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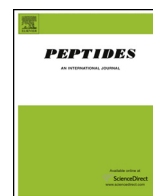
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Characterization of a far-red analog of ghrelin for imaging GHS-R in P19-derived cardiomyocytes

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ABSTRACT

Ghrelin and its receptor, the growth hormone secretagogue receptor (GHS-R), are expressed in the heart, and may function to promote cardiomyocyte survival, differentiation and contractility. Previously, we had generated a truncated analog of ghrelin conjugated to fluorescein isothiocyanate for the purposes of determining GHS-R expression in situ. We now report the generation and characterization of a far-red ghrelin analog, [Dpr^3(octanoyl), Lys^{19}(Cy5)]ghrelin (1–19), and show that it can be used to image changes in GHS-R in developing cardiomyocytes. We also generated the des-acyl analog, des-acyl [Lys^{19}(Cy5)]ghrelin (1–19) and characterized its binding to mouse heart sections. Receptor binding affinity of Cy5-ghrelin as measured in HEK293 cells overexpressing GHS-R1a was within an order of magnitude of that of fluorescein-ghrelin and native human ghrelin, while the des-acyl Cy5-ghrelin did not bind GHS-R1a. Live cell imaging in HEK293/GHS-R1a cells showed cell surface labeling that was displaced by excess ghrelin. Interestingly, Cy5-ghrelin, but not the des-acyl analog, showed concentration-dependent binding in mouse heart tissue sections. We then used Cy5-ghrelin to track GHS-R expression in P19-derived cardiomyocytes. Live cell imaging at different time points after DMSO-induced differentiation showed that GHS-R expression preceded that of the differentiation marker aMHC and tracked with the contractility marker SERCA 2a. Our far-red analog of ghrelin adds to the tools we are developing to map GHS-R in developing and diseased cardiac tissues.

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1. Introduction

Ghrelin is a growth-hormone releasing peptide that mediates its effects through the growth hormone secretagogue receptor, GHS-R1a. Knockout mouse models have identified the importance of GHS-R signaling in energy homeostasis [12], feeding behavior leading to body weight control [29] and stressor-induced metabolic changes [22]. In addition to effects on whole-body metabolism, there is a growing body of evidence to support the hypothesis that ghrelin exerts effects on the cardiovascular system, and in particular, directly on cardiomyocytes. In fact, ghrelin is synthesized in and secreted from human cardiomyocytes [6], binds in a specific and saturable manner to human left ventricular tissue [8], and stimulates contractility in isolated rat ventricular myocytes through increasing Ca2+ current via GHS-R [26]. Additionally, ghrelin administration inhibits cardiomyocyte apoptosis [1] and promotes differentiation of human embryonic stem cells to mature cardiomyocytes through the extracellular signal-regulated kinase (ERK 1/2) signaling pathway [4], but it is not clear if these effects are mediated through GHS-R.

The expression of GHS-R itself may reflect the progression of cardiac pathology. GHS-R expression is up-regulated in left ventricular tissue from patients with congestive heart failure [3] and atherosclerosis [8], as well as in the hearts of rats during the hyperdynamic phase of sepsis [28]. These results indicate that GHS-R signaling is altered in cardiovascular disease, and point toward the use of ghrelin in pharmacologic therapy of heart disease. Therefore, given the importance of GHS-R signaling and expression in the heart, we are developing ghrelin analogs for the purpose of generating agents for in situ and in vivo imaging of cardiac GHS-R. We have previously designed a fluorescent ghrelin analog consisting of a fluorescein moiety attached through a lysine to the N-terminal 18 amino acids of ghrelin (fluorescein-ghrelin(1–19)) [20] and have shown that it specifically binds to GHS-R1a in mouse heart tissue.

While this analog can be used in fixed cells and tissues, fluorescein is not useful for in vivo imaging, due to its propensity to photobleach, its lack of depth penetration and high degree of tissue autofluorescence in the fluorescein excitation wavelength. To obviate these issues, we report in the present study the synthesis and characterization of a far-red probe, Cy5-ghrelin(1–19), and demonstrate its use in tracking GHS-R expression in live differentiating cardiomyocytes.

2. Materials and methods

2.1. Synthesis of Cy5-ghrelin and Cy5-des-acyl ghrelin

2.1.1. [Dpr³(octanoyl)Lys¹⁹(Cy5)]ghrelin (1–19)

The 19 amino acid analog of ghrelin, GS-Dpr-FLSPEHQRVQQRKESK-amide, was synthesized using an automated peptide synthesizer on Rink Amide MBHA resin (0.1 mmol). All amino acids bear standard protecting groups with the exception of residue 3, diaminopropanoic acid (Dpr), and residue 19, lysine, that contain orthogonal side chain protecting groups allyloxycarbonyl (alloc) and 4-methyltrityl (mtt), respectively. While still on resin, Dpr³(alloc) was selectively deprotected with phenylsilane (2 mmol, 247 μL) and tetrakis(triphenylphosphine)palladium (0) (0.035 mmol, 40 mg) under nitrogen. The resulting free amine was coupled to octanoic acid (0.3 mmol, 247 μL) using standard FMOC procedures. Lys¹⁹(mtt) was selectively deprotected using 2% trifluoroacetic acid, 5% triisopropylsilane in dichloromethane, followed by coupling to Cy5 succinimidyl ester (0.025 mmol, 19 mg) in the presence of N,N-diisopropylethylamine (0.6 mmol, 100 μL). Once fully functionalized, Dpr³(octanoyl), Lys¹⁹(Cy5) ghrelin (1–19) was cleaved from the resin using 95% trifluoroacetic acid, 2.5% triisopropylsilane and 2.5% water (2 mL). The crude peptide was precipitated using ice cold tert-butylmethylether (20 mL) and the dark blue crude solid was then isolated by centrifugation. The solid was dissolved in 10% acetonitrile in water, frozen in dry ice and lyophilized overnight to yield a dark blue powder. Purification was performed by reverse-phase C18 HPLC (gradient 25–60% MeCN in water) to obtain a 7% yield (5.1 mg) of blue powder (89% purity). Mass spectrometry electrospray ionization (MS (ESI)): m/z calcd 1489.2, found 1489.4 [M+2H]$^{2+}$.

2.1.2. Des-acyl [Lys¹⁹(Cy5)]ghrelin (1–19)

The 19 amino acid control analog of ghrelin, GSSFLSPEHQRVQQRKESK-amide, was synthesized using an automated peptide synthesizer on Rink Amide MBHA resin with all amino acids bearing standard protecting groups with the exception of residue 19, lysine, which is protected by the orthogonal protecting group, 4-methyltrityl. Lys¹⁹(mtt) is selectively deprotected using 2% trifluoroacetic acid, 5% triisopropylsilane in dichloromethane, followed by coupling to Cy5 succinimidyl ester (0.025 mmol, 19 mg) in the presence of N,N-diisopropylethylamine (0.6 mmol, 100 μL). Once fully functionalized, [Lys¹⁹(Cy5)]ghrelin (1–19) was cleaved from the resin using 95% trifluoroacetic acid, 2.5% triisopropylsilane and 2.5% water (2 mL). The crude peptide was precipitated using ice cold tert-butylmethylether (20 mL) and the dark blue crude solid was isolated by centrifugation. The solid is dissolved in 10% acetonitrile in water, frozen in dry ice and lyophilized overnight to yield a dark blue powder. Purification was performed by reverse-phase C18 HPLC (gradient 20–60% MeCN in water) to obtain a 7% yield (4.8 mg) of blue powder (97% purity). MS (ESI): m/z calcd 1426.6, found 1427.0 [M+2H]$^{2+}$.

2.2. Receptor binding assays

HEK293 cells (a kind gift from Dr. Andy Babwah, Western University) were grown in MEM supplemented with non-essential amino acids solution and 10% fetal bovine serum (FBS; Life Technologies). To generate HEK293/GHS-R1a cells, cells were grown to 70% confluency in a 75 cm flask and transfected with GHS-R1a-pcDNA3.1 plasmid (Missouri S&T cDNA Resource Center; www.cdna.org) using a modified calcium phosphate transfection procedure [21]. Receptor binding was measured by the competitive binding of [^{125}I] ghrelin with increasing concentrations of the synthesized Cy5-ghrelin analogs using a previously published method [23]. Briefly, cells were harvested 48 h after transfection and frozen at a concentration of 2×10^6 cells/mL. On the day of the experiment, cells were thawed at room temperature, pelleted by centrifugation and resuspended in binding buffer (25 mM HEPES, 5 mM MgCl$_2$, 1 mM CaCl$_2$, 2.5 mM EDTA, 0.4% BSA, plus mini complete protease inhibitor (Roche), pH 7.4). Approximately 50,000 cells were incubated for 20 min at 37 °C in binding buffer, 15 pM [^{125}I] ghrelin (Perkin Elmer) and increasing concentrations of each Cy5-ghrelin analog (10^{-10}–1×10^{-5} M). Cells were pelleted, rinsed with ice-cold 50 mM Tris–HCl (pH 7.4) to remove unbound activity and radioactivity was determined in a gamma counter. Binding curves and IC$_{50}$ values determined using GraphPad Prism 5 software (San Diego, CA) as previously described [20]. Each sample was analyzed in triplicate and assays were repeated twice for each Cy5-ghrelin analog.

2.3. Live imaging of Cy5-ghrelin(1–19) in HEK293/GHS-R1a cells

HEK 293 cells were transiently transfected with the cDNA encoding GHS-R1a in order to visualize the binding of Cy5-ghrelin(1–19) in situ. Both wt HEK and HEK/GHS-R1a cells were plated at a density of 1×10^6 cells/well in a 6-well plate, and serum starved overnight. The following day, 10 μM Cy5-ghrelin(1–19) or 10 μM Cy5-des-acyl ghrelin(1–19) was added to the dishes (500 μL/well), and cells were incubated at 37 °C for 30 min, and subsequently rinsed in serum-free media. For blocking studies, HEK/GHS-R1a cells were incubated with 10 μM Cy5-ghrelin(1–19) in the presence of 40 μM unlabeled ghrelin (1–28). Live cell images were captured using an inverted fluorescent microscope and final images were prepared using ImageJ.

2.4. Binding of Cy5-ghrelin(1–19) and Cy5-des-acyl ghrelin(1–19) to heart tissue in situ

Mice were treated in accordance with the guidelines set by the Animal Use Subcommittee of the Canadian Council on Animal Care at Western University. Adult male or female C57BL/6 mice were sacrificed using CO$_2$ and the hearts were immediately removed and fixed in 4% paraformaldehyde (in PBS) overnight. Whole hearts were then equilibrated in 30% sucrose in PBS, snap-frozen in Tissue-Tek OCT using dry-ice cooled ethanol, and subsequently cryosectioned at 8 μm. To assess binding of both acylated and non-acylated ghrelin, sections were incubated for 30 min at room temperature with various concentrations (0–20 μM) of Cy5-ghrelin(1–19) or Cy5-des-acyl ghrelin(1–19) alone. To determine the specificity of binding, sections were incubated with 10 μM Cy5-ghrelin(1–19) and either 100 μM ghrelin (1–28) or 100 μM hexarelin. Slides were then rinsed 3 × 5 min in PBS, incubated in Hoechst 34445 as previously described [19] to visualize nuclei, and mounted with glass coverslips. Slides were viewed under a fluorescent microscope using the Cy5 and DAPI filters. Five random fields of view were captured for each of 5 slides per tissue per mouse. Average fluorescence intensities were determined using ImageJ, as described previously [18].
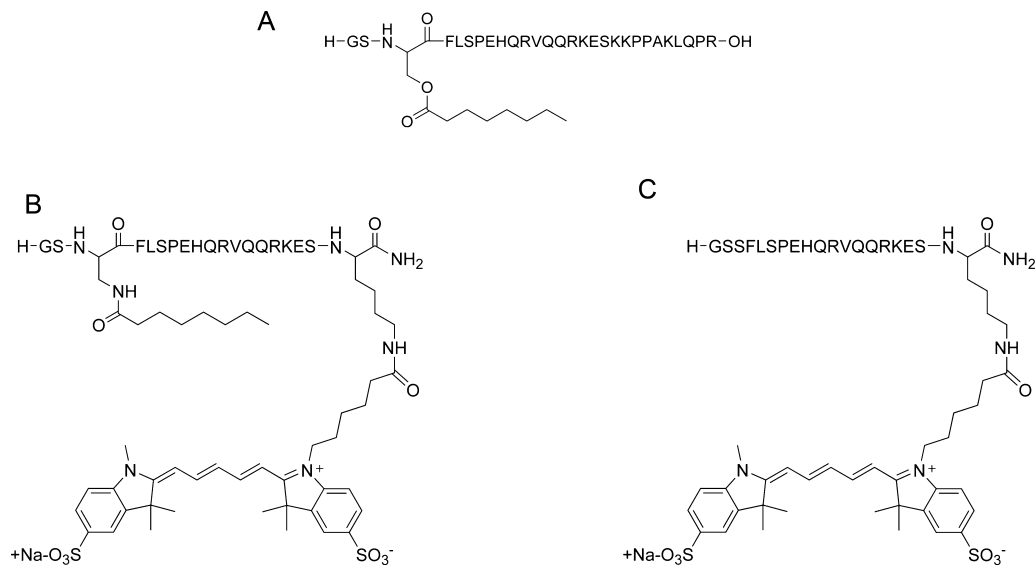
Fig. 1. Structures of Cy5-ghrelin analogs. (A) Structure of wild-type human ghrelin, showing the octanoyl modification on the side chain of Ser[3]. (B) Structure of [Dpr[3](octanoyl), Lys[19](Cy5)]ghrelin (1–19). The 19 amino acid analog of ghrelin, GS-DprFLSPEHQRVQQRKESK-amide, was synthesized using Dpr[3] in place of Ser[3]. The Cy5 moiety was coupled to Lys[19]. (C) Structure of des-acyl [Lys[19](Cy5)]ghrelin (1–19). The Ser[3] bears no octanoyl modification.

2.5. Differentiation of P19 cells and live cell imaging

P19 embryonic carcinoma cells were purchased from ATCC, and grown in alpha-MEM/10% FBS (Life Technologies). To track differentiation, P19 cells were stably transfected with a cDNA construct designed to drive RFP expression only in differentiated cardiomyocytes, as follows: the alpha myosin heavy chain (αMHC) promoter region (kind gift from Dr. J. Robbins, Cincinnati Children's Hospital) was fused to the *fluc-mrfp-sr39tk* trifusion reporter (TFR) fragment [24] (kind gift from Dr. S. Gambhir, Stanford) in the pBS vector (L. Hoffman, unpublished results). The resulting plasmid, pBS/αMHC-TFR, was digested with Not1 restriction enzyme to release the αMHC-TFR sequence, which was then inserted into the Not1 restriction sites of pcDNA3.1. Correct orientation of the fragment was confirmed by sequencing. The pcDNA3.1/αMHC-TFR construct was transfected into P19 cells using Lipofectamine 2000 (Life Technologies) and cells were selected for 3 weeks in 400 μg/mL hygromycin B (Life Technologies). For differentiation, spontaneous aggregation of P19 cells was performed by passaging the cells into 10 cm dishes coated with 0.1% agarose/alpha-MEM/10% FBS/0.8% DMSO to prevent adhesion of the cells. On day 4, embryoid bodies (cell aggregates) were transferred to 6-well cell culture dishes containing coverslips coated with 0.2% gelatin, and were maintained in alpha-MEM/10% FBS/0.8% DMSO. Medium was refreshed every 48 h. Beating cardiac myocytes were first observed at day 15 and by day 25 approximately 10% of the cells were beating synchronously.

For live imaging of GHS-R, P19 cells at several time points of differentiation (day 0, day 15 and day 28) were plated at a density of 1×10^6 cells/well in a 6-well plate, and serum starved overnight. The following day, 10 μM Cy5-ghrelin(1–19) alone, or with 100 μM ghrelin (1–28) for blocking studies, was added to the dishes and cells were incubated at 37 °C for 30 min, and subsequently rinsed in serum-free media. Live cell images were captured in the RFP (excitation 563 nm/emission 582 nm) and Cy5 (excitation 650 nm/emission 670 nm) wavelengths using an inverted fluorescent microscope and final images were prepared using ImageJ (National Institutes of Health, Bethesda, MD). Brightness and contrast were adjusted to improve image quality, and was equally applied to all pixels across all images. To amplify areas of coincident pixels and reduce background, the RFP and Cy5 images at day 25 were multiplied in ImageJ and green pseudocolor was applied.

2.6. Western Blot analysis

Immediately after the imaging session, cells were rinsed 2× in PBS and lysed in 0.5 mL ice-cold phospho-safe extraction reagent (EMD Millipore) plus mini complete protease inhibitor cocktail for Western Blot analysis, as previously described [17]. Fifty micrograms of protein were separated on a 4–12% gradient NuPAGE gel (Life Technologies), transferred to nitrocellulose membranes and the following antibodies were used: anti-GHS-R1a (Abcam, 1:1000); and anti-GAPDH (Abcam, 1:1000) to assess protein loading. Bands were visualized using SuperSignal chemiluminescent substrate (Pierce) and images were captured using GeneSnap software, as described previously [17].

2.7. Immunohistochemistry for differentiation markers

In parallel experiments, P19 cells at the same time points of differentiation were fixed and processed for immunohistochemistry, as described previously [16] using the following antibodies: anti-connexin 43 (Sigma, 1:100), anti-cardiac troponin (Abcam, 1:100) and anti-SERCA 2a (Abcam, 1:100) at days 15 and 28. Alexa 488-IgG (Life Technologies, 1:500) was used to visualize cx-43 antibody, Alexa 647-IgG (1:500) was used to visualize the c-troponin antibody, and Alexa 594-IgG (1:500) was used to visualize the SERCA 2a antibody. Images were captured and reconstructed using deconvolution microscopy as described previously [18]. Regions containing both non-beating and beating cells were captured from 5 fields of view from each of 3 wells per time point.

2.8. Statistical analysis

For quantified images, a one-way ANOVA followed by a post hoc Student's *t*-test was used to determine statistical significance. For comparisons of binding at varying concentrations of peptide, a two-way ANOVA and Student's *t* test were used. Significance was set at $p < 0.05$.

3. Results

3.1. Receptor binding assays in HEK–GHS-R cells

We characterized the binding of both Cy5-ghrelin and Cy5-des-acyl ghrelin to GHS-R1a (structures shown in Fig. 1) with an in vitro binding assay using HEK 293 cells over-expressing GHS-R1a that we generated. Table 1 shows that the binding affinity of Cy5-ghrelin to GHS-R1a was within an order of magnitude of that of wild-type ghrelin, and comparable to our previously reported value for fluorescein-ghrelin(1–19) [20]. In contrast, Cy5-des-acyl ghrelin did not show any appreciable binding to GHS-R1a.

3.2. Microscopy in live HEK–GHS-R cells with block and without receptor and with des-acyl ghrelin

To assess the specificity of Cy5-ghrelin for imaging GHS-R1a, HEK/GHS-R1a cells were incubated with Cy5-ghrelin alone or in the presence of excess ghrelin, and imaged with fluorescence microscopy. The robust fluorescence observed in HEK/GHS-R1a cells incubated with Cy5-ghrelin (Fig. 2, panel A) was completely abrogated by ghrelin (panel B), indicating displacement from GHS-R1a. Further confirmation of the specificity of binding was demonstrated by the lack of fluorescence in wild-type HEK cells (Fig. 2, panel C). Localized fluorescence, suggestive of receptor binding and internalization, was detected in cells expressing GHS-R1a (Fig. 2, panel D).

Table 1
Receptor binding affinities for ghrelin analogs.

| Peptide	IC_{50} (nM)
Ghrelin (1–28)	2.5 ± 1
Fluorescein-ghrelin(1–19)	9.5 ± 2.6[a]
Cy5-ghrelin(1–19)	25.8 ± 3.4
Cy5-des-acyl ghrelin(1–19)	>1000

Values are means from two experiments.
[a] From Ref. [18].

3.3. Images of mouse heart tissue sections with block and with des-acyl ghrelin

We then characterized the binding of Cy5-ghrelin and Cy5-des-acyl ghrelin to sections of mouse cardiac tissue. Although Cy5-des-acyl ghrelin did not bind GHS-R1a, there are reports of des acyl ghrelin binding to and inducing signaling in isolated cardiomyocytes [1,10], so we investigated the use of the Cy5-des-acyl ghrelin to visualize its binding to heart tissue. As expected, tissue sections incubated with 10 μM Cy5-ghrelin showed robust fluorescence, which was almost completely displaced by excess ghrelin (Fig. 3A). In contrast, Cy5-des-acyl ghrelin showed no binding to mouse cardiac tissue at the same concentration as Cy5-ghrelin. In order to further investigate the binding of both Cy5-ghrelin and des-acyl ghrelin to murine cardiac tissue, sections were incubated with increasing concentrations of both peptides, and the fluorescence intensity was quantified. Cy5-ghrelin bound
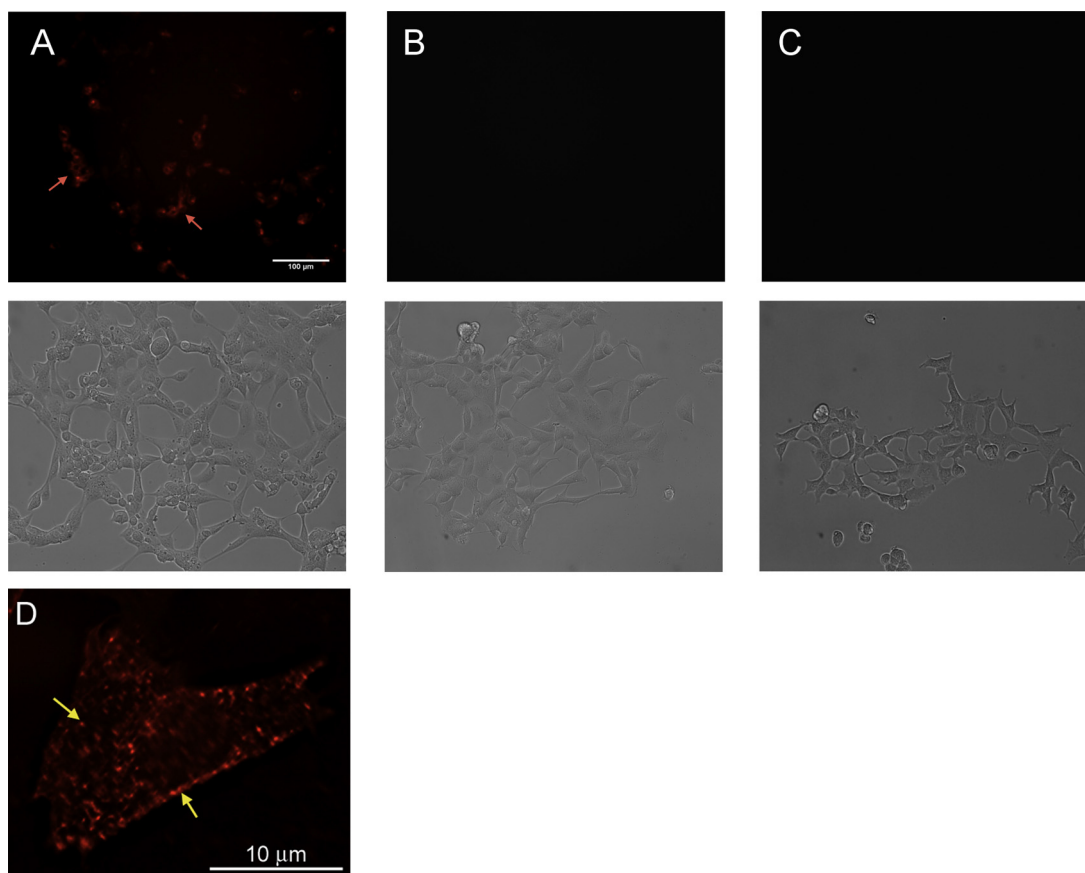
Fig. 2. Cy5-ghrelin binds in a specific manner to GHS-R1a. (A) Live HEK 293/GHS-R1a cells were incubated with 10 μM Cy5-ghrelin(1–19) for 30 min, and subsequently rinsed in serum-free media and live cell images were captured. (B) Live HEK/GHS-R1a cells were incubated with 10 μM Cy5-ghrelin(1–19) in the presence of 40 μM unlabeled ghrelin (1–28). There was no fluorescence in the presence of unlabeled ghrelin. (C) Live wt HEK 293 cells were incubated with 10 μM Cy5-ghrelin(1–19) for 30 min. No binding of Cy5-ghrelin was observed, indicating that no nonspecific binding occurred. Images are representative of 10 fields of view per well (n = 3). Scale bar for A–C is 100 μm. (D) High-magnification image of a HEK 293/GHS-R1a cell incubated with 10 μM Cy5-ghrelin(1–19) for 30 min. Localized Cy5 fluorescence is suggestive of membrane binding and internalization (arrows). Scale bar is 10 μm.
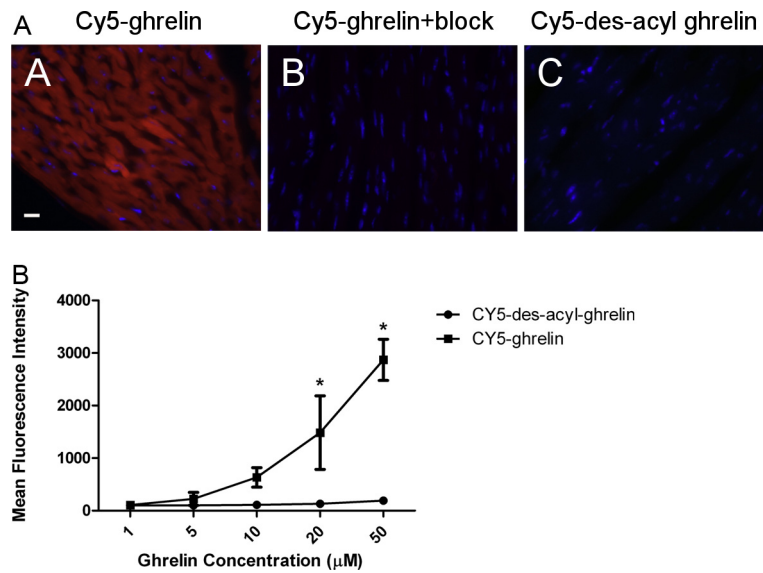
Fig. 3. Cy5-ghrelin binds to GHS-R1a on mature mouse cardiomyocytes. Frozen embedded mouse hearts were cryosectioned at 8 μm. (A) Sections were incubated with 10 μM Cy5-ghrelin(1–19) alone (panel A), or in the presence of 100 μM ghrelin (1–28) (panel B) to determine the specificity of binding. Unlabeled ghrelin prevented the binding of Cy5-ghrelin. In panel C, sections were incubated with 10 μM Cy5-des-acyl ghrelin(1–19) alone, which resulted in very little fluorescence. Images are representative of five random fields of view for each of 5 slides per tissue per mouse ($n = 4$). Scale bar for all images is 20 μm. (B) Sections were incubated for 30 min with various concentrations (0–50 μM) of Cy5-ghrelin(1–19) or Cy5-des-acyl ghrelin(1–19) alone. Mean fluorescence intensity increased dramatically with increasing concentrations of Cy5-ghrelin, but did not increase significantly with increasing concentrations of Cy5-des-acyl ghrelin. Values are mean ± SEM ($n = 4$). *$p < 0.01$ between Cy5-ghrelin and Cy5-des-acyl ghrelin. SEM bars are present on all data points, but may be too small to be visualized in some cases.

murine cardiac tissue in a concentration-dependent manner, whereas Cy5-des-acyl ghrelin showed greatly reduced binding at the same concentrations (Fig. 3B).

3.4. Using Cy5-ghrelin to detect GHS-R expression during differentiation

Having determined that Cy5-ghrelin binds to adult mouse cardiac tissue via GHS-R1a, we investigated its utility in detecting changes in GHS-R1a expression during cardiac differentiation. The P19 cell line is an embryonic carcinoma cell line that can be induced to differentiate into beating cardiomyocytes by treatment with DMSO [7] (Suppl. Video 1). In order to track differentiation over time, we transfected cells with a trifusion imaging reporter gene containing RFP whose expression is driven by the αMHC promoter. Since αMHC transcription is activated only in mature cardiomyocytes, RFP fluorescence should be detectable only in fully differentiated cells. In live P19 cells, there is no RFP signal in scattered, undifferentiated cells at day 0, very weak fluorescence in non-beating embryoid bodies at day 15 and very robust fluorescence in beating cardiomyocytes at day 25 (Fig. 4), thus demonstrating differentiation in live cells. These same cells were also incubated with Cy5-ghrelin. Interestingly, a few cells showed Cy5 fluorescence at day 15, before RFP fluorescence was detectable. However, there was strong Cy5 fluorescence at day 25, and that pattern of Cy5 fluorescence coincided with that of RFP, as demonstrated when the RFP and Cy5 images were multiplied (Fig. 4). Both RFP and Cy5 fluorescence were seen at the stage where cells were beating (Suppl. Videos 2 and 3).

3.5. P19 cells gain expression of GHS-R1a during induced cardiomyocyte differentiation

To confirm that P19 cells were differentiating and expressing GHS-R, cells treated with DMSO at day 0, day 15 and day 25 were assessed for GHS-R expression by Western Blot, and cells were examined for expression of differentiation markers by immunofluorescence microscopy. There was no expression of GHS-R1a at day 0, very faint expression at day 15, and robust expression at day 25 (Fig. 5A), coinciding with Cy5-ghrelin imaging. We then examined cellular architecture using cardiac troponin as a marker of cardiomyocyte differentiation and connexin-43 as an indicator that cardiomyocytes were interconnected and forming a functional syncitium. At day 15 after DMSO treatment, there was some defined and localized fluorescence of cardiac troponin and connexin-43, which appeared to increase at day 25 (Fig. 5B), indicating that cells were differentiating to a cardiomyocyte phenotype. Finally, cells were immunostained for SERCA 2a to mark the onset of contractility. Robust SERCA 2a immunofluorescence was observed only at day 25 (Fig. 5B).

4. Discussion

Structure–activity relationship studies using varying lengths of the peptide ghrelin have determined the minimum active core that is responsible for ghrelin receptor affinity [2,16,27]. Ghrelin can be truncated to as small as a pentapeptide containing the five N-terminal amino acid residues and still maintain high-affinity receptor binding. Additionally, the octanoyl modification on the side chain of Ser[3] is crucial for binding activity as it acts like an anchor, inserting itself into the ghrelin receptor and facilitating ligand binding [5]. In the native peptide this unique feature bears an ester linkage to the octanoyl that is sensitive to ester hydrolysis in vivo. In order to increase peptide stability, we replaced Ser[3] with the unnatural amino acid diaminopropanoic acid (Dpr), which replaces the ester linkage with an amide linkage. It has been shown that this modification results in improved stability while having no effect on GHS-R1a binding affinity [13]. We have previously synthesized two analogs of ghrelin, 14-mer and 19-mer, to incorporate these important structural features as well as further modifications to bear non-radioactive isotopes, ^{19}F and Re [25] and an optical dye [20]. Our results demonstrate the flexibility permitted by the structure of ghrelin in designing molecules for imaging GHS-R1a. We now report the synthesis of a 19-mer ghrelin analog containing the
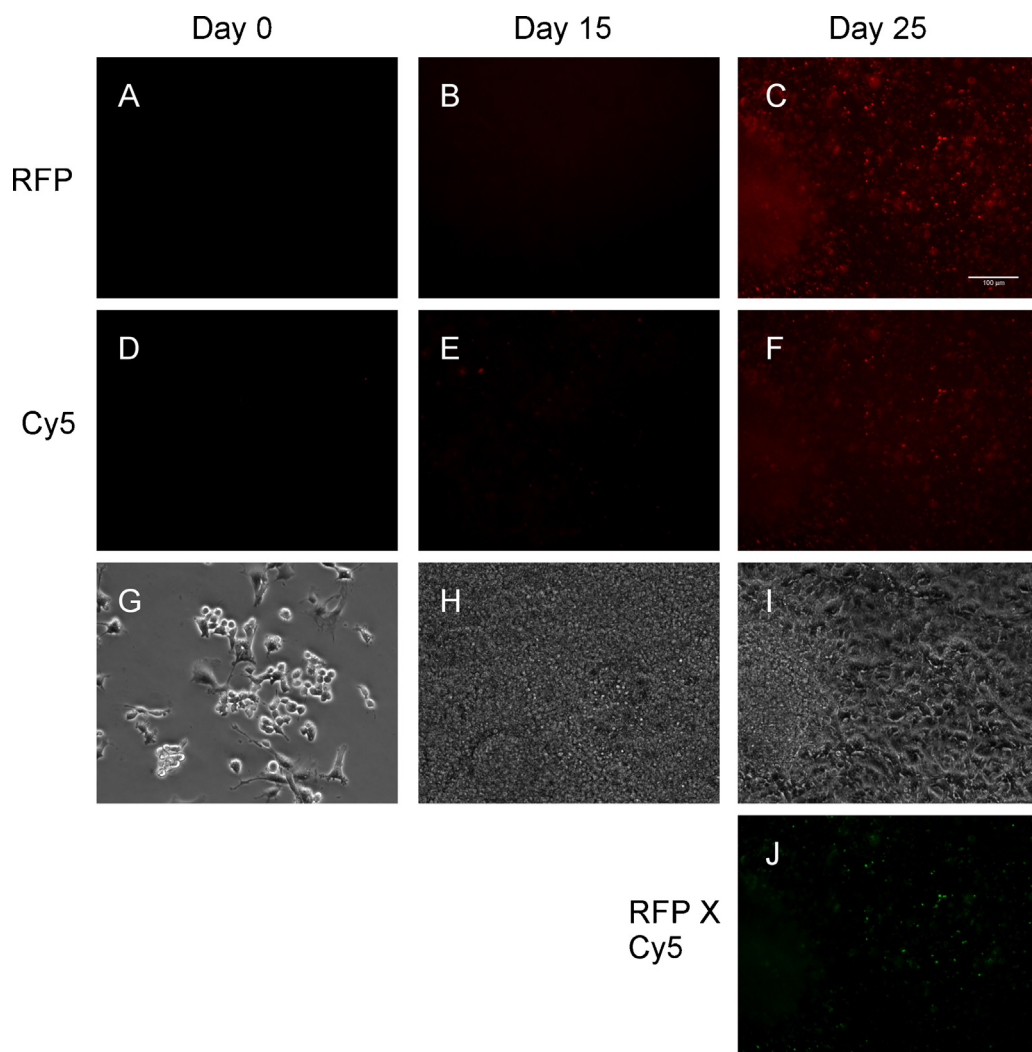
Day 0 Day 15 Day 25

Fig. 4. Using Cy5-ghrelin to track GHS-R1a expression during cardiomyocyte differentiation. Live P19 cells expressing αMHC-TFR were incubated with 10 μM Cy5-ghrelin(1–19) before (day 0) or after (day 15, day 28) addition of DMSO to induce differentiation into beating cardiomyocytes. In panels A–C, RFP fluorescence is observed only at day 28. Cy5-ghrelin binds to a few cells at day 15 (panel E), and there is robust Cy5 fluorescence at day 28 (panel F). Panels G and H show corresponding bright-field images. Images are representative of 5 fields of view in each of 3 wells of cells at each time point. To show areas of coincident RFP- and Cy5-positive pixels in the absence of background, images at day 25 were multiplied in Image J (panel H). Scale bar for all images is 100 μm.

first 19 N-terminus residues with substitutions at Ser[3](octanoyl) to contain Dpr[3](octanoyl), to replace the ester linkage, and Lys[19] to integrate the far-red dye, Cy5. The binding of Cy5-ghrelin shows strong affinity for GHS-R1a and the IC50 value, while not as favorable as what we reported for fluorescein ghrelin [20], is still in the nanomolar range. Therefore, it appears that any organic fluorescent dye can be incorporated at the C-terminus of truncated ghrelin analogs while preserving high-affinity binding to GHS-R1a. Since structure–function studies indicate receptor binding is intact down to 5-mer peptides, it may be possible to develop GHSR imaging agents with even lower molecular weight and with improved stability for in vitro and in vivo use. Furthermore, these GHSR targeted ligands could be appended to nanoparticles or other carrier molecules for efficient receptor targeting and improved imaging signal.

The design of our Cy5-ghrelin analogs demonstrates the ability to add any fluorescent cyanine dye at Lys[19], maintain receptor binding affinity and have an emission wavelength appropriate for any given study. Additionally, the photostability and negligible spectral overlap between the Cy family of dyes permit two- or three-color imaging, as has been done for single molecule FRET [11]. For example, it might be interesting to use Cy3-ghrelin and Cy5-des acyl ghrelin to simultaneously investigate binding sites on cardiomyocytes. Alternatively, Cy7, whose emission and excitation spectra are in the near-infrared range, can be conjugated to ghrelin using our design for in vivo fluorescence imaging.

Development of fluorescent ghrelin analogs allowed us to investigate the binding of des-acyl ghrelin to GHS-R1a and to murine cardiac tissue. Our results show that Cy5-des-acyl ghrelin does not bind to GHS-R1a. These results are consistent with previous reports that show that the octanoic acid modification on Ser[3] is required for binding of ghrelin to GHS-R1a [2,15]. However, our results also show that Cy5-des-acyl ghrelin does not bind to mouse heart tissue in situ. These results are at variance with a report showing two distinct binding sites for des-acyl ghrelin in HL-1 cells and isolated murine cardiomyocytes [10]. An alternate explanation for their binding data is that the abundance of low-affinity binding sites is much greater than that of high-affinity binding sites. The biphasic competitive binding curves suggests that this is indeed the case. Moreover, binding of [^{125}I-Tyr[4]]des-acyl ghrelin does not appear to reach saturation, suggesting nonspecific binding or a high dissociation rate. If the majority of des-acyl ghrelin binding sites on cardiomyocytes are of low affinity, this generally means a high dissociation rate, and therefore the des-acyl binding site may not be
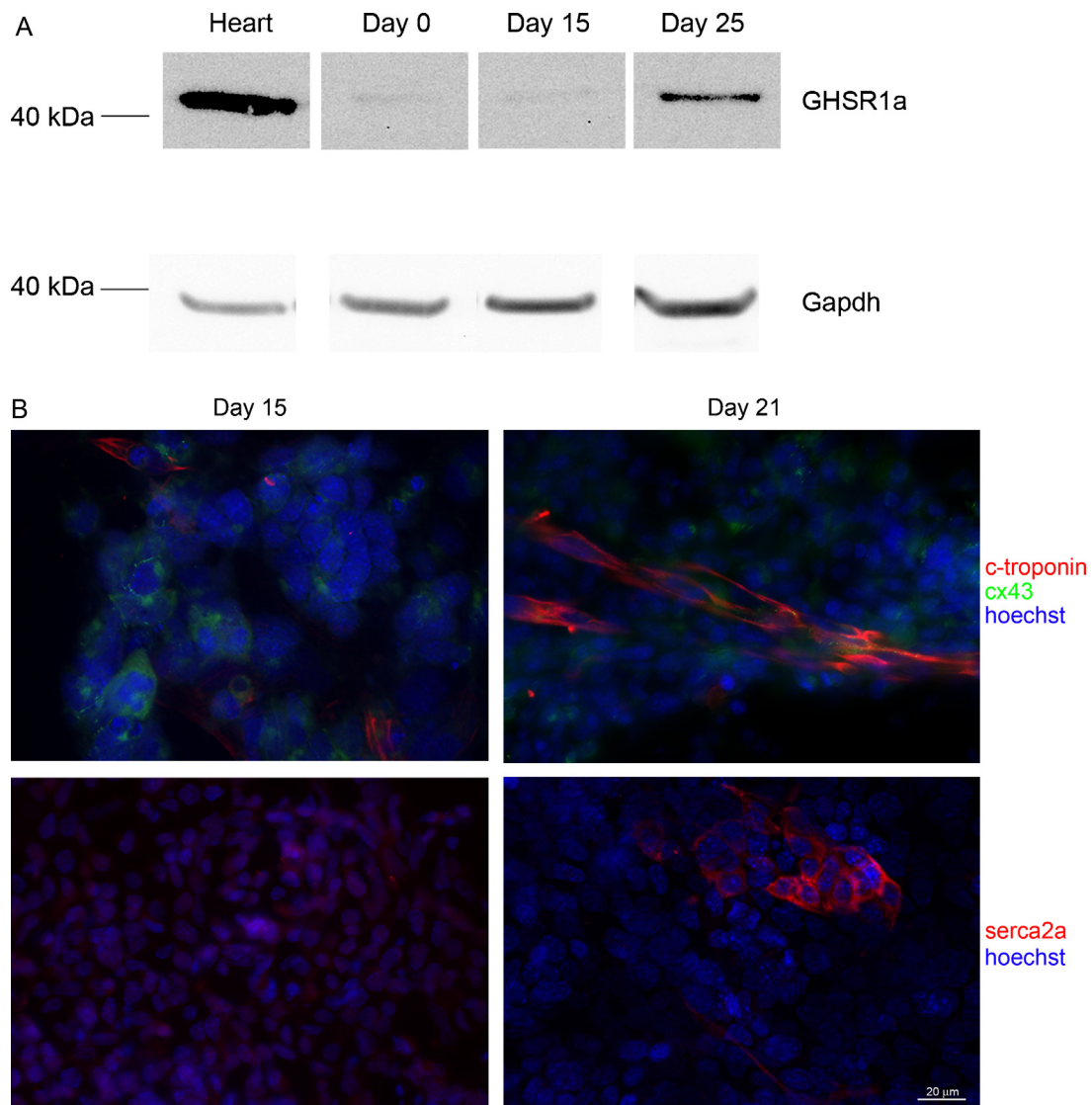
Fig. 5. GHS-R1a expression anticipates onset of cardiomyocyte contractility. (A) Cell lysates from P19/αMHC-TFR cells were collected at the indicated time points. Fifty micrograms of protein were separated on a 4–12% gradient SDS-PAGE gel and membranes were probed for GHS-R (upper panel) and GAPDH (lower panel). The images are representative of two independent experiments. (B) P19/αMHC-TFR cells were grown and fixed on coverslips at the indicated time points. Cells were incubated with the indicated antibodies and images were collected from 5 fields of view from each of 3 coverslips. Note the elongated morphology of c-troponin staining in cells at day 25 after differentiation. The expression of SERCA 2a coincides with the onset of beating, as shown in Supplemental Videos 1–3.

captured by the Cy5 ligand. The presence of the Cy5 moiety may also affect the binding of des-acyl ghrelin to such a low-affinity receptor. If the Cy5 moiety decreases des-acyl ghrelin binding affinity by 8-fold, as is the case with octanoylated ghrelin, then is it entirely possible that binding sites are not being detected. Therefore, Cy5-des-acyl ghrelin may be used as a tool to further investigate the nature of the des-acyl ghrelin binding sites on cardiomyocytes, and other tissues.

Using our fluorescent ghrelin analog to examine cardiac GHS-R expression will provide a useful tool in determining the distribution and function of GHS-R in the normal mouse heart. A recent report has described the use of a monoclonal antibody against GHS-R to determine receptor distribution in primary rat hippocampal and cortical neurons [9]. The fluorescent peptide analogs could also be used to map GHS-R distribution in normal, developing and diseased heart, both in situ and in vivo, and additionally, can be used to determine the dynamics of receptor activation, such as internalization and trafficking. As well, the question of the roles of ghrelin vs. des-acyl ghrelin in cardiomyocyte function can be examined.

Cardiomyocyte contraction in normal isolated cardiomyocytes and inhibition of sympathetic activity appear to be mediated through ghrelin activation of GHS-R1a [14,26]. Both actions could contribute either directly or indirectly to the promotion of cardiomyocyte survival. However, in studies examining signaling pathways in survival and differentiation, des-acyl ghrelin has the same anti-apoptotic effects as ghrelin in an embryonic cardiomyocyte cell line H9c2 [1], and both ghrelin and des-acyl ghrelin promote differentiation through ERK 1/2 activation in undifferentiated hESCs [4]. Interestingly, that study showed that hESCs gradually express GHS-R as they differentiate, similar to our results with P19 cells. Therefore, our fluorescent ghrelin probes will be useful in ascertaining whether des-acyl ghrelin really does bind to stem cells or cardiac progenitors in vivo to induce differentiation.

Another advantage of the Cy5-ghrelin analog is that it allows for identification of GHS-R expression at the individual cell level. By Western Blot alone, GHS-R expression in P19-derived cardiomyocytes is evident only after day 28 of differentiation. However, imaging with Cy5-ghrelin shows defined and discrete fluorescence

in a significant number of cells at d15. These cells do not show RFP fluorescence, indicating that GHS-R expression precedes αMHC expression. Our results suggest that GHS-R may be a marker of the early stages of cardiomyocyte differentiation. In particular, since GHS-R expression appeared before the cells started to beat, it may be that the appearance of GHS-R1a presages the onset of contractility in cardiomyocytes.

In summary, we have synthesized a far-red analog of ghrelin that binds specifically to GHS-R1a and to mature cardiomyocytes. This analog can be used to track GHS-R expression during differentiation in P19-derived cardiomyocytes. Studies using our Cy5-ghrelin(1–19) imaging agent to determine GHS-R1a expression in differentiating and diseased cardiac tissue are currently underway.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.peptides.2014.01.011.

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