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Abstract—Hexarelin, a synthetic hexapeptide of the growth hormone–releasing peptide (GHRP) family with strong growth hormone (GH)–releasing activity, features protecting activity against postischemic ventricular dysfunction in hearts from GH-deficient and senescent rats. To document whether hexarelin action is mediated through specific cardiac receptors, perfusion of Langendorff rat hearts with hexarelin and binding studies were carried out. In the Langendorff rat heart system, hexarelin induced a dose-dependent increase in coronary perfusion pressure. Nifedipine, chelerythrine, and bisindolylmaleimide partially inhibited the vasoconstriction induced by hexarelin, suggesting that this effect was mediated at least in part by L-type Ca^{2+} channels and protein kinase C. In contrast, diclofenac and 1-(7-carboxyheptyl)imidazole were without effect, suggesting that prostaglandins and thromboxanes were not involved in the coronary vasoconstriction induced by hexarelin. To characterize the hexarelin binding sites in the rat heart, [^{125}I]Tyr-Bpa-Ala-hexarelin was used as photoactivatable radioligand in saturation and competitive binding studies. We specifically labeled a hexarelin receptor with an M_r of 84 000 in rat cardiac membranes. Saturation binding curves revealed a single class of binding sites with a K_d of 14.5 nmol/L and a density of 91 fmol/mg of protein. Competition binding studies gave an IC_{50} of 2.9 μmol/L for hexarelin; MK-0677 and EP51389, both potent GH secretagogues, did not displace the binding of the photoactivatable derivative from rat cardiac membranes. Interestingly, both compounds were devoid of any vasoconstrictive activity. These results suggest the existence of a new class of hexarelin receptor in the heart, whose role in the regulation of the coronary vascular tone is yet to be determined. **(*Circ Res*. 1999;85:796-802.)**

Key Words: growth hormone–releasing peptide ■ receptor ■ heart ■ photoaffinity labeling ■ Langendorff perfused heart

Growth hormone–releasing peptides (GHRPs), which consist of a family of small synthetic peptides modeled from Met-enkephalin,[1] and their nonpeptidyl derivatives[2] have been shown to release growth hormone (GH) both in vitro and in vivo by direct action on the pituitary gland.[3,4] The GH releasing action of GHRPs is mediated through the interaction of these ligands with a G protein–coupled receptor, which has been cloned recently from porcine, human, and rat libraries,[5–7] as well as with their receptor subtype identified by the photolabeling approach.[8] Besides this stimulatory effect on GH secretion, it has been demonstrated that hexarelin, an hexapeptide member of the GHRP family, features protective activity against postischemic dysfunction of perfused hearts isolated from GH-deficient[9] and senescent rats[10] receiving the peptide subcutaneously as a 2-week pretreatment. This beneficial effect induced by hexarelin is not followed by any apparent stimulation of the growth hormone/insulin-like growth factor-1 (GH/IGF-1) axis. The effect is not likely related to the cardiovascular actions of GH/IGF-1 through interaction with specific receptors expressed at the myocardial level,[11,12] suggesting a direct influence of hexarelin on cardiac function. The primary aims of the present study were to evaluate whether hexarelin exerts a direct action on the cardiac function using the perfused Langendorff heart and to document the cellular signaling pathways involved. The secondary aim was to identify and to characterize the new GHRP receptor from the rat heart by the photoaffinity labeling approach using a photoactivatable derivative of hexarelin.

Materials and Methods

Experimental Protocol With Langendorff Hearts

Male Sprague-Dawley rats (300 to 325 g) were purchased from Charles River (Montreal, Canada). Animal use was in accordance with the Canadian council on animal care guidelines. Rats were

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necrotized with CO_2 until a complete loss of consciousness and promptly decapitated. Their heats were quickly excised and mounted on the experimental Langendorff setup as described by Bouchard et al.[13] A dose-response curve to hexarelin (10 nmol/L to 30 μmol/L) was charted by successive infusions of increasing concentrations of the peptide. The infusions were maintained for 5 to 10 minutes, which was enough to reach steady state. A washout period of 10 minutes was allowed after the first dose-response curve. A second dose-response curve for the same agent was drawn 30 minutes after beginning an infusion with either diclofenac (1 μmol/L), 1-(7-carboxyheptyl) imidazole (1-7 CHI, 10 μmol/L), chelerythrine (1 μmol/L), bisindolylmaleimide I, HCl (1 μmol/L), nifedipine (1 μmol/L), or their respective vehicles. Drug-induced changes in coronary resistance were evaluated by computing the percentage of change in coronary perfusion pressure, measured immediately before each drug infusion and after a new steady state.

Membrane Preparations

All animals were anesthetized with sodium pentobarbital, and their hearts were promptly removed and placed in ice-cold saline buffer. Cardiac membranes were prepared according to Harigaya et al.[14] Membranes from bovine anterior pituitaries were prepared according to Ong et al.[8]

Receptor Binding and Photolabeling With [^{125}I]-Tyr-Bpa-Ala-Hexarelin

The radioiodination procedure of the photoactivatable ligand and the receptor binding assays were performed as described by Ong et al.[8] For saturation binding assays, incubations were set in the presence of increasing concentrations of the radiolabeled photoactivatable hexarelin derivative (from 1.5 to 45 nmol/L). Nonspecific binding was defined as binding not displaced by 10 μmol/L hexarelin. For competition binding assays, incubations were performed in the presence of a fixed concentration of radioiodinated photoactivatable hexarelin derivative (0.33 nmol/L) and increasing concentrations of competitive ligands from 1 nmol/L to 10 μmol/L. After autoradiography, bands corresponding to the specifically labeled protein of M_r 84 000 were cut out, and radioactivity was counted in a γ counter to establish saturation and competition curves.

Deglycosylation of Photolabeled GHRP Receptor With *N*-Glycosidase F

Deglycosylation of the photolabeled GHRP receptors from cardiac and pituitary membranes was performed as previously described.[8]

Autoradiographic Distribution of GHRP Binding Sites in the Rat Heart

Rat hearts were processed for cryostat sectioning. Consecutive 6-μm-width sections were mounted on gelatinized glass slides. Sections were stained with hematoxylin-eosin for histological determination. Consecutive unstained sections were rehydrated by incubation with PBS. Sections used for the total binding were incubated with [^{125}I]Tyr-Bpa-Ala-hexarelin (0.3 nmol/L) for 60 minutes at room temperature. The nonspecific binding was determined in sections incubated in the presence of 10 μmol/L hexarelin. All the sections were then exposed to UV light for 15 minutes and washed with PBS. Tissue sections were placed in cassettes for autoradiography. The densitometry analysis of the autoradiogram was performed using Quantity One software (PDI) for the quantification of the total and nonspecific binding.

Data Analysis

The effects of the different drugs on hexarelin-induced vasoconstriction and the data analysis of densitometry were performed by ANOVA. Radioligand saturation and competition curves were analyzed with ALLFIT for Windows software.[15,16]

An expanded Materials and Methods section is available online at http://www.circresaha.org.

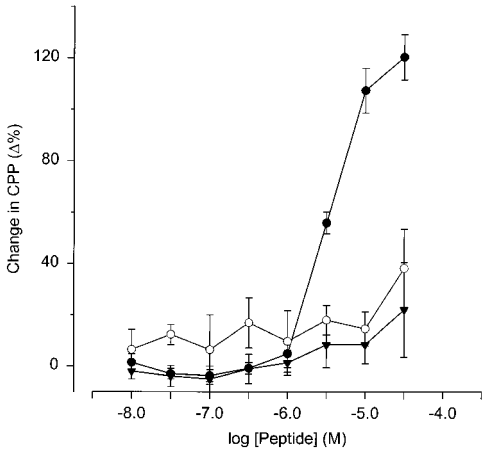

Figure 1. Changes in coronary perfusion pressure (CPP in Δ%) induced by GH secretagogues. Isolated rat hearts were perfused with increasing concentrations of hexarelin (●, n=5), MK-0677 (○, n=3), and EP51389 (▼, n=3), and perfusion pressure was monitored to calculate coronary perfusion resistance.

Results

Change in Coronary Resistance Induced by GHRPs

Figure 1 summarizes the dose-response curves for changes in the coronary resistance at various concentrations of peptidyl and nonpeptidyl GHRP derivatives infused. Coronary resistance measured just before hexarelin infusion was 5.1 ± 0.4 mm Hg · min^{-1} · mL^{-1} for a coronary flow rate of 6.8 ± 0.7 mL · min^{-1} · g^{-1}. At a concentration of 30 μmol/L, hexarelin infusion induced an increase in coronary resistance at 11.0 ± 0.7 mm Hg · min^{-1} · mL^{-1} for a coronary flow rate of 6.8 ± 0.7 mL · min^{-1} · g^{-1}. Coronary perfusion pressure (+120%) was increased maximally at 30 μmol/L hexarelin. The increase in the coronary resistance induced by hexarelin contrasted with that obtained with EP51389, a truncated GHRP derivative, and the peptidomimetic MK-0677, as maximal coronary resistance was obtained at 8.6 ± 0.9 and 6.7 ± 0.4 mm Hg · min^{-1} · mL^{-1} for EP51389 and MK-0677, respectively. Hexarelin did not affect left ventricular end-diastolic pressure. However, both dP/dt_{max} and dP/dt_{min} were reduced by ≈40% at the highest concentration of hexarelin studied (data not shown). Heart rate was not modified even by the highest dose of hexarelin (data not shown).

Signaling Pathways Involved in the Change of Coronary Resistance

The role of Ca^{2+} influx in the increase of coronary resistance induced by hexarelin was assessed after preperfusion with 1 μmol/L nifedipine, an L-type Ca^{2+} channel inhibitor. Nifedipine evoked a significant decrease in coronary resistance increased by 30 μmol/L hexarelin. The changes in the coronary perfusion pressure were (Δ%) 36.1 ± 5.4 and 127.2 ± 7.8 in the presence and absence of 1 μmol/L nifedipine, respectively, as shown in Figure 2.

The role of protein kinase C (PKC) in mediating the increase in coronary resistance was evidenced by a selective inhibitor of PKC, chelerythrine.[17] This compound significantly reduced the coronary perfusion pressure induced by

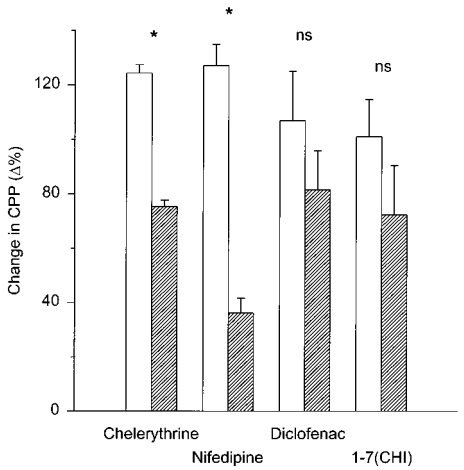

Figure 2. Signaling pathways involved in hexarelin-induced increase in coronary perfusion pressure. Isolated rat hearts were perfused with 30 μmol/L hexarelin alone (open bars) or 30 μmol/L hexarelin in the presence of either 1 μmol/L chelerythrine, 1 μmol/L nifedipine, 1 μmol/L diclofenac, or 10 μmol/L 1-7 CHI (hatched bars). ns indicates not significant. n=5 to 7 per group. *$P<0.05$.

30 μmol/L hexarelin. The changes in coronary perfusion pressure were (Δ%) 76.5±2.4 and 124.4±3.0 in the presence and the absence of 1 μmol/L chelerythrine, respectively, as shown in Figure 2. The role of PKC was confirmed by the use of the highly selective inhibitor of this enzyme, bisindolylmaleimide I, HCl.[18] At a concentration of 1 μmol/L, this compound significantly reduced the coronary vasoconstriction induced by 10 μmol/L hexarelin (changes in coronary perfusion pressure were (Δ%) 17.6±7.6 and 123.0±14.0 in the presence and the absence of the inhibitor respectively, n=4).

To rule out the contribution of contractile prostaglandins and thromboxanes in the coronary vasoconstriction induced by hexarelin, isolated rat hearts were preperfused with 1 μmol/L diclofenac, a cyclooxygenase inhibitor, or 10 μmol/L 1-(7-carboxyheptyl) imidazole (1-7 CHI), an inhibitor of thromboxane synthase.[19] Neither compound significantly affected the coronary vasoconstriction induced by hexarelin (Figure 2).

Covalent Photolabeling of GHRP Binding Sites With [^{125}I]-Tyr-Bpa-Ala-Hexarelin

Covalent photolabeling of rat cardiac membranes with [^{125}I]-Tyr-Bpa-Ala-hexarelin revealed a single specific binding site with an M_r of 84 000 visualized by autoradiography after SDS-PAGE (Figure 3, top). The saturation isotherm generated by the radioactive signal detected in the band at M_r 84 000 is reported in Figure 3, bottom. It is best fitted to a single class of binding sites with a dissociation constant of 14.5 nmol/L and an estimate for the number of binding sites of 91 fmol/mg of protein.

The specificity of covalent photolabeling of rat cardiac membranes with the photoactivatable hexarelin derivative was assessed by competitive binding studies with hexarelin, its truncated analog EP51389, and the nonpeptidyl derivative MK-0677, as shown in Figure 4, top. Analysis of the competition curves gave IC_{50} values of 2.9 μmol/L for

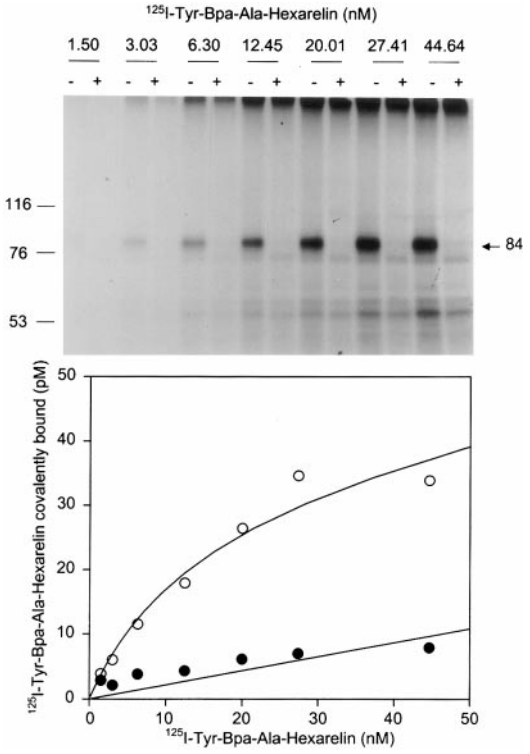

Figure 3. Covalent saturation of the GHRP receptor in rat cardiac membranes. Top, Autoradiography of the photolabeled proteins separated by SDS-PAGE. Bottom, Saturation isotherm of the specifically labeled 84-kDa protein. ○, Total binding; ●, Nonspecific binding.

hexarelin and more than 10 μmol/L for EP51389 and MK-0677. These results contrasted with those obtained with the same competitive ligands for binding of the photoactivatable radioligand to the GHRP receptor subtype in bovine anterior pituitary membranes, which featured an M_r of 57 000.[8] The IC_{50} of hexarelin, EP51389, and MK-0677 inhibiting the covalent binding of [^{125}I]-Tyr-Bpa-Ala-hexarelin to the pituitary GHRP receptor was 0.6, 1.5, and 20 μmol/L, respectively (Figure 4, bottom). All three ligands, which are potent growth hormone secretagogues, appeared to bind to GHRP binding sites in rat cardiac membranes with a lower affinity than that observed for pituitary GHRP binding sites. Interestingly, the tripeptide analog EP51389, which displaced the radioligand binding with the same IC_{50} as hexarelin in bovine pituitary membranes, was without effect in rat cardiac membranes.

It is well-known that a network of nonadrenergic, noncholinergic perivascular nerve fibers supplying the coronary vasculature releases vasoactive neuropeptides, as demonstrated in immunocytochemical studies.[20] The main neuropeptides identified in nerves associated with coronary blood vessels, namely neurotensin, calcitonin gene-related peptide (CGRP), substance P, and neuropeptide Y (NPY), did not inhibit the covalent photolabeling of the M_r 84 000 protein by the photoactivatable derivative of hexarelin, as shown in Figure 5. Furthermore, the vasoactive peptidic hormones endothelin 1, angiotensin II, and arginine-vasopressin, for which specific receptors are found within the heart, did not compete with the photolabeling of hexarelin

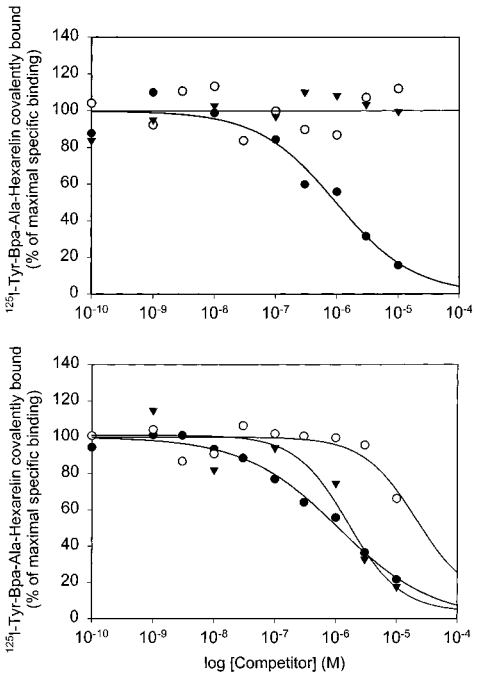

Figure 4. Competition curves of GH secretagogues for the binding of [^{125}I]-Tyr-Bpa-Ala-hexarelin in rat cardiac (top) and bovine pituitary (bottom) membranes. A fixed concentration of [^{125}I]-Tyr-Bpa-Ala-hexarelin was incubated with rat cardiac and bovine pituitary membranes in the presence of increasing concentrations of competitor. ●, hexarelin; ○, MK-0677; and ▼, EP51389.

binding sites (angiotensin II and arginine-vasopressin; data not shown).[21–23]

Deglycosylation of GHRP Binding Sites

Figure 6 illustrates that incubation of the photolabeled receptor from cardiac membrane preparations with *N*-glycosidase F for 24 hours at 25°C increased its mobility from M_r 84 000 to 57 000 (Figure 6B). The photolabeled receptor from bovine pituitary membranes submitted to the same incubation conditions displayed only a slight increase in mobility from M_r 57 000 to 53 000 (Figure 6A). The ability of *N*-glycosidase F to reduce the apparent molecular size of the photolabeled receptor from both cardiac and pituitary membrane preparations confirmed the glycoprotein nature of GHRP receptors.

Distribution of GHRP Receptors in the Heart and Among Species

The compartmental distribution of GHRP receptor in the rat heart has been documented. As shown in Figure 7, the density

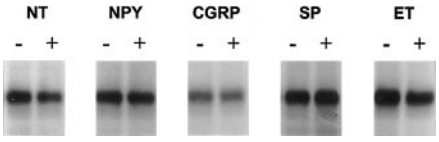

Figure 5. Effect of various neuropeptides on the binding of [^{125}I]-Tyr-Bpa-Ala-hexarelin to rat cardiac membranes. A fixed concentration of [^{125}I]-Tyr-Bpa-Ala-hexarelin was incubated with rat cardiac membranes in the absence (−) or presence (+) of 10 μmol/L of the competitive peptides. NT indicates neurotensin; NPY, neuropeptide Y; CGRP, calcitonin gene-related peptide; SP, substance P; and ET, endothelin 1.

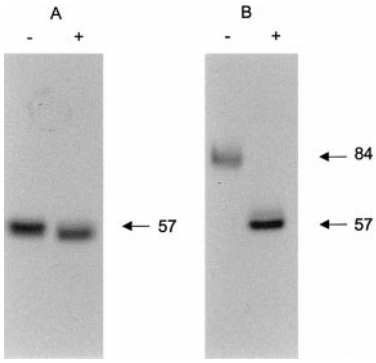

Figure 6. Glycosylation state of the pituitary and cardiac GHRP receptors. Photolabeled receptors from bovine pituitary membranes (A) and rat cardiac membranes (B) were purified by SDS-PAGE and incubated in the absence (−) or presence (+) of *N*-glycosidase F.

of the signal corresponding to the specifically photolabeled protein was found to be higher in ventricles than in atria. Cardiac GHRP receptors were not only expressed in cardiac membranes from the rat but also in the hearts of hamsters, dogs, and pigs as revealed in Figure 8. Although the unique photoaffinity-labeled band at M_r 84 000 was detected in membranes from rat and hamster hearts, membrane preparations from canine and porcine hearts featured two specific photolabeled bands (at M_r 84 000 and 60 000 for the dog and at M_r 74 000 and 58 000 for the pig).

Autoradiographic Distribution of GHRP Binding Sites in the Rat Heart

The densitometry analysis of the total binding on four replicates of rat heart sections gave optical densities of 24.25±2.19 (OD×mm^2) (n=4). The optical densities of the tissue sections relative to the nonspecific binding were 18.00±1.02 (OD×mm^2). The difference in the optical densities from the total and nonspecific binding defined as specific binding represented 25% of the total signal and was found to be significant ($P<0.05$). The specific hexarelin binding was homogeneously distributed within the heart section as shown in Figure 9.

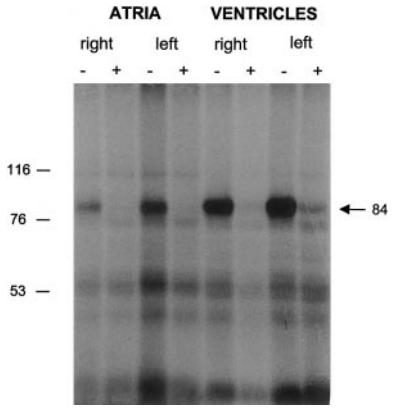

Figure 7. Compartmental distribution of GHRP binding sites in rat heart. Membranes from right and left atria and ventricles from rat heart were incubated with [^{125}I]-Tyr-Bpa-Ala-hexarelin in the absence (−) or presence (+) of 10 μmol/L hexarelin.

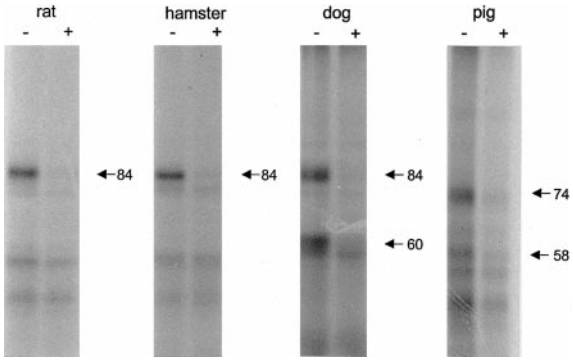

Figure 8. GHRP receptor in cardiac membranes from various species. Membranes from rat and hamster hearts and dog and porcine left ventricles were incubated with [^{125}I]-Tyr-Bpa-Ala-hexarelin in the absence (−) or presence (+) of 10 μmol/L hexarelin.

Discussion

It is now well-known that GHRPs and peptidomimetics are endowed with strong GH releasing activity in a variety of mammalian species.[1–3] This effect occurs most likely through interaction with a G protein–coupled receptor cloned recently from porcine, human, and rat pituitary libraries. This 366 amino acid protein of M_r 41 000 with seven transmembrane spanning domains is most likely coupled through activation of $G_{\alpha 11}$ to phosphoinositol metabolism.[5] GHRPs may also interact with the M_r 57 000 receptor subtype identified in pituitary membranes.[8] Using the Langendorff perfused heart, we have revealed an unexpected cardiovascular effect of hexarelin, a hexapeptide member of the GHRP family. Hexarelin presents a selective dose-response curve of increasing coronary perfusion pressure in the rat heart. This contrasts with the dose-response curves obtained with the peptidomimetic spiroindoline derivative MK-0677 and the shorter analog of GHRP, EP51389. Given that both compounds are known to be potent GH secretagogues, there is the possibility of a different structure-activity profile for GHRP binding sites within the heart. The signal transduction pathways mediating the cardiac effect of hexarelin seem to involve L-type Ca^{2+} channels, because the increase in coronary perfusion pressure induced by the hexapeptide is strongly altered by nifedipine. However, the contribution of other types of Ca^{2+} channels could not be excluded. Furthermore, coronary vasoconstriction in response to hexarelin appears to also involve PKC, because both chelerythrine and bisindolylmaleimide partially inhibit the vasoconstrictive effect induced by hexarelin. The reduced vasoconstriction induced by hexarelin in the presence of the above-mentioned inhibitors suggests that other transduction pathways potentially involving tyrosine kinases may be linked with the vasoconstrictive effect observed.[24] The vasoconstrictive response to hexarelin is probably endothelium independent, because the inhibitors of prostaglandin and thromboxane synthesis, diclofenac and 1-7 CHI, respectively, did not affect the increase in coronary perfusion pressure. Hexarelin does not display any chronotropic effect, as heart rate remained unchanged throughout the perfusion study. However, both dP/dt_{max}, an index of myocardial contractility, and dP/dt_{min}, which reflects the ability of the heart to relax, were reduced at the highest concentration investigated. Although we cannot rule out a direct action of hexarelin on cardiomyocytes, this effect is most probably caused by the strong vasoconstriction itself, which can impair myocardial perfusion and result in myocardial hypoxia. These results suggest that the effect of hexarelin might be mediated through GHRP binding sites on vascular smooth muscle cells. To characterize these binding sites, attempts at equilibrium binding using the radiolabeled hexarelin derivative [^{125}I]Tyr-Ala-hexarelin were unsuccessful because of the hydrophobicity of the GHRP derivative that contributes to the high level of nonspecific binding.[25,26] The covalent photolabeling approach with benzophenone as the photoreactive group featuring higher stability than other diazoaryl or arylazide derivatives[27] has allowed the identification of a new class of GHRP binding sites in the rat heart. Effectively, the results of the saturation binding assays have shown that the photoactivatable hexarelin derivative binds to the cardiac membrane protein at M_r 84 000 with a high affinity. However, according to the results of competition binding curves, the majority of these binding sites seem to have a lower affinity for hexarelin, given that the ED_{50} value of hexarelin to displace the photoactivatable ligand was found to be in the micromolar range.

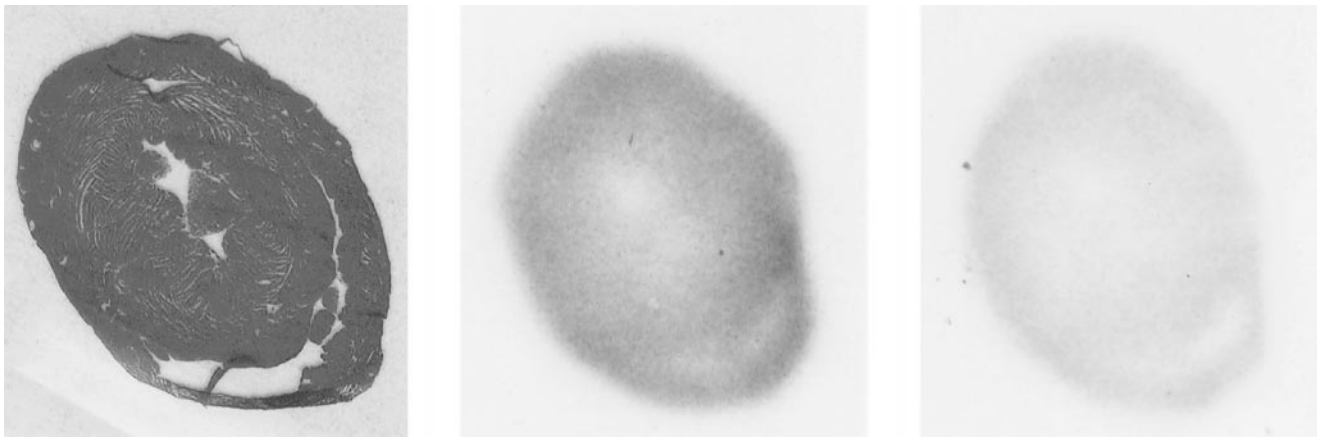

Figure 9. Histological and autoradiographic distribution of hexarelin binding in the rat heart. Consecutive sections (6 μm) of rat heart prepared as described in Materials and Methods are represented. Left, Hematoxylin-eosin staining. Middle, Total binding. Right, Nonspecific binding.

The rank order of potencies of GHRP derivatives to reduce the binding of the photoactivatable hexarelin derivative is well correlated with the rank order of potencies of these ligands in increasing the coronary perfusion pressure in the Langendorff perfused heart system. The difference in ED_{50} values of the peptidyl and nonpeptidyl derivatives of hexarelin, EP51389 and MK-0677, in competing with photoactivatable hexarelin derivative for binding to cardiac and pituitary membranes[8] suggests that the cardiac GHRP binding sites are distinct from those identified in the pituitary. Moreover, these cardiac GHRP binding sites appear to be highly glycosylated, contrasting with pituitary GHRP receptors identified recently by the photolabeling approach. These binding sites are also distinct from vasoactive neuropeptide receptors expressed in the coronary vasculature, because neither NPY, neurotensin, CGRP, nor substance P competes for binding of the photoactivatable ligand. Furthermore, the photoaffinity-labeled signal is not altered by circulating vasoactive hormones such as endothelin 1, angiotensin II, or arginine-vasopressin, for which specific receptors are found in the cardiac vasculature.

The regional distribution of the photoactivatable hexarelin derivative has shown that GHRP binding sites are mostly confined to the ventricles, contrasting with the low density observed in the atria. The autoradiographic distribution of this hexarelin derivative reveals that specific binding is present throughout the rat heart sections. However, the resolution of the technique does not allow the cellular localization of the GHRP binding sites in the rat heart. Considering that significant binding is present throughout the thickness of the heart section, the presence of GHRP binding sites on the microvascular system and/or the cardiomyocytes might be expected.

These cardiac GHRP binding sites are found to be widely distributed among various species. Although a unique form of the GHRP receptor at M_r 84 000 was detected in the cardiac membranes from rodents, two forms of the receptor are expressed in membranes from dog and porcine hearts, which most probably reflect different degrees of glycosylation. The ability of endoglycosidase F to reduce the apparent molecular mass of the photolabeled cardiac GHRP receptor from M_r 84 000 to 57 000 suggests that this receptor contains *N*-linked oligosaccharides. The difference in sensitivity of the cardiac GHRP receptor to *N*-glycosidase F compared with that from the pituitary indicates a distinct glycosylation pattern of this protein.

Recent studies have demonstrated the cardiovascular effect of GH and its local effector IGF-1 through the interaction with specific receptors expressed at the myocardial level.[11,12] GH and IGF-1 have been found to play a critical role in cardiac remodeling and inotropism.[28,29] However, hexarelin, a GHRP derivative, appears to have a direct effect on vascular tone, contrasting with the pharmacological profile of GH and IGF-1.

In conclusion, we reported for the first time the existence of specific GHRP binding sites in the mammalian heart, using a photoactivatable hexarelin derivative. We demonstrated that these cardiac GHRP receptors are distinct from the pituitary GHRP receptors involved in GH secretion. Hexarelin appeared to bind with low affinity to these cardiac receptors, inducing potent but reversible coronary vasoconstriction in the perfused Langendorff rat heart. Our results suggest the putative existence of a specific peptidic hormone as endogenous ligand for the cardiac GHRP receptor characterized. This new hormone may be involved in the regulation of vascular tone within the heart.

Acknowledgments

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