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[^{125}I-*His*^{9}]-Ghrelin, a novel radioligand for localizing GHS orphan receptors in human and rat tissue; up-regulation of receptors with atherosclerosis

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1 Ghrelin is the recently identified endogenous ligand for the cloned growth hormone secretagogue receptor (GHS-R). We have characterized for the first time the binding of human [^{125}I-*His*^{9}]-ghrelin to normal human and rat tissue and demonstrated expression of this 'orphan' receptor that has previously been predicted to exist from mRNA. Furthermore, we have discovered that [^{125}I-*His*^{9}]-ghrelin density is significantly increased in atherosclerosis.

2 [^{125}I-*His*^{9}]-Ghrelin bound to non-diseased human heart (left ventricle) with an association rate constant (k_{obs}) of 0.16 ± 0.004 min^{-1}, a dissociation rate constant of 0.068 ± 0.0005 min^{-1} (kinetically derived K_D of 0.1 nM; $n = 5$ individuals $\pm$ s.e.mean), a K_D of 0.43 ± 0.08 nM and B_{max} of 7.8 ± 0.9 fmol mg^{-1} protein ($n = 6$ individual $\pm$ s.e.mean).

3 Specific [^{125}I-*His*^{9}]-ghrelin binding was to the human vasculature including aorta, coronary, pulmonary, arcuate arteries in the kidney and saphenous veins. In rat tissues, binding sites were also localized to the vasculature in peripheral tissues as well as the granular layer of the cerebellum in the CNS.

4 [^{125}I-*His*^{9}]-Ghrelin binding was significantly up-regulated (3–4 fold) in both atherosclerotic coronary arteries and saphenous vein grafts with advanced intimal thickening, compared with normal vessels ($P < 0.05$).

5 Our results suggest that the native receptor for [^{125}I-*His*^{9}]-ghrelin may be widely distributed in the human cardiovascular system. Furthermore, changes in the density of this proposed ghrelin receptor implicates this new transmitter system in the development of atherosclerosis and may therefore represent a novel therapeutic target in the treatment of cardiovascular disease.

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Keywords: Growth hormone secretagogue receptor; orphan receptor; ghrelin; human heart; human coronary artery; saphenous vein grafts; atherosclerosis; ghrelin receptor; kidney; lung

Abbreviations: DCM, dilated cardiomyopathy; GH, growth hormone; GHRH, growth hormone releasing hormone; GHS, growth hormone secretagogue; GHS-R, growth hormone secretagogue receptor; IHD, ischaemic heart disease

Introduction

Ghrelin is a 28 amino acid peptide originally discovered in the rat stomach and has been proposed as the previously unidentified cognate endogenous ligand for the 'orphan' growth hormone secretagogue receptors (GHS-R). The Ser^{3} residue has been *n*-octanoylated, a unique post-translational modification amongst naturally occurring peptides which is essential for binding of ghrelin to the GHS-R (Kojima *et al*., 1999). Using a rat ghrelin cDNA library, human ghrelin cDNA was identified and the amino acid sequence was predicted to be identical to rat ghrelin apart from two residues, which represent minor changes (Kojima *et al*., 1999).

Ghrelin was originally reported to induce an increase in intracellular Ca^{2+} in cells expressing GHS-R (Kojima *et al*., 1999). Since ghrelin can be detected in human plasma at a concentration of 120 fmol ml^{-1}, it is possible that ghrelin once produced and secreted from the stomach, circulates in the blood stream to bind to specific tissues expressing GHS-R (Kojima *et al*., 1999). In addition, the synthesis of ghrelin by the mouse kidney has been recently documented, suggesting ghrelin may also function as a locally produced autocrine/paracrine mediator (Mori *et al*., 2000). Clinical studies in human volunteers have shown that ghrelin strongly stimulated growth hormone (GH) release (Arvat *et al*., 2000; Peino *et al*., 2000; Takaya *et al*., 2000). Furthermore, administration of ghrelin into animals resulted in an increase in food intake and weight gain (Tschop *et al*., 2000; Wren *et al*., 2000).

The deduced sequence of GHS-R comprises 366 amino-acids with the predicted structure of a seven transmembrane G protein coupled receptor (Howard *et al*., 1996) that is distinct from the growth hormone releasing hormone (GHRH) receptor, since GHRH did not bind to GHS-R (Smith *et al*., 1999). Labelled synthetic growth hormone secretagogues (GHS) such as hexarelin have been shown to

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bind to a specific region in the human brain, for example the hypothalamus and pitutary (Muccioli *et al*., 1998). In agreement, mRNA encoding GHS-R has also been detected in the rat brain (Guan *et al*., 1997) as well as peripheral tissues such as the heart and lung (Kojima *et al*., 1999). However, the binding and anatomical distribution of [^{125}I-*His*^{9}]-ghrelin has not been determined in human or animal tissue.

The purpose of this study was firstly to label the endogenous peptide human ghrelin and to characterize the binding of the resulting radioligand in human cardiovascular tissue as well as determining the anatomical distribution by quantitative autoradiography. We chose to use human cardiac tissue for these experiments since the original description of the pairing of the GHS-R with ghrelin, reported detectable mRNA encoding this receptor in these tissues. Secondly, in order to elucidate a possible pathophysiological role, we compared normal and diseased cardiac tissue to seek evidence (if any) for a change in receptor density. In the vasculature, we focused on atherosclerosis in coronary arteries and the related process, accelerated atherosclerosis of saphenous vein graft. Finally, to determine whether the binding of [^{125}I-*His*^{9}]-ghrelin (human) was specific to human tissue or whether the receptor protein might also be expressed in other species, we examined those rat tissues where molecular studies reported mRNA encoding GHS-R and therefore the presence of the receptor protein might be expected. Preliminary data were presented to the British Pharmacological Society (Katugampola *et al*., 2001a).

Methods

With informed consent and local ethical approval, sections of human left ventricle free wall, right atrial, aortic and pulmonary tissues were obtained from patients with histologically normal hearts not required for further transplantation (Table 1) (21–45 years). Epicardial coronary arteries, left ventricle and right atrial tissue were obtained from individuals (21–52 years) following heart transplants for dilated cardiomyopathy. Samples of epicardial coronary artery containing advanced atheromatous lesions, occluded saphenous vein bypass grafts, left ventricle and right atrial tissues were from individuals undergoing cardiac transplantation for ischaemic heart disease (48–69 years). Saphenous veins were from patients receiving coronary artery by-pass grafts (54–71 years). Histologically normal renal tissue (containing cortex and medulla) was obtained following nephrectomy (32–74 years). Normal lung tissue was from patients (34–53 years) undergoing lobectomy for carcinoma. Experiments were carried out using tissue from three or more individuals.

Tissues were from female Sprague-Dawley rats (300–350 g). Animals were killed by an overdose of sodium pentobarbitone followed by exsanguination.

Kinetic studies

Kinetic studies were carried out as described for characterization of endothelin receptors in human tissue (Davenport *et al*., 1998). Briefly, for association studies 30 μm sections of non-diseased human left ventricular tissue were incubated in assay buffer comprising (mM) Tris 50, EDTA 10, EGTA 10 and 4-(2-aminoethyl)benzenesulphonylfluoride (AEBSF) 1, pH 7.2 for 0–150 min with 0.15 nM [^{125}I-*His*^{9}]-ghrelin. Hexarelin (1 μM) defined non-specific binding. Dissociation experiments were performed on sections of non-diseased human left ventricle by incubating with 0.15 nM [^{125}I-*His*^{9}]-ghrelin for 25 min, before sections were washed in an excess of buffer at room temperature for increasing time periods.

Table 1 Patient details

Patient	*Sex*	*Age*	*Diagnosis*	*Drugs*
1	M	43	CF	
2	F	45	CF	
3	M	43	CF	
4	F	41	CF	
5	M	21	CF	
6	M	33	CF	
7	M	21	DCM	1, 5, 6
8	M	52	DCM	1, 9, 10
9	M	49	DCM	1, 3, 4, 8
10	M	25	DCM	2, 3, 9
11	M	35	DCM	1, 2, 10
12	M	42	DCM	5, 6, 9
13	M	50	DCM	1, 3, 5, 8
14	F	48	IHD	1, 2, 4, 9
15	M	50	IHD	1, 2, 4, 6, 7
16	M	53	IHD	1, 2, 3, 4, 6, 7, 9
17	M	55	IHD	1, 3
18	M	69	IHD	1, 2, 4, 7, 9
19	M	50	IHD	1, 2, 7, 9
20	F	64	IHD	2, 3, 7
21	M	54	CABG	1, 2, 7, 9
22	F	64	CABG	3, 5, 7
23	F	71	CABG	2, 5, 11
24	M	32	Pheochromocytoma	12
25	M	51	Hypernephroma	5
26	M	74	Hypernephroma	5
27	M	34	Lobectomy for carcinoma	N.A.
28	M	51	Lobectomy for carcinoma	N.A.
29	M	53	Lobectomy for carcinoma	N.A.

CF, cystic fibrosis; DCM, dilated cardiomyopathy; IHD, ischaemic heart disease; CABG, coronary artery bypass graft, N.A., not available. Drugs: 1, ACE inhibitors; 2, diuretics; 3, nitrates; 4, calcium antagonist; 5, β-blockers; 6, warfarin; 7, lipid lowering agents; 8, anti-oxidants; 9, aspirin; 10, AT_1 receptor blockers; 11, antibiotics; 12, α-adrenoreceptor blocking agents.

pH dependence

Cryostat sections (30 μm) of non-diseased human left ventricle were incubated with 0.15 nM [^{125}I-*His*^{9}]-ghrelin in assay buffer with 10 different pH values varying over the range pH 5–pH 9.

Saturation binding assays

Following optimization of binding conditions, 30 μm cryostat sections of human tissue were pre-incubated in assay buffer for 15 min. Saturation binding curves with 12 points were constructed using increasing concentrations (0.01–1.5 nM) of [^{125}I-*His*^{9}]-ghrelin. Non-specific binding was defined using an excess (1 μM) of hexarelin. Following a 25 min incubation at

room temperature the sections were rapidly washed in 50 mM Tris (4°C, pH 7.4). Protein concentration was determined using the Biorad DC 96-well microtiter plate system (Biorad Laboratories, Hertfordshire, U.K.).

Competition binding studies

Competition binding studies were carried out using the assay conditions described above. Sections of non-diseased human left ventricular tissue were labelled with a fixed concentration (0.15 nM) of [^{125}I-*His*^{9}]-ghrelin in the presence and absence of unlabelled peptides. Non-specific binding was defined by incubating adjacent sections with 1 μM hexarelin. For full competition experiments with hexarelin, sections of non-diseased human left ventricular tissue were labelled with 0.15 nM [^{125}I-*His*^{9}]-ghrelin in the presence of increasing concentrations of hexarelin (10 pM – 1 μM). Non-specific binding was determined by the incubation of 1 μM hexarelin.

In vitro *receptor autoradiography*

GHS-R were detected autoradiographically with the method adopted for the endothelin receptors (Maguire & Davenport, 1999). Cryostat tissue sections were incubated with 0.15 nM [^{125}I-*His*^{9}]-ghrelin in the absence or presence of hexarelin (1 μM) under the conditions described above. Dried sections were apposed to radiation sensitive film (Hyperfilm βmax, Amersham Pharmacia Biotech, Bucks, U.K.) for 4 days together with ^{125}I microscale standard (Amersham Pharmacia Biotech, Bucks, U.K.). Adjacent sections were stained with haematoxylin and eosin to facilitate histological identification.

Data analysis

The results from binding experiments were analysed as previously described (Maguire *et al*., 1996) using the iterative, non-linear curve fitting programmes EBDA and LIGAND in the KELL package (Biosoft, Cambridge, U.K.). All values are expressed as mean ± s.e.mean. Kinetic analysis of [^{125}I-*His*^{9}]-ghrelin binding provided estimates for the observed association (k_{obs}) and dissociation rate constants. These were in turn used to derive values for the association rate constant and the kinetically determined K_D. Individual saturation binding experiments were analysed with EBDA (McPherson, 1983) to obtain initial estimates. The resulting data files were co-analysed with LIGAND (Munson & Rodbard, 1980) to obtain values of ligand affinity (K_D) and receptor density (B_{max} expressed as fmol mg^{-1} protein). A two-site model was accepted only if it resulted in a significantly better fit as judged by an *F*-test. K_D values were compared using the Mann-Whitney *U*-test with a significance of $P<0.05$ and B_{max} values were compared using Student's unpaired *t*-test with a significance value of $P<0.05$.

Materials

[^{125}I-*His*^{9}]-Ghrelin (2000 Ci mmol^{-1}) (Figure 1) was prepared (Amersham Pharmacia Biotech Bucks, U.K.) from the unlabelled material ghrelin (Peptide Institute, Osaka, Japan) by direct iodination with sodium [^{125}I]-iodide using the chloramine-T method (Amersham Pharmacia Biotech Bucks, U.K.) which labelled the (*His*^{9}) residue and was purified to be carrier free by reverse phase high performance liquid chromatography. Hexarelin (His-*D*-2-methyl-Trp-Ala-Trp-*D*-Phe-Lys-NH_2) was obtained from Peninsula laboratories (Belmont, CA, U.S.A.) and other peptides from Peptide Institute (Osaka, Japan). All other chemicals including EGTA [ethylene glycol-bis-(β-aminoethyl ether) N,N,N,N-tetraacetic acid], EDTA (ethylenediaminetetracetic acid) and AEBSF were obtained from Sigma-Aldrich (Poole, Dorset, U.K.).

Results

Receptor-ligand characterization

Kinetic studies At a concentration of 0.15 nM, [^{125}I-*His*^{9}]-ghrelin bound in a time-dependent manner to sections of human left ventricle, reaching equilibrium binding by 20 min (Figure 2), with an association rate constant (k_{obs}) of 0.16 ± 0.004 min^{-1} and a half time for association ($t_{1/2}$) of 4 min. The binding of [^{125}I-*His*^{9}]-ghrelin to sections of non-diseased human left ventricle was reversible at room temperature and analysis of the data indicated dissociation

Figure 1 Amino acid sequence of the radiolabelled [^{125}I-*His*^{9}]-ghrelin with the *n*-octanyl modification on the Ser^{3} residue. [^{125}I-*His*^{9}]-Ghrelin was prepared by direct iodination with sodium [^{125}I]-iodide using the chloramine-T method, which labelled the *His*^{9} residue.

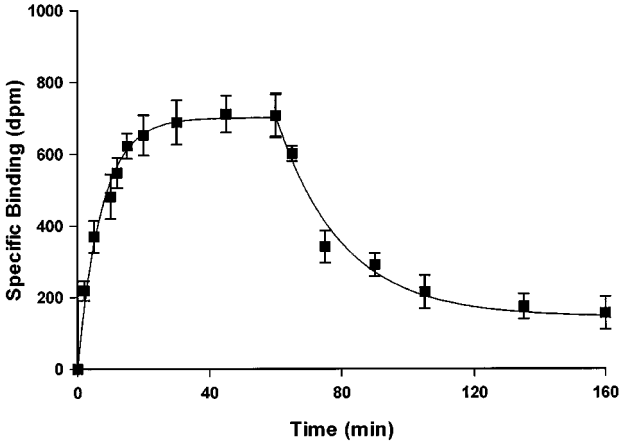

Figure 2 Time-dependent association and dissociation of [^{125}I-*His*^{9}]-ghrelin binding to sections of human left ventricle with an association rate constant of 0.16 ± 0.004 min^{-1}. [^{125}I-*His*^{9}]-Ghrelin dissociated from a single site with a dissociation rate constant of 0.068 ± 0.0005 min^{-1}. Tissue sections were incubated for 25 min and washed in excess of Tris buffer for differing time points. Data points are mean ± s.e.mean from five individuals.

from a single site with a dissociation rate constant of 0.068 ± 0.0005 min^{-1}. The half time for dissociation ($t_{1/2}$) was 11 min (Figure 2). Kinetically derived K_D of 0.1 nM was comparable to the value obtained from saturation studies in human left ventricle.

pH dependence [^{125}I-*His*^{9}]-Ghrelin binding to non-diseased human left ventricle was pH dependent, with highest specific binding being achieved over the range pH 6.75–7.25 and optimum binding at pH 7.2. At pH 6.5 the specific binding decreased by approximately 30% and at pH 7.5 the specific binding was reduced by approximately 40%.

Saturation studies in normal human cardiovascular tissue Over the concentration range tested (0.01–1.5 nM) [^{125}I-*His*^{9}]-ghrelin binding was saturable (Figure 3) to all human cardiovascular tissues examined with 60–65% specific binding. [^{125}I-*His*^{9}]-Ghrelin bound to sections of non-diseased human left ventricle and right atrial tissue with no significant difference in ligand affinity and similar receptor density (Table 2). For each tissue, a one site fit was preferred to a two site model and the Hill coefficients were close to unity. In the vasculature, [^{125}I-*His*^{9}]-ghrelin bound with sub-nanomolar affinity to elastic arteries (aorta and pulmonary), muscular arteries (coronary) and veins (saphenous) with no significant difference in ligand affinity comparing arteries and veins (Table 2).

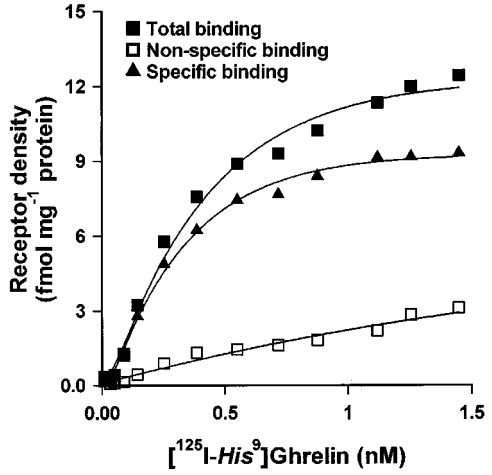

Figure 3 Saturation binding curve for [^{125}I-*His*^{9}]-ghrelin. Increasing concentrations of radioligand (0.01–1.5 nM) were incubated with sections of human left ventricle for 25 min at room temperature. Curve represents a typical experiment with a dissociation constant (K_D) of 0.35 nM and maximal receptor density (B_{max}) of 8.8 fmol mg^{-1} protein.

Competition studies At a concentration of 1 μM, hexarelin as well as unlabelled ghrelin competed for 0.15 nM [^{125}I-*His*^{9}]-ghrelin with 60% specific binding. Hexarelin competed for the [^{125}I-*His*^{9}]-ghrelin binding site with a K_D of 10.1 ± 0.22 nM ($n = 3$ individuals $\pm$ s.e.mean). Other structurally unrelated peptides (endothelin-1, angiotensin II, atrial natriuretic peptide, orexin-A, adrenomedullin, and apelin) at 1 μM concentration did not compete for the [^{125}I-*His*^{9}]-ghrelin binding site in non-diseased human left ventricle.

Alteration of GHS-R density with disease

[^{125}I-*His*^{9}]-Ghrelin bound to sections of diseased human left ventricle and right atrial tissue with no significant difference in ligand affinity and comparable receptor density to non-diseased cardiac tissue (Table 2), with no indication of a change in receptor density amongst the individuals examined. However, compared to non-diseased coronary artery, GHS-R density was significantly up-regulated in atherosclerotic coronary arteries (Table 2) ($P < 0.05$). Arterialization following saphenous vein grafting into the coronary circulation resulted in a significant increase in GHS-R density compared with non-diseased saphenous veins ($P < 0.05$).

Autoradiographical localization of GHS-R in human tissue

In human cardiovascular tissue [^{125}I-*His*^{9}]-ghrelin binding sites were localized to cardiac myocytes of human left ventricular and right atrial tissue (Figure 4A,B) within the heart and to the media and intimal smooth muscle layers of all human blood vessels examined. Specific binding sites were

Table 2 Dissociation constant (K_D), maximal density of receptors (B_{max}) and Hill coefficients (n_H) for [^{125}I-*His*^{9}]-ghrelin binding to non-diseased and diseased human cardiovascular tissue

Tissue	K_D (nM)	B_{max} (fmol mg^{-1} protein)	n_H	n
Left ventricle – non-diseased	0.43 ± 0.08	7.8 ± 0.9	0.95 ± 0.06	6
Left ventricle – DCM	0.44 ± 0.02	7.8 ± 0.8	1.09 ± 0.04	7
Left ventricle – IHD	0.37 ± 0.05	5.9 ± 0.7	1.05 ± 0.04	6
Right atria – non-diseased	0.48 ± 0.12	9.7 ± 2.2	1.03 ± 0.04	5
Right atria – DCM	0.50 ± 0.07	9.1 ± 1.8	1.05 ± 0.5	7
Right atria – IHD	0.45 ± 0.10	6.4 ± 1.1	1.03 ± 0.06	6
Coronary artery – non-diseased	0.22 ± 0.08	6.2 ± 2.1	0.96 ± 0.03	3
Atherosclerotic coronary artery	0.44 ± 0.07	28.8 ± 6.9†	0.99 ± 0.07	4
Saphenous vein – non-diseased	0.29 ± 0.10	7.2 ± 2.7	1.04 ± 0.05	3
Saphenous vein grafts	0.25 ± 0.03	23.7 ± 1.7*	0.89 ± 0.04	4
Aorta – non-diseased	0.43 ± 0.19	17.5 ± 2.5	1.06 ± 0.05	3
Pulmonary artery – non-diseased	0.57 ± 0.08	24.4 ± 7.1	1.03 ± 0.09	3

Values represent mean $\pm$ s.e.mean ($n = 3$–7) individuals. †$P < 0.05$ Student's unpaired *t*-test, compared to non-diseased coronary artery. *$P < 0.05$ Student's unpaired *t*-test, compared to non-diseased saphenous vein. DCM, dilated cardiomyopathy; IHD, ischaemic heart disease.

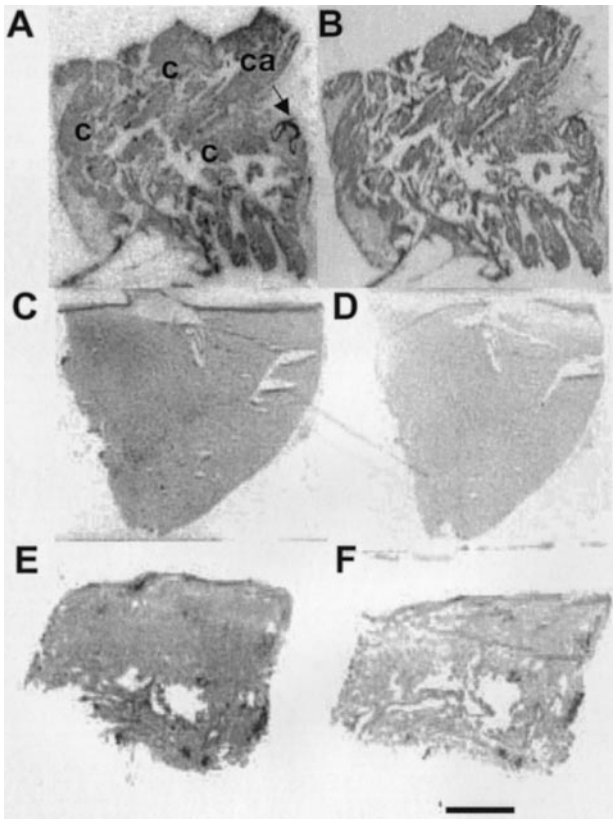

Figure 4 Autoradiographical localization of growth hormone secretagogue receptors in human right atrial tissue section using 0.15 nM [^{125}I-*His*^{9}]-ghrelin (A) Total binding in the absence of 1 μM hexarelin (B) section stained with heamatoxylin and eosin. c = cardiac myocytes, ca = coronary artery (C) total binding of human kidney section containing cortex (c) and medulla (m) (D) non-specific binding of human kidney section in the presence of 1 μM hexarelin (E) total binding representing a section of human lung tissue (F) non-specific binding of human lung tissue in the presence of 1 μM hexarelin. Scale bar = 2 mm.

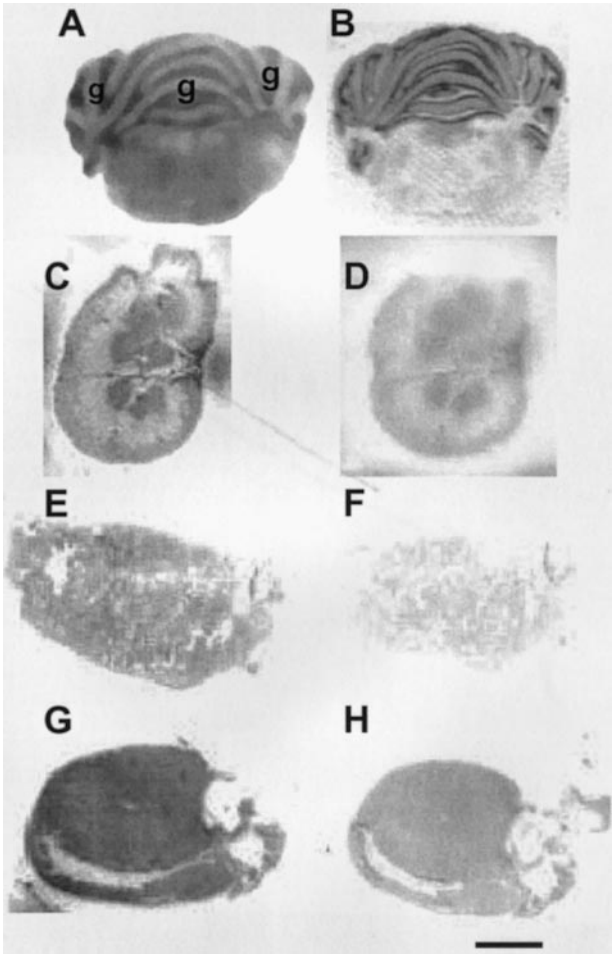

Figure 5 (A) Autoradiographical localization of growth hormone secretagogue receptor to rat brain with high density in the granular layer (g) of the rat cerebellum (B) section stained with heamatoxylin and eosin (C) total binding to a section of rat kidney (D) non specific binding defined in the presence of 1 μM hexarelin (E) total binding to a section of rat lung (F) corresponding non-specific binding in the presence of 1 μM hexarelin (G) total binding to a section of rat heart (H) non-specific binding defined in the presence of 1 μM hexarelin. v = ventricle, a = atria. Scale bar = 2 mm.

also detected in small intra-myocardial coronary arteries and internal mammary artery. In sections of human kidney (Figure 4G,D) containing both cortex and medulla, [^{125}I-*His*^{9}]-ghrelin bound to the vasculature, particularly to the arcuate arteries at the cortico-medullary junction with no detectable binding in other structures such as the glomeruli. Specific [^{125}I-*His*^{9}]-ghrelin binding was also detected in human lung parenchyma (Figure 4E,F).

[^{125}I-His^{9}]-Ghrelin binding and autoradiographical localization of GHS-R in rat tissue

[^{125}I-*His*^{9}]-Ghrelin (human) bound to a single site in both the atria and ventricle of the rat heart, in a specific and saturable manner with a K_D of 0.24 ± 0.03 nM, B_{max} of 4.8 ± 0.8 fmol mg^{-1} protein, Hill slope of 0.89 ± 0.03 ($n = 3$) and to rat kidney tissue with a K_D of 0.31 ± 0.01 nM, B_{max} of 2.8 ± 0.2 fmol mg^{-1} protein, Hill slope of 1.05 ± 0.04 ($n = 3$). In rat tissues examined, [^{125}I-*His*^{9}]-ghrelin binding sites were detected with high density confined to the granular layer of the cerebellum (Figure 5A,B). GHS-R protein was identified in kidney (confined to the renal vasculature) (Figure 5C,D), lung (Figure 5E,F) and in sections of heart in both the atria and ventricle (Figure 5G,H).

Discussion

We have demonstrated for the first time the binding of [^{125}I-*His*^{9}]-ghrelin to GHS-R, to establish the expression of the previously paired orphan receptor protein, predicted to exist from molecular studies of the mRNA. We characterized the binding in normal human heart and used these initial findings to determine the anatomical distribution of the receptors in human and rat tissue. Finally, we have discovered that GHS-R density is increased in human atherosclerosis, which may well have pathophysiological implications.

[^{125}I-*His*^{9}]-Ghrelin binding to human cardiovascular tissue was saturable, reversible and specific, characteristic of a ligand-receptor interaction. Within the concentration range

used in saturation experiments, the radioligand bound with high affinity. Hill slopes were close to unity indicating that [^{125}I-*His*^{9}]-ghrelin bound with a single affinity, with no evidence for receptor subtypes. In agreement, kinetic studies showed that the radioligand also bound rapidly to a single site at room temperature. Binding was reversible with the ligand dissociating from a single site. None of the structurally unrelated peptides tested competed for the binding of [^{125}I-*His*^{9}]-ghrelin in human heart, but as expected unlabelled ghrelin and the synthetic GHS, hexarelin, competed equally with 60–65% specific binding, indicating that both the synthetic GHS and the endogenous unlabelled ligand compete for the same receptor population. The density of receptors in the human myocardium was comparable to another recently adopted orphan receptor APJ (6 fmol mg^{-1} protein; Katugampola *et al*., 2001b). However, this density is lower than that for endothelin receptors in the human left ventricle (65 fmol mg^{-1} protein; Molenaar *et al*., 1993). [^{125}I-*His*^{9}]-Ghrelin bound with a single and comparable high affinity to GHS-R in the rat heart suggesting that the radiolabelled analogue of human ghrelin (which differs by two amino-acid from the rat sequence) is also a suitable ligand for the characterization of GHS-R in animal models.

The synthetic GHS agonist, hexarelin, causes vasoconstriction (Bodart *et al*., 1999) and has positive inotropic effects in human hearts with an increase in left ventricle ejection fraction (Bisi *et al*., 1999). Initial studies found that ghrelin induces an increase in intracellular Ca^{2+} in GHS-R expressing cells (Kojima *et al*., 1999) and may therefore have similar vasoconstrictor and inotropic actions to other endogenous peptides such as endothelin-1 and angiotensin-II. Intriguingly, evidence is emerging that chronic administration of hexarelin to growth hormone deficient rats is cardioprotective (Berti *et al*., 1998; De-Gennaro *et al*., 2000; Rossoni *et al*., 2000; Tivesten *et al*., 2000), suggesting a further potential function for ghrelin. Ghrelin has been reported to circulate in humans at a plasma concentration much higher (120 pmol l^{-1}) than other cardiovascular peptides such as endothelin (typically about 5 pmol l^{-1}; Davenport *et al*., 1990) or adrenomedullin ($\sim$10 pmol l^{-1}; Ohta *et al*., 1999), but comparable to the circulating vasoactive atrial natriuretic peptide ($\sim$90 pmol l^{-1}; Yandle *et al*., 1993). At present its unclear whether locally produced or circulating endogenous peptide may activate the receptors that we have visualized using [^{125}I-*His*^{9}]-ghrelin. The *n*-octanyl group is essential for ligand binding and removal of this group abolishes activity (Kojima *et al*., 1999). In agreement, we found that incubating with AEBSF to protect the *n*-octanyl group from peptide degradation, is essential for [^{125}I-*His*^{9}]-ghrelin binding to native human cardiovascular tissue.

The binding of [^{125}I-*His*^{9}]-ghrelin was localized to both human and rat tissue using autoradiography. GHS-R were detected in cardiac myocytes of human ventricular and atrial tissue in agreement with the presence of mRNA encoding this receptor detected by RT–PCR (Kojima *et al*., 1999). In the vasculature, binding was detected in the medial layer (comprising predominantly smooth muscle cells) of epicardial coronary arteries with lower densities localizing to small intramyocardial coronary arteries, as expected from the reported vasoactive properties of the peptide.

Saturation studies revealed that the density of ghrelin binding (with no change in affinity) was significantly increased in coronary arteries containing atherosclerotic lesions as well as occluded saphenous vein recovered at the time of transplantation. Intimal proliferation of the smooth muscle may be a characteristic of both conditions. This is in contrast with other vasoactive peptides such as apelin (Katugampola *et al*., 2001b), where there is no change or endothelin-1, where there is significant down-regulation of the receptors in the intimal smooth muscle layer (Bacon *et al*., 1996; Katugampola & Davenport, 2000). It is not known whether the endogenous ligand production is increased and might be expected to enhance receptor mediated actions or if there is a compensatory decrease with coronary artery disease. The pathophysiological importance of this increase in GHS-R density in atherosclerosis and saphenous vein grafts remains to be determined. However, this change in receptor density implicates a role for ghrelin in intimal hyperplasia and atherosclerosis. In addition, the localization of GHS-R to the vasculature present in a range of other human tissues including the lung, and kidney suggests ghrelin may have a vasoactive role in a number of vascular beds.

In conclusion our results suggest that the native receptor for [^{125}I-*His*^{9}]-ghrelin may be widely distributed in the human cardiovascular system. Furthermore, changes in the density of this proposed ghrelin receptor implicates this new transmitter system in the development of atherosclerosis and may therefore represent a novel therapeutic target in the treatment of cardiovascular disease.

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