G-protein-coupled receptors in human atherosclerosis: comparison of vasoconstrictors (endothelin and thromboxane) with recently de-orphanized (urotensin-II, apelin and ghrelin) receptors

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ABSTRACT

Endothelin (ET)-1 and thromboxane (Tx) levels are increased in human atherosclerosis. One of the aims of this study was to understand how receptors for a peptide mediator (ET-1) with a long physiological half life, would differ from a lipid mediator (TxA2), with a short physiological half life, in human coronary artery disease (CAD). Secondly, to determine if receptor protein is present in human coronary artery vascular smooth muscle for the recently adopted peptide orphan receptors for urotensin-II, apelin and ghrelin. The ETA receptor subtype predominated in the medial smooth muscle layer of both non-diseased coronary artery (NCA) and CAD. However, this subtype was present at relatively low density in the proliferated intimal layer of CAD. The ETB receptor protein was not altered with CAD, compared with NCA. Tx receptor density was significantly (P < 0.05) increased in both the media and intima of CAD, compared with NCA. There was no alteration in receptor density, on the medial smooth muscle for urotensin-II and apelin with CAD. Interestingly, receptor density for the novel vasodilator peptide ghrelin was significantly (P < 0.05) increased (approx. 4 fold) with CAD, compared with NCA. The alteration of receptor density with disease for Tx and ghrelin provides novel therapeutic targets for the treatment of atherosclerosis. In conclusion, while some GPCR are altered, others remain unchanged with human atherosclerosis. The increase in vasoconstrictor Tx receptor density with disease suggests the importance of Tx receptor antagonism. Intriguingly, the increase in receptor density for the novel vasodilator ghrelin, identified from post-genomic research, may potentially be beneficial with human atherosclerosis.

INTRODUCTION

Elevated plasma levels of the vasoconstrictors endothelin (ET) [1] and thromboxane (Tx) [2] in cardiovascular disease can lead to an increase in coronary vascular tone. The co-mitogenic properties associated with these two mediators can also lead to narrowing of the coronary vessels and thereby causing further reduction in blood flow to the heart. The understanding and therapeutic control of human coronary vascular smooth muscle...
growth is of considerable interest as it appears to be a major contributing factor for atherosclerosis.

Vascular smooth muscle cell proliferation is associated with structural modifications, a process known as phenotypic modulation [3]. The contractile cells exhibit great sensitivity to growth factors and start to proliferate with increased synthesis of extracellular matrix components. Non-diseased coronary arteries (NCA) have little or no intima but advanced thickening of the intimal layer, which has undergone phenotypic modification is apparent in atherosclerotic coronary arteries [4].

Although the symptoms of coronary artery disease (CAD) maybe partly ameliorated by current drug therapies (angiotensin-converting enzyme inhibitors, angiotensin-type I receptor antagonist, β-blockers), they are not cured, suggesting further receptor systems, including ET and Tx could be involved. Furthermore, approximately 100 novel receptors have been predicted to exist from the human genome [5]. However, it is not yet known how many proteins these genes encode, the physiological functions of these proteins and perhaps interesting their pathophysiological role. Some of these may encode ‘orphan’ G-protein-coupled receptors (oGPCR) which are of particular interest as GPCR are the target of more than 50% of the current drugs that are already available [6]. Following de-orphanization, we have studied a number of these receptors in human tissues and three peptide receptor systems of particular interest were urotensin II [7], apelin [8] and ghrelin [9]. The mRNA from molecular biological studies and/or functional studies in animal tissues predicted a cardiovascular localization/function for these three receptors in animal tissue, but the role in human tissue is unknown.

The GPCR are characterized by their ability to respond to endogenous agonist to modulate receptor number. Alteration in receptor number measured by ligand binding can inform us of potential changes in function of a transmitter system with disease. Therefore, the objective of this study was to understand how known GPCR (ET, peptide with long physiological half-life; Tx, lipid mediator with short half-life) and recently paired oGPCR (urotensin-II, apelin and ghrelin) are modulated by human atherosclerosis.

**METHODS**

With local ethical approval, human epicardial coronary arteries were obtained, from patients transplanted for ischaemic heart disease (16 men, 4 women; 45–69 years) or dilated cardiomyopathy (18 men, 7 women; 25–68 years), who have histologically normal coronary arteries [10]. Receptor autoradiography and saturation studies were carried out on 30-μm cryostat sections. For autoradiography studies, the concentrations of radioligands were selected to label approximately 33% of receptors. All radioligands were from Amersham Pharmacia Biotech, U.K. (2000 Ci/mmol), except [125I]-BOP (Tx receptor agonist; 2200 Ci/mmol; Cayman Chemicals, U.S.A.).

**In vitro receptor autoradiography for ET receptors**

Sections of human tissues were incubated in assay buffer containing 50 mM Hepes, pH 7.4, 5 mM MgCl₂ and 0.3% BSA for 120 min as described previously [11]. Total binding was determined by incubating sections with 0.1 nM [125I]-labelled ET-1. Non-specific binding was defined using 1 μM ET-1. The proportion of ET₅₆ and ET₆₈ receptors were defined using 1 μM BQ3020 (ET₅₆ selective agonist) and 1 μM FR139937 (ET₅₆ selective antagonist) respectively.

**In vitro receptor autoradiography for Tx receptors**

Following optimization of binding conditions, tissue sections were incubated in assay buffer containing 20 mM Hepes buffer containing 140 mM NaCl, 5 mM MgCl₂ and 5 mM KCl (pH 7.4). Total binding was defined with the Tx receptor agonist, 0.1 nM [125I]-BOP. Non-specific binding was determined with 1 μM SQ29548 (Tx receptor antagonist).

**In vitro receptor autoradiography for urotensin receptors**

Following optimization of binding conditions, sections were incubated for 1 h in 20 mM Tris/HCl buffer, pH 7.4, containing 5 mM MgCl₂ and 0.2% BSA with 0.25 nM [125I]-labelled urotensin-II (human) to define total binding. Non-specific binding was determined using human urotensin-II.

**In vitro receptor autoradiography for apelin receptors**

Once the optimal binding conditions were established, tissues were incubated in buffer containing 50 mM Hepes, 1 mM EDTA, 0.3% BSA, 100 mM NaCl, 5 mM MgCl₂ and 10 mM KCl (pH 7.4) for 30 min with 0.15 nM [125I]-labelled (Pyr⁴)apelin-13 (human) without 1 μM (Pyr⁴)apelin-13 (total binding) and with 1 μM (Pyr⁴) apelin-13 (non-specific binding). Dried sections were exposed to radiation sensitive film for 4 days. Developed images were quantified using computer-assisted densitometry [12].

**Saturation binding studies for the identification of ghrelin receptors**

Following optimization of binding conditions, tissue sections were incubated for 25 min in assay buffer...
comprising 50 mM Tris, 10 mM EDTA, 10 mM EGTA and 1 mM 4-(2-aminoethyl)benzenesulphonylfluoride, pH 7.2 with increasing concentrations (0.01–1.5 nM) of $[^{125}]$I-labelled His$^8$-ghrelin (human). Non-specific binding was defined with 1 μM ghrelin. Protein concentration was determined and receptor densities were compared using unpaired Student’s $t$ test with a significance value of $P < 0.05$.

RESULTS

Autoradiographical analysis of ET receptor subtypes in human NCA and CAD

High density of $[^{125}]$I-labelled ET-1 binding was demonstrated in the vascular smooth muscle of the media of both NCA and CAD ($75.26 \pm 5.36$ and $86.92 \pm 8.15$ amol/mm$^2$; $n = 8–12$ individuals; mean ± S.E.M.). In contrast to the media, the smooth muscle cells of the thin neointima of NCA and the proliferated intima of CAD had fewer ET-1 binding sites (Figure 1a). ET$_A$ receptors were predominantly localized to the media of both NCA and CAD, and these receptors were significantly down-regulated in the thickened intimal layer of CAD ($P < 0.001$). There was no significant difference in ET$_A$ receptor density in NCA compared with CAD in both the media as well as the intima. ET$_B$ receptors were of very low density (compared with ET$_A$ receptors) on the medial layer of both NCA and CAD and virtually absent on the proliferated intimal smooth muscle layer of CAD. Perivascular nerves represented a high density of ET$_B$ receptor-binding sites.

Autoradiographical analysis of Tx receptors in NCA and CAD

Tx receptors were identified on both the media and intimal (when detectable) smooth muscle layers of NCA and CAD (Figure 1b). Tx receptor density was significantly ($P < 0.05$) increased with CAD, on both the medial and intimal smooth muscle layers, compared with NCA ($n = 7–12$ individuals; mean ± S.E.M.). In CAD, significantly fewer ($P < 0.05$) Tx receptors were localized to the intimal layer, compared with the medial smooth muscle.

Figure 1 Receptor densities in normal and diseased human coronary arteries

Quantitative autoradiography results showing the density of (a) ET and (b) Tx binding to the media and intimal smooth muscle layers, (c) apelin and urotensin-II receptors in the medial layer and (d) saturation-binding studies showing the maximal ghrelin receptor density of human NCA (□) and atherosclerotic CAD (■). Results are from $n = 3–12$ individuals; data are mean ± S.E.M. *$P < 0.05$, Student’s unpaired $t$ test, compared with non-diseased.
Autoradiographical analysis of the recently paired peptide orphan GPCR for urotensin-II and apelin in human NCA and CAD

In human cardiovascular tissue, $^{125}$I-labelled urotensin-II binding was detected in vessels, including the medial smooth muscle layer of epicardial NCA and CAD (Figure 1c). There was no difference in the density of medial urotensin-II receptors between normal ($14.2 \pm 5$ amol/mg protein; $n = 4$ individuals; mean $\pm$ S.E.M.) and CAD ($16.8 \pm 4$ amol/mg protein; $n = 3$). Quantitative receptor autoradiography identified apelin receptors to be present in the vascular smooth muscle cells, at very low density, in human epicardial NCA and CAD (Figure 1c). There was no significant difference in the density of medial apelin receptors between NCA and CAD.

The novel vasodilatory peptide receptor, ghrelin, in human NCA and CAD

Radioligand binding and in vitro receptor autoradiography identified ghrelin receptors in the media and intimal smooth muscle layers of human epicardial NCA and CAD. The maximal density of ghrelin receptors was significantly ($P < 0.05$) increased (appro $4$ fold) in CAD ($28.8 \pm 6.9$ fmol/mg protein) with advanced intimal thickening, compared with NCA ($6.9 \pm 2.1$ fmol/mg protein; Figure 1d). However, there was no alteration in ligand affinity, comparing CAD ($K_r 0.44 \pm 0.07$ nM) with NCA ($K_r 0.22 \pm 0.08$ nM).

DISCUSSION

This study has described the density and distribution of ET (long physiological half-life, peptide ligand) receptors and compared this with Tx (short half-life, lipid mediator) in the different phenotypic states of human coronary artery. The identification of receptor protein in vascular smooth muscle cells for the recently paired orphan peptide receptors for urotensin-II, apelin and ghrelin, suggest as yet undetermined vasoactive properties for these peptides. Human urotensin-II and apelin receptor densities in the medial smooth muscle layer, were not altered with CAD. Interestingly, ghrelin receptor density (with no change in affinity) was significantly increased with CAD, compared with NCA. This may suggest a potentially beneficial role for this novel potent vasodilator peptide in human CAD.

We have shown that some GPCR systems are altered with atherosclerosis and others remain unchanged. The increase in vasoconstrictor Tx receptor density implicates the need for specific Tx receptor antagonism for the treatment of atherosclerosis. Intriguingly, the increase in receptor density for the novel vasodilator ghrelin, may potentially be beneficial with human atherosclerosis. By comparison of a number of different receptor systems, we have documented a post-genomic strategy to successfully identify novel receptors in human cardiovascular tissue and one of which has provided a novel therapeutic target for the treatment of atherosclerosis.

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REFERENCES

14 Hasegawa, K., Fujiiwara, H., Doyama, K. et al. (1994) Endothelin-1-selective receptor in the arterial intima of patients with hypertension. Hypertension 23, 288–293