# Anti-Inflammatory Activity of Ghrelin in Human Carotid Artery Cells

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*Abstract*—Receptors for eicosanoids such as prostaglandin $E_2$, prostacyclin and thromboxane $A_2$, as well as the ghrelin receptor polypeptide (GHS-R1b), can all regulate ghrelin (GHS-R1a) receptor activity, by the process of hetero-oligomerization, when heterologously expressed in human embryonic kidney (HEK 293) cells. To determine if such regulation might occur in inflammatory diseases of the vasculature, we incubated human coronary artery endothelial cells and human coronary artery smooth muscle cells with lipopolysaccharide and determined mRNA expression levels of these proteins using real-time PCR. Acute inflammation increased GHS-R1a mRNA in smooth muscle cells and increased cyclo-oxygenase-2 mRNA in endothelial cells; both these changes were attenuated by pretreatment of cells with ghrelin. Lipopolysaccharide did not affect expression of GHS-R1b or prostanoid receptor mRNA. Therefore, hetero-oligomerization of GHS-R1a with GHS-R1b or prostanoid receptors is unlikely to influence GHS-R1a activity in the vasculature; at least under conditions of acute vascular inflammation.

**KEY WORDS:** Human coronary artery; ghrelin; inflammation; prostanoid receptors.

## INTRODUCTION

There is increasing evidence to show that the appetite-regulating hormone ghrelin [1] additionally behaves as an endothelium-independent vasorelaxant peptide [2] and can display anti-inflammatory actions within the immune system [3]. Ghrelin receptor (GHS-R1a) expression is increased in atherosclerotic coronary artery [4], and ghrelin may thus have the therapeutic potential to correct endothelial dysfunction associated with atherosclerosis and metabolic syndrome in a GHS-R1a-dependent manner [5, 6].

GHS-R1a is a G protein-coupled receptor whose cell surface expression can be modulated by the dominant-negative activity of the truncated ghrelin receptor polypeptide (GHS-R1b) [7]. In addition, we have shown that cell surface expression of ghrelin receptors is also affected by co-transfection of human embryonic kidney (HEK 293) cells with the prostacyclin (IP) receptor, the prostaglandin $E_2$ ($PGE_2$) receptor ($EP_{3-I}$) subtype, and the thromboxane $A_2$ (TPα) receptor [8]. These prostanoid receptors are also vasoactive and can modulate inflammatory responses [9, 10]. Importantly, ghrelin receptors and these prostanoid receptors are all associated with atherosclerotic plaques [4, 11]. A lack of IP receptors or a decrease in prostacyclin production produces a prothrombotic state [12] allowing for a dominant role for TP receptors [13]. In addition to TP receptors, $EP_3$ receptors are also vasoconstrictors [14] and can stimulate platelet aggregation [15].

The ghrelin receptor shows a high degree of agonist-independent activity in cell-based assays [7]. Therefore, because GHS-R1b, $EP_{3-I}$, IP and/or TP receptors can decrease the cell surface expression of GHS-R1a [8], they might be expected to decrease both constitutive and agonist-dependent activity and thus

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influence the functional activity of GHS-R1a in atherosclerosis. Therefore, we aimed to look for conditions under which the expression of these receptors could be increased or decreased, and thus might be predicted to influence ghrelin receptor function through the formation of hetero-oligomers. Because of the lack of suitable antibodies capable of distinguishing ghrelin receptors from GHS-R1b, then ghrelin receptor expression cannot be reliably assessed using methods based on immunodetection. Therefore, for this initial investigation, we have determined the mRNA expression of these proteins of interest by using real-time polymerase chain reaction (PCR) methods on human vascular cells exposed to the inflammatory stimulus lipopolysaccharide (LPS). We chose human coronary artery endothelial cells (HCAECs) and human coronary artery smooth muscle cells (HCASMCs) because of the pathogenic consequences of atherosclerosis in this vascular bed. Although LPS is a commonly used inflammatory agent for studies on vascular smooth muscle cells, studies using endothelial cells tend to use interleukin-1$\beta$, tumour necrosis factor-$\alpha$ (TNF-$\alpha$) and/ or interferon-$\gamma$ (INF-$\gamma$). However, LPS and TNF-$\alpha$ produce similar effects on gene expression profiles in human endothelial cells [16], and both exert overlapping but non-identical effects on gene expression profiles in endothelial cells and vascular smooth muscle cells [17].

We show here that LPS increased GHS-R1a mRNA expression in HCASMCs and increased cyclo-oxygenase-2 (COX-2) mRNA expression in HCAECs; both these responses were attenuated by ghrelin. However, acute treatment of human carotid artery cells with LPS did not affect GHS-R1b, EP$_3$, IP or TP receptor mRNA expression. Therefore, hetero-oligomerization of GHS-R1a with GHS-R1b or these prostanoid receptors is unlikely to regulate GHS-R1a activity in the vasculature.

## MATERIALS AND METHODS

### Materials

Ghrelin was purchased from Phoenix Biotech (Beijing, China). DNase I (Amp Grade), oligo-d(T)$_{20}$ primer, dNTPs, Superscript III First-Strand Synthesis SuperMix, SYBR GreenER qPCR Supermix for iCycler, and all RT-PCR primers were from Invitrogen (Carlsbad, CA, USA). RNeasy Mini kit was purchased from Qiagen (Hong Kong). GelRed$^{TM}$ was purchased from Biotium (Hayward, CA, USA). All other compounds were supplied by Invitrogen (Carlsbad, CA, USA) or Sigma Chemical Co. (St. Louis, MO, USA).

### Cell Culture

Cryopreserved cells were purchased from Lonza-Walkersville, Inc. (Walkersville, MD, USA) and cultured according to the company's instructions. HCAECs were cultured in EGM (Endothelial Growth Medium) supplemented with EGM-MV BulletKit containing 1 µg/ml hEGF (human epidermal growth factor), 0.01 mg/ml hydrocortisone, 50 µg/ml gentamicin, 5% FBS (fetal bovine serum) and 0.012 mg/ml BBE (bovine brain extract). HCASMCs were cultured in SmGM-2 (Smooth Muscle cell Growth Medium-2) medium supplemented with SmGM-2 BulletKit containing 0.5 ng/ml hEGF, 5 µg/ml insulin, 2 ng/ml hFGF (human fibroblast growth factor), 50 µg/ml gentamicin and 5% FBS. Cells were cultured in 75 cm$^2$ culture flasks and maintained at 37°C in 5% $CO_2$ atmosphere. Medium was changed every 48 h, and the cells were subcultured at about 70–80% confluence. All experiments were performed at cell passage 4–6 for HCAECs and 5–7 for HCASMCs.

### Stimulation of Cells with LPS

Subconfluent HCAECs and HCASMCs were plated in six-well plates at a density of $10^5$ cells/well, in duplicate. After overnight incubation in reduced serum medium (0.5% FBS), ghrelin (100 nM) or medium was added 4 h before LPS (100 ng/ml, *E. coli* 055:B55) or medium, and cells were incubated for a further 4 h. The medium was then removed and cells were washed once with PBS before processing further.

### Detection of Target Proteins by Real-Time Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)

RNA was extracted from HCAECs and HACSMCs with RNeasy kit according to manufacturer's instructions. The extracted RNA was digested with DNase to remove any contaminating DNA, and Superscript III First-Strand Synthesis SuperMix was used to produce cDNA. All primers were designed using Primer 3 [18] (see Table 1) except for GHS-R1b which were according to Viani *et al*. [19]. The primers for the EP$_{3-I}$ receptor isoform will detect both EP$_{3-Ia}$ and EP$_{3-Ib}$ [20] while the primers for TP will detect both TP$\alpha$ and TP$\beta$ isoforms.

Real-time PCR was performed using SYBR GreenER qPCR supermix in IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules,

**Table 1.** Primer Sequences for mRNA Analysis by Real-Time PCR

| Gene | Primer sequence (5′ to 3′) | Amplicon length (bp) | GenBank accession number |
|------|---------------------------|---------------------|--------------------------|
| GHS-R1a | Forward: TCCTACTTCCGTTTGTGGTG | 154 | NM_198407 |
| | Reverse: GATGGAGTCACGACGGTAGT | | |
| GHS-R1b | Forward: TCGTGGGTGCCTCGCT | 66 | NM_004122 |
| | Reverse: GCTGAGACCCACCCAGCA | | |
| EP$_3$ | Forward: TTTTCCAACGGGGTCAATAC | 151 | NM_000957 |
| | Reverse: TAGAGTCAGGTCACGGGTCA | | |
| EP$_{3\text{-}I}$ | Forward: CGTAAGAAAGACGAAGAGGC | 159 | NM_000957 |
| | Reverse: GTTGATACGTAGGTCGAGGT | | |
| IP | Forward: TCGTCGCCTAGCACTCTC | 156 | NP_000951 |
| | Reverse: GAAGGTCGCTGAGTTCGAGA | | |
| TP | Forward: AGGTCCCATTACAAACACCC | 160 | NP_001051 |
| | Reverse: GAGGAGGAAGGAGTGGAAGG | | |
| COX-1 | Forward: CCTTGTCGTTCTTGGTCTCG | 153 | NM_000962 |
| | Reverse: GAACTGGCGATGGTCACACT | | |
| COX-2 | Forward: GTCGTTGGCATCTACGAGT | 158 | NM_000963 |
| | Reverse: CGTCAACAAGGTCTGTTCGT | | |
| Ghrelin | Forward: GACGAACTGGAGGTAGAAGG | 153 | NM_001134941 |
| | Reverse: GCCTCTTCCCAGAGGATGTC | | |
| GAPDH | Forward: CCAGTTACTTCCCCAGTAAC | 159 | NM_002046 |
| | Reverse: GCTCTAGGGAGGTTTTAGTT | | |

CA, USA). Each reaction was 20 μl containing 10 μl SYBR GreenER qPCR supermix, 0.8 μl cDNA, 2 μM forward and reverse primers. Conditions for real-time PCR were 50°C for 2 min, 95°C for 8 min 30 s then followed by 45 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. PCR products were resolved on an agarose gel (1.5%), containing GelRed$^{\text{TM}}$ and the DNA bands were visualized using a ChemiDoc XRS imaging system (Bio-Rad Laboratories, Hercules, CA, USA).

Analysis of real-time PCR data was performed using the $2^{-\Delta\Delta Ct}$ method [21]. This relative quantification method compares the PCR signal of the transcript in the treatment groups (LPS) with that of the control (no LPS) group. For all groups, PCR data has been normalised against an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), to account for variations in RNA added to the reaction mixture. To quantify mRNA expression levels, the cloned DNAs for GHS-R1a, GHS-R1b, EP$_{3\text{-}I}$, IP and TP genes were used in constructing standard curves, which were made by plotting the cycle threshold *versus* the log of known concentrations [22]. Details of these cDNAs have been reported previously [7, 8].

## Statistical Analysis

Values reported are means±SEM. Comparisons between groups were made using Students' *t* test or ANOVA with Dunnett's *post hoc* test, as appropriate. Statistical significance was taken as $P<0.05$.

## RESULTS

### Expression of Target mRNAs in HCAECs and HCASMCs

GHS-R1a and GHS-R1b mRNA were readily detectable in both HCAECs and HCASMCs (Fig. 1), with GHS-R1b mRNA expression being 5-fold higher in HCAECs compared to HCASMCs ($P<0.001$). In addition, the amount of GHS-R1b mRNA is significantly higher ($P<0.001$) than GHS-R1a mRNA in HCAECs. IP and TP mRNA were similarly expressed in both cell types, with slightly greater copy number of IP mRNA (Fig. 1). The EP$_{3\text{-}I}$ receptor isoform represented 9% of total EP$_3$ receptor expression in HCASMCs, but no EP$_3$ mRNA could be detected in HCAECs. In addition, any expression of ghrelin mRNA in either HCAECs or HCASMCs was below the limit of detection of our assay. In contrast, we could readily detect ghrelin mRNA in human erythroleukemia (HEL) cells, confirming reports by De Vriese *et al.* [23], (data not shown).

### Regulation of Target mRNA Expression in HCAECs and HCASMCs by LPS

Ghrelin inhibited LPS-stimulated cytokine production when added to macrophages 4 h before LPS [3]. Therefore, human coronary artery cells were
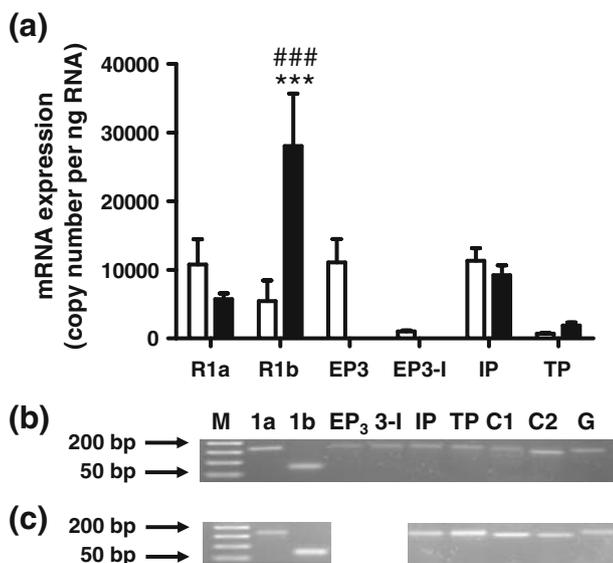
**Fig. 1.** Comparison of receptor mRNA expression in HCASMCs and HCAECs. RNA was extracted from unstimulated control HCASMCs (*open bars*) and HCAECs (*filled bars*) and mRNA expression determined using real-time PCR. **a** mRNA expression shown as means±SEM from three independent experiments, for GHS-R1a (*R1a*), GHS-R1b (*R1b*), all isoforms of EP3 (*EP3*), EP3-1 (*EP3-I*), IP and TP receptors. ***$P<0.001$ comparing GHS-R1b in HCAECs and HCASMCs (Students' *t* test). ###$P<0.001$ comparing GHS-R1a and GHSR1b in HCAECs (1-way ANOVA with Dunnett's post hoc test). PCR products from HCASMCs (**b**) and HCAECs (**c**) run on agarose gel showing base pair marker (*M*), GHS-R1a (*1a*), GHS-R1b (*1b*), EP3 (*EP3*), 3-I (*EP3-I*), IP, TP, COX-1 (*C1*), COX-2 (*C2*) and GAPDH (*G*).

incubated with and without ghrelin for 4 h before addition of 100 ng/ml LPS for a further 4 h. Incubation of control HCASMCs with LPS led to a significant 2.8±0.5-fold increase in GHS-R1a mRNA expression (Fig. 2a), which was inhibited by 45% in cells pretreated with ghrelin (100 nM). In contrast, LPS produced a mere 1.7±0.6-fold increase in GHS-R1a mRNA expression in HCAECs (Fig. 2b). In both cell types, LPS produced a slight, but not statistically significant, increase in GHS-R1b mRNA expression, but this was not affected by pretreatment of cells with ghrelin.

LPS had no effect on expression of EP3, IP or TP mRNA in HCASMCs (Fig. 2a) or on IP and TP mRNA in HCAECs (Fig. 2b). COX-2 mRNA expression was also determined to confirm an active inflammatory response to LPS. While there was no change in COX-1 or COX-2 mRNA expression in HCASMCs (Fig. 2a), LPS produced a statistically significant 2.1±0.3-fold increase in COX-2 mRNA

expression (Fig. 2b), which was inhibited by 61% in cells pretreated with ghrelin.

## DISCUSSION

The present study has identified that GHS-R1a mRNA is co-expressed with its truncated ghrelin receptor polypeptide (GHS-R1b) in both smooth muscle cells and endothelial cells of the human carotid artery, with significantly more GHS-R1b mRNA in endothelial cells than in smooth muscle cells. Furthermore, HCAECs express significantly more GHS-R1b than GHS-R1a mRNA. Given that GHS-R1a and GHS-R1b are capable of forming hetero-oligomers when expressed in HEK 293 cells [7], these results indicate that the dominant-negative role of GHS-R1b may have some functional consequence. For example, if the expression of GHS-R1b mRNA was down-regulated in HCAECs, with a corresponding decrease in GHS-R1b protein expression, then one might predict increased cell surface expression of GHS-R1a. In an attempt to discover the potential for such an outcome, we first need to find a situation in which the relative expression levels of GHS-R1a and GHS-R1b are changed. We show herein that an acute inflammatory stimulus, i.e. 4 h incubation of cells with LPS, significantly increased GHS-R1a mRNA expression in HCASMCs but not in HCAECs. In the absence of any marked change in GHS-R1b mRNA, we would not predict any role for this dominant-negative ghrelin receptor polypeptide in conditions of acute inflammation in HCASMCs. In addition, while the relative excess of GHS-R1b mRNA may negatively regulate GHS-R1a expression in HCAECs by the process of hetero-dimerization, LPS had no significant effect on GHS-R1a or GHS-R1b mRNA expression. Therefore, treatment of these endothelial cells with LPS is unlikely to alter this relationship.

LPS has also been shown to increase GHS-R1a at the mRNA and protein level in rat aortic smooth muscle cells, where it is thought to result in the increased vascular reactivity to ghrelin during the hyperdynamic phase of sepsis [24]. It is interesting therefore that our pretreatment of HCASMCs with ghrelin attenuated the upregulation of GHS-R1a mRNA by LPS. In this regard, ghrelin would be considered as an anti-inflammatory agent, by regulating responses to LPS.

Human microvascular endothelial cells [25], but not rat smooth muscle cells [24], are a possible source of anti-inflammatory ghrelin, but any expression of
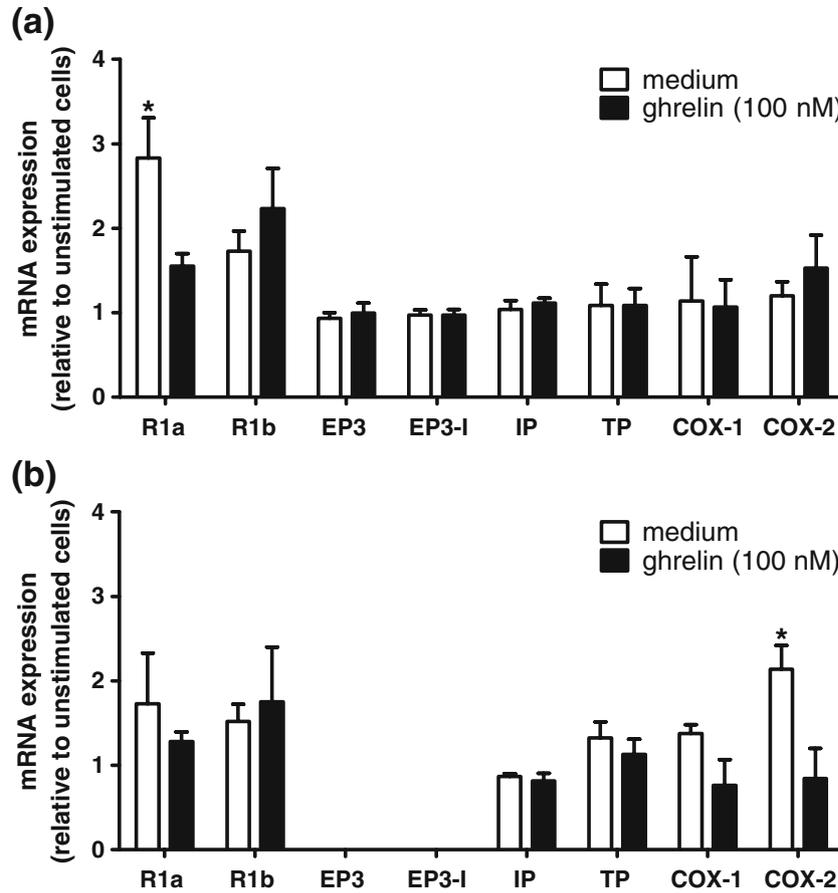
**Fig. 2.** LPS decreases GHS-R1a mRNA expression in HCASMCs and increases COX-2 mRNA expression in HCAECs. RNA was extracted from **a** HCASMCs and **b** HCAECs following 4 h treatment with LPS (100 ng/ml; *open bars*) and mRNA expression relative to the unstimulated control group (no LPS) determined using real-time PCR. Ghrelin (100 nM) was added 4 h before LPS (*filled bars*). Data shown are means±SEM from three independent experiments. *$P<0.05$ compared to control group for each mRNA assayed (one-way ANOVA with Dunnett's *post hoc* test).

ghrelin mRNA in either HCAECs or HCASMCs was below the limit of sensitivity of our assay. Under conditions of systemic or localised inflammation, one would expect accumulation of inflammatory cells; macrophages, neutrophils and T cells are therefore alternative potential sources of ghrelin. It is also probable that the inflammatory cells targeted to athero-sclerotic plaques are the source of any cardioprotective ghrelin [26], and this conclusion is worthy of further investigation.

The ability of ghrelin to restrain vascular smooth muscle cell GHS-R1a expression during vascular inflam-mation is intriguing. Homologous regulation of GHS-R mRNA has been reported in isolated cells; ghrelin can decrease GHS-R1a mRNA expression in human adre-nocortical carcinoma cell lines [27] and in porcine isolated somatotrophes [28], yet up-regulates GHSR gene transcription in black seabream *Acanthopagrus schlegeli* [29]. Moreover, there are biphasic and tissue-specific changes in GHSR mRNA levels in response to GHSs in isolated tissues [30]. Therefore, it remains to be determined if the anti-inflammatory action of ghrelin in coronary artery cells requires GHS-R1a mRNA to be up-regulated or down-regulated. In transfected cell systems, ghrelin receptors are internalized constitutively [31] and ghrelin produces up to 75% loss of cell surface receptors within 20 min [32]. However, the role of constitutive internalization and homologous regulation

of cell surface receptor expression and function in primary cultures of cardiovascular cells is unknown.

Ghrelin is a directly-acting vasodilator peptide [2] yet stimulates proliferation of human aortic endothelial cells [33]. And, in human aortic smooth muscle cells, ghrelin inhibits angiotensin II-induced proliferation and contraction and has been proposed to be a protective factor against vascular damage [34]. Whether or not these actions of ghrelin are all mediated by GHS-R1a receptors remains problematic since there are at least three functional receptors for ghrelin reported in the cardiovascular system: GHS-R1a, GHS-Ru (unknown) and the glycoprotein type B scavenger receptor CD36 [35]. In addition, the ghrelin receptor antagonist D-Lys (3)-GHRP-6 has a $pA_2$ value of 5.78 in human aortic smooth muscle cells [34] and a $pA_2$ value of 7.91 in human aortic endothelial cells [33]. Such a discrepancy in $pA_2$ values suggests that ghrelin may be acting on different receptors in endothelial and vascular smooth muscle cells.

We have shown previously that cell surface expression of GHS-R1a is affected by co-transfection of HEK 293 cells with $EP_{3-I}$, IP or TP receptor cDNA [8]. As these prostanoid receptors are also vasoactive and can modulate inflammatory responses [9, 10], we used real-time PCR to determine their co-expression in vascular smooth muscle and endothelial cells from human carotid artery. The present study has identified that GHS-R1a is expressed along with $EP_3$, $EP_{3-I}$, IP and TP receptors in HCASMCs, but that $EP_3$ receptors are absent in HCAECs. This latter observation confirms previous suggestions that $EP_3$ receptors, identified by immunohistochemical analysis, are present in vascular smooth muscle cells but absent from endothelial cells [20]. Of the eight $EP_3$ receptor isoforms, $EP_{3-I}$ is a relatively commonly expressed isoform, but in HCASMCs it represented merely nine percent of total $EP_3$ mRNA. Our studies also demonstrated that IP and TP mRNA expression was similar in both cell types, but that IP mRNA exceeded TP mRNA. However, in human coronary artery cells, we found no evidence for altered expression of $EP_3$, $EP_{3-I}$, IP or TP mRNA after acute exposure to LPS. Therefore, the functional activity of GHS-R1a is unlikely to be regulated by hetero-oligomerization with these prostanoid receptors during conditions of acute inflammation. Nevertheless, we cannot exclude the possibility of GHS-R1a regulation during the later phases of inflammation. We found that LPS significantly increased COX-2 mRNA expression in HCAECs after 4 h, and that ghrelin prevented this

effect. Thus, we have additional evidence for an anti-inflammatory effect of ghrelin in endothelial cells. As LPS would be expected to increase the production of both $PGE_2$ and prostacyclin by HCAECs after 12 h [36], then this increased prostanoid production might regulate EP and IP receptor function at a later time point.

Clearly, our conclusions are predicated on there being a direct relationship between the expression of mRNA and protein in our two cell types, and this presents a potential shortcoming to this study. Primary cultures of vascular smooth muscle and endothelial cells undergo a phenotypic change with time, therefore need to be used over a short period of time. With so many proteins of interest in this project, we chose the most sensitive assay of mRNA expression for our initial study. Our novel finding that acute inflammation increased GHS-R1a mRNA in HCASMCs should be confirmed by larger scale studies looking at LPS-stimulated GHS-R1a protein expression over time, however we are currently limited by tools of sufficient specificity to pursue this goal. As we have shown herein, both HCAECs and HCASMCs express GHS-R1a and GHS-R1b mRNA. However, in our hands, none of the commercially available antibodies readily detected GHS-R1a in HCAECs or HCASMCs and none could distinguish between GHS-R1a or GHS-R1b when transiently expressed in HEK 293 cells (data not shown). Radio-labelled ghrelin is currently not an option because although it has been used to detect GHS-R1a in human atherosclerotic arteries [4] and would give selectivity due to its failure to detect GHS-R1b [7], radiolabelled ghrelin will bind to at least two other binding proteins in the cardiovascular system [29].

In conclusion, we show here that human coronary artery smooth muscle cells express GHS-R1a, GHS-R1b, $EP_3$, $EP_{3-I}$, IP and TP mRNA while human coronary artery endothelial cells lack $EP_3$ receptor mRNA. Thus, smooth muscle cell $EP_3$ receptors may facilitate the adverse effects mediated by thromboxane $A_2$ in atherosclerosis. Acute inflammation increased GHS-R1a mRNA in HCASMCs and increased COX-2 mRNA in HCAECs; both these changes were attenuated by pretreatment of cells with ghrelin. Hence ghrelin displays anti-inflammatory activity in both vascular smooth muscle cells and endothelial cells of a vascular bed known to be susceptible to damage due to vascular inflammation. Just how ghrelin coordinates these effects through the GHS-R1a receptor warrants further investigation. Acute treatment of human carotid artery cells with LPS did not however affect expression

of ghrelin receptor polypeptide (GHS-R1b) mRNA or prostanoid EP$_3$, IP or TP receptor mRNA. Therefore, hetero-oligomerization of GHS-R1a with GHS-R1b or these prostanoid receptors is unlikely to influence GHS-R1a activity in the vasculature; at least under conditions of acute vascular inflammation.

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