. 107).

Skeletal muscle satellite cells are mononucleated myoblasts, which, upon muscle diseases or direct injury, undergo proliferation and eventually differentiate to

form new muscle fibers in order to allow muscle regeneration. *In vivo*, differentiation of skeletal muscle involves first the growth factor-sustained expansion of the population of skeletal myoblasts, and then cell cycle exit and initiation of terminal differentiation, which involves expression of contractile proteins and formation of multinucleated syncitia by myocytes fusion. The extracellular signals triggering growth arrest and the molecular mechanisms involved in the induction of myoblasts differentiation and fusion still remain to be fully elucidated (for review see 107).

In vitro, muscle differentiation steps can be reproduced with myoblastic satellite-derived cell lines, such as the C2C12 murine myoblast cells. C2C12 myoblasts proliferate in presence of 10% FCS (growth medium, GM), and undergo differentiation when cultured in 2% horse serum (differentiation medium, DM).

10.4. Presentation of the article by Filigheddu *et al.* (Mol. Biol. Cell, 2007)

In this article, we demonstrated that both ghrelin and des-acyl ghrelin stimulate proliferating C2C12 skeletal myoblasts to differentiate and to fuse into multinucleated myotubes *in vitro* through activation of p38 MAP kinase. Consistently, both ghrelin and des-acyl ghrelin inhibit C2C12 proliferation in growth medium. In addition, the we demonstrated that ectopic expression of ghrelin in C2C12 enhances the differentiation and fusion of these myoblasts in differentiation medium. Finally, C2C12 cells are analyzed for expression for expression of GHSR-1a and are shown not to express its mRNA. Indeed, C2C12 contain a common high-affinity binding site recognized by both acylated and des-acylated ghrelin, suggesting that the described activities on C2C12 are likely to be mediated by a novel yet unidentified receptor for both ghrelin forms.