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Lipopolysaccharide induces H1 receptor expression and enhances histamine responsiveness in human coronary artery endothelial cells

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Summary

Histamine is a well-recognized modulator of vascular inflammation. We have shown that histamine, acting via H1 receptors (H1R), synergizes lipopolysaccharide (LPS)-induced production of prostaglandin I_2 (PGI_2), PGE_2 and interleukin-6 (IL-6) by endothelial cells. The synergy between histamine and LPS was partly attributed to histamine -induced expression of Toll-like receptor 4 (TLR4). In this study, we examined whether LPS stimulates the H1R expression in human coronary artery endothelial cells (HCAEC) with resultant enhancement of histamine responsiveness. Incubation of HCAEC with LPS (10–1000 ng/ml) resulted in two-fold to four-fold increases in H1R mRNA expression in a time-dependent and concentration-dependent fashion. In contrast, LPS treatment did not affect H2R mRNA expression. The LPS-induced H1R mRNA expression peaked by 4 hr after LPS treatment and remained elevated above the basal level for 20–24 hr. Flow cytometric and Western blot analyses revealed increased expression of H1R protein in LPS-treated cells. The specific binding of [^{3}H]pyrilamine to H1R in membrane proteins from LPS-treated HCAEC was threefold higher than the untreated cells. The LPS-induced H1R expression was mediated through TLR4 as gene silencing by TLR4-siRNA and treatment with a TLR4 antagonist inhibited the LPS effect. When HCAEC were pre-treated with LPS for 24 hr, washed and challenged with histamine, 17-, 10- and 15-fold increases in PGI_2, PGE_2 and IL-6 production, respectively, were noted. Histamine-induced enhancement of the synthesis of PGI_2, PGE_2 and IL-6 by LPS-primed HCAEC was completely blocked by an H1R antagonist. The results demonstrate that LPS, through TLR4 activation, up-regulates the expression and function of H1R and amplifies histamine-induced inflammatory responses in HCAEC.

Keywords: endothelial cells; endotoxin/lipopolysaccharide; H1 receptor; inflammation; innate immunity

Introduction

Histamine is a major constituent of mast cells, and is well-recognized for its involvement in allergic and hypersensitivity reactions.[1] The *de novo* synthesis of histamine from histidine is catalysed by the enzyme histidine decarboxylase. Histamine plays a key role in the regulation of vasodilatation and bronchoconstriction [2] and in the physiological functions and inflammatory responses in a variety of cell types.[3,4] Recent reports have documented the presence of increased numbers of mast cells in the atherosclerotic lesions and in the coronary arteries of patients

Abbreviations: COX, cyclo-oxygenase; EGM-2 MV, endothelial cell growth medium; EIA, enzyme immunoassay; H1R, histamine H1 receptor; H2R, histamine H2 receptor; HCAEC, human coronary artery endothelial cells; PGE_2, prostaglandin E_2; PGI_2, prostaglandin I_2; siRNA, small interfering RNA.

with ischaemic heart disease.[5] Furthermore, the elevated levels of histamine noted in the coronary circulation of patients with variant angina support the possible involvement of this vasoactive amine in cardiovascular disease.[6] The ability of histamine to stimulate the synthesis of many inflammatory cytokines and prostaglandins by endothelial cells[7–9] suggests that it can elicit inflammatory responses in the vessel wall.

The regulation of cellular responses by histamine is initiated through four different subtype receptors (H1, H2, H3 and H4) belonging to the G-protein-coupled receptor family.[10] The H1 receptors (H1R) are expressed in endothelial cells and smooth muscle cells, and by signalling through these receptors histamine modulates inflammatory and hypersensitivity responses.[11,12] The H2R are involved in the stimulation of gastric acid secretion in the gut and in the modulation of the synthesis of cytokines in a variety of cell types.[13,14] The H3R are generally located in the brain, where they act as pre-synaptic autoreceptors in histamine-containing neurons,[15,16] and H4R are highly expressed in the bone marrow and in leucocytes, and are moderately expressed in spleen, thymus, lung, small intestine, colon and heart tissues.[10,17,18]

A role for infection in the progression of atherosclerosis has been proposed, and both bacterial and viral pathogens are widely recognized as inflammatory stimulants.[19–21] Of particular interest is the role of microbial pathogens such as *Chlamydia pneumoniae*, *Helicobacter pylori* and cytomegalovirus. Lipopolysaccharide (LPS) is a major constituent of the Gram-negative bacterial cell wall and is a strong inducer of inflammatory responses in endothelial cells via Toll-like receptor 4 (TLR4) activation.[22] Such responses to microbial pathogens via pattern recognition receptors on endothelial cells are vital for the host defence mechanisms but their persistent and amplified activation may lead to chronic vascular inflammation. Previous reports from our laboratory have demonstrated that histamine and LPS act in synergy to enhance the production of interleukin-6 (IL-6), IL-8, prostaglandin E_2 (PGE_2) and PGI_2 by endothelial cells.[9,23,24] This synergy between histamine and LPS was partially attributed to histamine-mediated increase in the expression of functionally active TLR4.[23] It is also possible that LPS-induced expression of H1R may contribute to the synergy between histamine and LPS. The objective of the present study was to determine whether LPS stimulates the expression of H1R in human coronary artery endothelial cells (HCAEC) and enhances histamine responsiveness in these cells. The data presented in this report demonstrate, for the first time, that LPS is capable of stimulating the expression of functionally active H1R in HCAEC, as shown by the increased mRNA and protein expression, enhanced specific binding of H1R ligand, [^{3}H]pyrilamine, to membrane proteins, and amplified H1R-mediated production of PGI_2, PGE_2 and IL-6 by LPS-primed HCAEC.

Materials and methods

Materials

The HCAEC, endothelial cell growth medium (EGM-2 MV), trypsin–versene mixture, and trypsin-neutralizing solution were purchased from Lonza (Walkersville, MD). Enzyme immunoassay (EIA) kits for 6-keto $PGF_1\alpha$, PGE_2 were purchased from Cayman Chemicals (Ann Arbor, MI). Histamine dihydrochloride, cetirizine hydrochloride, promethazine and *Escherichia coli* (0111 : B4) LPS, non-enzymatic cell dissociation buffer were purchased from Sigma-Aldrich (St Louis, MO). The TLR4 antagonist, TAK-242, was purchased from InvivoGen (SanDiego, CA) and H1R (A-20), β-actin, donkey anti-goat IgG horseradish peroxidase (HRP), goat anti-mouse IgG HRP antibodies, Western blot luminol reagent and small interfering RNA (siRNA) for TLR2 and TLR4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Radioimmunoprecipitation assay buffer (RIPA buffer) and protease inhibitor cocktail were products of Thermo Scientific (Rockford, IL). Recombinant human tumour necrosis factor-α (TNF-α; $2{\cdot}0 \times 10^5$ U/μg) was purchased from R&D Systems (Minneapolis, MN). RNeasy minikit was purchased from Qiagen (Gaithersburg, MD). High-Capacity cDNA Reverse Transcription kit, SYBR Green PCR Master Mix kit, and all the gene-specific primers were purchased from Applied Biosystems (Foster City, CA). Transfection reagent siPort Amine, and negative control siRNA were obtained from Ambion and [^{3}H]pyrilamine was purchased from Perkin-Elmer Life Sciences (Boston, MA).

Culture of HCAEC

The HCAEC were grown in EGM-2MV containing 1 μg/ml hydrocortisone acetate, 50 ng/ml gentamycin, 50 μg/ml amphotericin B and the recommended concentrations of human epidermal growth factor, vascular endothelial growth factor, human fibroblast growth factor-β, recombinant insulin-like growth factor-1, ascorbic acid and 5% fetal bovine serm (FBS), as described previously.[9,23] At confluence, the cells were detached from the culture flasks using trypsin–versene mixture, washed twice, and resuspended in fresh EGM-2MV. The cells used in all experiments were between three and six passages.

Real-time quantitative RT-PCR

After incubating HCAEC monolayers with medium or the stimulating agents, supernatant was removed from the cell culture dish and total RNA was isolated using an RNeasy mini kit, according to the manufacturer's protocol. Total RNA was reverse-transcribed into first-strand cDNA using a High-Capacity cDNA Reverse Transcription kit following the manufacturer's procedure. The primers used for

SYBR Green real-time PCR were designed using the PRIMER EXPRESS software v3.0 (Applied Biosystems). The sequences of primers used in the real-time PCR analyses of various genes are as follows; H1R forward primer 5′-GCCGTCGTCATGCCTATGA-3′ and reverse primer 5′-GAGAGGACGGCCCAGTGA-3′; H2R forward primer 5′-CCATCCTGCATGACACCAAA-3′ and reverse primer 5′-TCTCCGCTTCCCAGGTTTT-3′; and β-actin forward primer 5′-CCAGCTCACCATGGATGATG-3′ and reverse primer 5′-ATGCCGGAGCCGTTGTC-3′.

Real-time PCR was performed using the ABI 7500 Real-Time PCR system (Applied Biosystems) equipped with a 96-well optical reaction plate. The amplification reactions were performed in 25 µl total volume containing SYBR Green PCR Master Mix with respective primers and 5 µl cDNA of each sample. All real-time experiments were run in triplicate and a mean value was used for the determination of mRNA levels. The mRNA levels for H1R and H2R from each treatment were normalized to the corresponding amount of β-actin mRNA levels. Negative controls, containing water instead of sample cDNA, were used in each real-time plate.

Flow cytometry

Mouse anti-human H1R monoclonal antibody, goat $F(ab')_2$ anti-mouse IgG–allophycocyanin and mouse IgG1 as isotype control for flow cytometry were purchased from R&D Systems. Both surface and intracellular expression of H1R was determined in HCAEC upon incubation with LPS at different time points. Briefly, the 90% confluent monolayers of HCAEC were treated with LPS (100 ng/ml) at 12 and 24 hr and at a further increased concentration of LPS (1000 ng/ml) for 24 hr. The cells were collected using non-enzymatic cell dissociation buffer and washed twice with staining buffer (PBS/0·1% sodium azide/1% FBS). Cells were resuspended in staining buffer, counted and divided so that approximately $2{\cdot}5 \times 10^5$ cells from each treatment could be stained for surface and intracellular expression of H1R protein separately. For surface staining, the cells were incubated with anti-human histamine H1R monoclonal antibody in the dark at room temperature for 30 min. After incubation, the cells were washed with staining buffer twice and incubated with goat $F(ab')_2$ anti-mouse IgG (H + L) conjugated with allophycocyanin for 30 min in the dark at 4°. The cells were then washed three times with staining buffer and fixed with 1% paraformaldehyde. For total staining, the cells, after washing with staining buffer, were incubated for 10 min with permeabilization buffer [50 mM PBS, 1% (vol./vol.) FBS, 0·1% (weight/vol.) and sodium azide, 0·1% (weight/vol.) saponin]. After incubation, the cells were washed and resuspended in 50 µl permeabilization buffer and further incubated with primary antibody as in surface staining followed by incubation with allophycocyanin-conjugated $F(ab')_2$ as secondary antibody. Mouse IgG was used as the isotype control. Analysis was conducted by counting 1×10^4 cells with a BD™ LSR II using BD FACSDIVA™ software v6 (BD Biosciences, San Jose, CA).

Western blot analysis

Confluent HCAEC monolayers were incubated with LPS (100 ng/ml) for 2–24 hr at 37°. The cells were washed with ice-cold PBS twice and then lysed with RIPA buffer containing 25 mM Tris–HCl (pH 7·6), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0·1% SDS in the presence of 1% (vol./vol.) protease inhibitor cocktail at 4°. Cell debris was removed by centrifugation of lysate at 14 000 ***g*** for 15 min at 4°. Aliquots of the supernatants normalized for protein concentrations were mixed with equal volumes of 2 × SDS sample buffer and boiled at 100° for 5 min. Samples were resolved on 10% SDS–PAGE and transferred onto a PVDF membrane. After blocking with 5% non-fat dry milk in buffer containing 20 mM Tris–HCl, 150 mM NaCl and 0·1% Tween-20 (TBST) for 90 min, membranes were washed four times in TBST and incubated with primary antibody for H1R (1 : 200), overnight at 4° in blocking buffer. After washing four times, membranes were incubated with HRP-conjugated secondary antibody (1 : 2000) for 90 min, and washed four times and the signals were detected using Western blot luminol reagent. Membranes were stripped off H1R antibodies with Western blot stripping buffer and stained for β-actin as described for normalization.

Radioligand binding assay

The HCAEC (2×10^6) were cultured in T75 flasks and treated with medium or medium plus LPS (100 ng/ml) for 24 hr and assayed for H1R binding according to the methods of Strakhova *et al.*[25] Cells were harvested non-enzymatically and suspended in ice-cold 50 mM Na^+/K^+ assay buffer (37·8 mM $Na_2HPO_4.7H_2O$, 12·2 mM KH_2PO_4, pH 7·4). Cells were then homogenized using a PRO homogenizer (setting 4 for 10 seconds). The crude homogenate was centrifuged twice (10 min at 48 000 × ***g*** at 4°), and the pellet was resuspended each time in 20 volumes of Na^+/K^+ buffer. The final pellet was resuspended in Na^+/K^+ buffer to yield a final concentration of 100 µg protein/tube. Binding assays were performed in polystyrene tubes in a final volume of 0·5 ml. Membranes were incubated with a near-saturating concentration (approximately 18 nM) of [^{3}H]pyrilamine ([pyrindyl-5-^{3}H]-(mepyramine), specific activity 25·8 Ci/mmol) in the absence or presence of 10 µM H1R antagonist promethazine for 30 min at 23°. The reaction was terminated by rapid filtration using a Brandel cell harvester and

Whatman GF/B filters pre-treated with 0·5% polyethyleneimine. Filters were subsequently washed three times with 3 ml ice-cold buffer (50 mM Tris–HCl; pH 7·4 at 23°), and placed in scintillation vials. After the addition of scintillation cocktail, vials were shaken, equilibrated for 6 hr, and radioactivity was determined by a scintillation counter. Protein concentration was determined using the BCA protein assay reagent (Pierce, Rockford, IL) with BSA used as the standard. Specific [^{3}H]pyrilamine binding was calculated by subtracting total binding from non-specific binding.

Transfection and siRNA

Gene-specific siRNA or negative control siRNA was introduced into HCAEC by using the siPORT Amine transfection reagent according to the manufacturer's instructions. Briefly, 100 µl of the transfection reagent mixture containing 1·5 µl siPORT Amine reagent, 100 nM siRNA, and OPTI-MEM I was added to each of the wells of a 12-well cell culture plate. Then, 900 µl HCAEC cell suspension containing $0{\cdot}8 \times 10^5$ cells was then added to the transfection mixture and incubated at 37° in 5% CO_2 for 24 hr. After the incubation, medium containing the transfection reagent was discarded, and the cells were maintained in fresh medium for another 24 hr. Then cells were subsequently treated with LPS (100 ng/ml) for 4 hr. Total RNA was extracted from the HCAEC monolayer for real-time PCR analysis.

TLR4 blocking with TAK-242

TAK-242 is a selective inhibitor of the signals initiated by the intracellular domain of TLR4 and 1 µM concentration of this compound has been shown to suppress the effect of LPS.[26] Therefore, TAK-242 was used to reconfirm the involvement of TLR4 in this study. The HCAEC monolayer was pre-treated with 1 µM TAK-242 for 1 hr and stimulated with LPS (100 ng/ml) for 4 hr. After the incubation, total RNA was isolated, cDNA was synthesized and H1R gene expression was analysed by real-time PCR.

EIA determinations of the production of PGI_2 and PGE_2

The HCAEC ($1{\cdot}0 \times 10^4$) were plated onto each of the wells of a 96-well cell culture plate and allowed to adhere for 16–18 hr in monolayer and treated with medium or three concentrations of LPS (10, 100 and 1000 ng/ml) and incubated at 37° in 5% humidified CO_2 for 24 hr. The supernatants were collected and the monolayer was washed with HBSS. The cells were then subsequently treated with histamine with or without H1R-specific antagonist, cetirizine hydrochloride, for another 24 hr. After 24 hr the supernatants were again collected and appropriately diluted, and assayed for 6-keto $PGF_1\alpha$ (for PGI_2) and PGE_2 levels by EIA.

IL-6 measurement

Interleukin-6 expression was measured using an ELISA kit (R&D Systems). The HCAEC ($1{\cdot}0 \times 10^4$) were plated onto the wells of a 96-well cell culture plate; the same experimental procedure was followed as in PGI_2 and PGE_2 determination, and the supernatants were collected for IL-6 measurement.

Statistical analysis

One-way analysis of variance (ANOVA) followed by Newman–Keuls test, one-way ANOVA followed by Bonferroni's test, two-way ANOVA followed by Bonferroni's test or Student's *t*-test was used as applicable as described in the respective figure legends. Data were expressed as mean ± SD and $P < 0{\cdot}05$ was considered significant. GRAPHPAD PRISM v.5.00 for Windows (GraphPad Software, San Diego, CA) was used to perform all the statistical analyses.

Results

LPS induces H1R but not H2R mRNA

To examine whether LPS regulates H1R and H2R gene expression in HCAEC, a kinetic study on its effect on mRNA expression was carried out using real-time quantitative RT-PCR. In our previous studies, an LPS concentration of 100 ng/ml was found to be sufficient to induce near-optimum induction of inflammatory responses in HCAEC.[24] However, in this study HCAEC were incubated with 100 and 1000 ng/ml LPS for different periods of time to determine their effect on the expression of H1R mRNA. As shown in Fig. 1(a), incubation of HCAEC with LPS (100 ng/ml) resulted in a time-dependent increase in H1R mRNA expression which peaked by 4 hr post-stimulation. The mRNA expression subsequently decreased but remained at significantly higher levels even after 8 and 20 hr. In contrast, the expression of H2R mRNA was not altered by LPS treatment at any of the time-points or doses of LPS tested. The kinetic profile of H1R gene expression was also studied using 1000 ng/ml LPS. The results depicted in Fig. 1(b) show a similar pattern of LPS-induced H1R expression even with increased concentration of LPS. Results presented in Fig. 1(c) demonstrate a concentration-dependent increase in LPS-induced H1R expression between 10 and 100 ng/ml at 4 hr treatment. Interestingly, TNF-α (200 U/ml) was found to stimulate the expression of both H1R and H2R mRNA in endothelial cells.

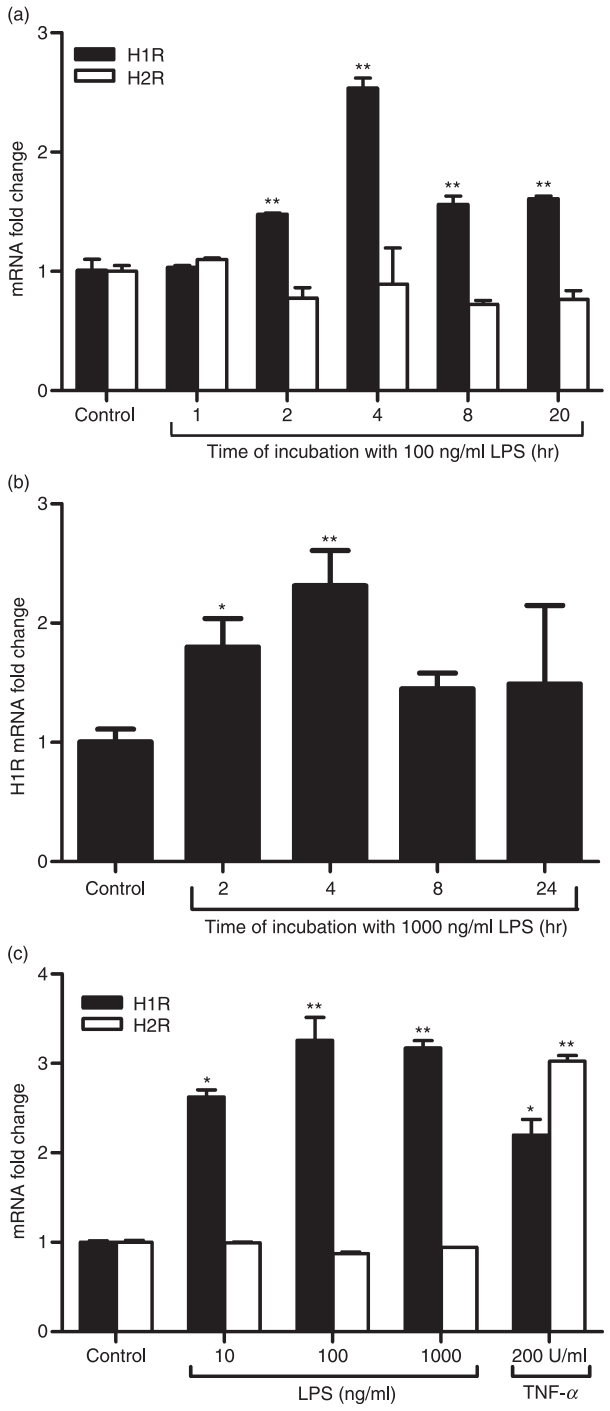

Figure 1. Lipopolysaccharide (LPS) induces histamine H1 receptor (H1R) mRNA, but not H2R mRNA, expression in human coronary artery endothelial cells (HCAEC). Monolayers of HCAEC were incubated with 100 ng/ml LPS (a) or 1000 ng/ml LPS (b) for the indicated times, or with different doses of LPS (10–1000 ng/ml) or tumour necrosis factor-α (TNF-α; 200 U/ml) for 4 hr (c). After the incubation, total RNA was extracted, reverse transcribed and analysed by real-time PCR using specific primers as described in the Materials and methods. The amplicons were normalized for β-actin and assigned a value of one for medium-treated cells. The magnitude of changes of mRNA expression in LPS or TNF-α-treated cells was calculated by comparing them with the message in untreated HCAEC. The results presented are representative of three to four experiments with similar results. Each value presented is the mean ± SD of triplicate determinations. Data analysis used a one-way ANOVA followed by Newman–Keuls multiple comparison test; $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}001$ when compared with untreated cells.

LPS induces H1R protein expression

To determine whether the newly expressed mRNA was translated into protein, we conducted flow cytometry analyses for the presence of cell surface or total H1R protein after incubating HCAEC with 100 ng/ml LPS for 12 and 24 hr. As shown in Fig. 2(a,b), incubation of HCAEC with 100 ng/ml LPS resulted in little or no change in the surface expression of H1R protein. On the other hand, when the cells were treated with 100 ng/ml LPS for 12 and 24 hr, a progressive increase in total expression of H1R protein was evident with significant increase noted at 24 hr (Fig. 2c,d). A similar increase in total H1R protein was noted when cells were treated with 1000 ng/ml LPS for 24 hr (Fig. 2e,f).

The LPS-induced expression of H1R protein was also confirmed by Western blot analysis using HCAEC treated with 100 ng/ml LPS for 2–24 hr. As shown in Fig. 2(g), an increase in H1R protein expression was evident as early as 2 hr after LPS treatment with peak expression noted at 8 hr.

LPS increases radioligand binding to H1R proteins

To evaluate the ability of the newly expressed H1R protein to bind to its ligand, the specific binding of [^{3}H] pyrilamine to membrane proteins isolated from HCAEC incubated with LPS for 24 hr was measured. The data presented in Fig. 3 demonstrate that specific binding of [^{3}H]pyrilamine to H1R was increased 3·4-fold after LPS treatment of HCAEC for 24 hr when compared with unstimulated controls. The competitive ligand-binding experiment was conducted only using a single concentration of [^{3}H]pyrilamine that would be near-saturating assuming a *K*d value of 1 nM, in the presence or absence of 10 μM promethazine. Because of the requirement for a large amount of membrane proteins, we were unable to perform a saturation study so we were unable to calculate the dissociation constant. Nevertheless, the present data demonstrate an increase in [^{3}H]pyrilamine binding in the LPS-treated cells. Although the data do not allow for the determination of whether that increase in binding results from an increase in the number of receptors, an increase in the affinity of the receptor, or both, the observation of increased H1R mRNA and protein, suggests that an increase in the number of receptors almost certainly contributes to the increase in binding.

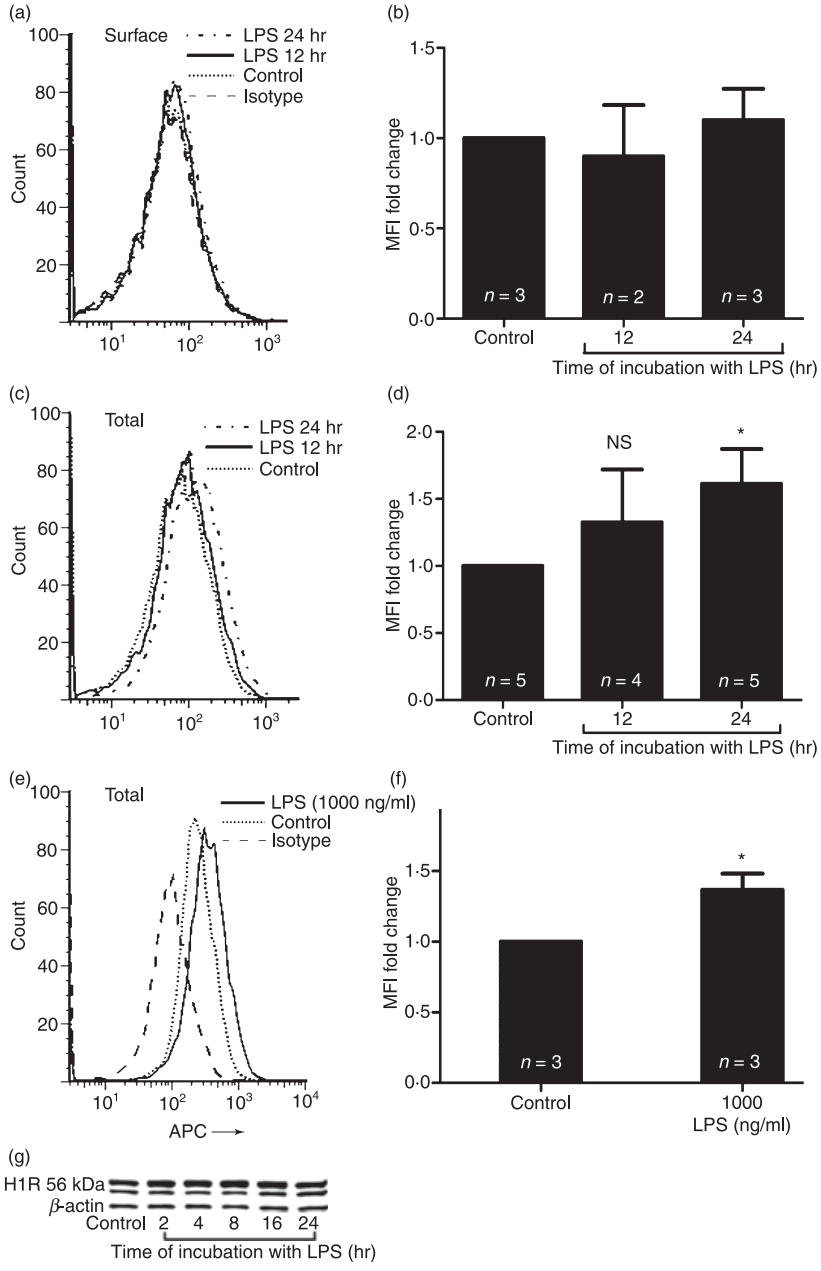

Figure 2. Lipopolysaccharide (LPS) stimulates the intracellular expression of histamine H1 receptor (H1R) in human coronary artery endothelial cells (HCAEC). (a–f) Monolayers of HCAEC were incubated with medium or LPS (100 ng/ml) for 12 or 24 hr and analysed for the expression of H1R on the surface, (a) or total (c) expression by flow cytometry as described in detail in the Materials and methods section. (b, d) Fold changes in mean fluorescence intensity (MFI) for the surface and total expression of H1R, respectively, for HCAEC treated with 100 ng/ml LPS compared with untreated cells. *n* indicates number of experiments. (e) HCAEC were treated with medium or LPS (1000 ng/ml) for 24 hr, and analysed for total H1R. (f) Fold changes in MFI for the total expression of H1R in treated with 1000 ng/ml LPS cells compared with untreated cells. One-way ANOVA followed by Bonferroni's multiple comparison test was used for data presented in (b) and (d) and Student's *t*-test was applied for data presented in (f). Data represented are mean ± SD; $^*P < 0{\cdot}05$. (g) Western blot analysis demonstrating the expression of H1R in HCAEC treated with 100 ng/ml LPS for 2–24 hr.

TLR4 siRNA but not TLR2 siRNA transfection blocks the H1R gene transcription

Toll-like receptors recognize pathogen-associated molecular patterns and activate the innate immune responses. It has been reported that LPS can be recognized by TLR2 as well as by TLR4.[27,28] To determine the involvement of these TLRs in the LPS-mediated H1R up-regulation, we transiently knocked down TLR2 or TLR4 using siRNA. The transient transfection of HCAEC with TLR2-specific and TLR4-specific siRNA resulted in decreased expression of TLR4 and TLR2 by 88% and 76%, respectively (Fig. 4a,b). The results presented in Fig. 4(c) show that TLR4 gene silencing by siRNA caused a 58% reduction in LPS-stimulated H1R mRNA expression as compared with mock-transfected control cells. In contrast, TLR2 gene knock-down did not inhibit LPS-mediated H1R mRNA expression (Fig. 4c). These results indicate that LPS-induced H1R mRNA expression was mediated through TLR4 activation.

TLR4 blocking with TAK-242 inhibits LPS-induced H1R expression

We also tested the effect of a pharmacological agent (TAK-242) that is known to block TLR4-mediated signalling.[26] Pre-incubation of HCAEC with TAK-242 1 hr before stimulation with LPS significantly reduced

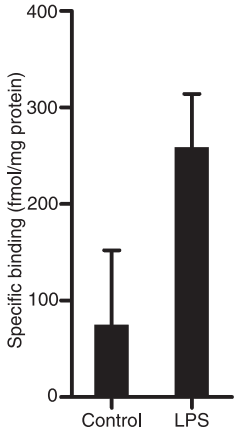

Figure 3. Lipopolysacchairde (LPS) -induced histamine H1 receptor (H1R) protein exhibits increased radioligand binding. Human coronary artery endothelial cell (HCAEC) monolayers were treated with LPS (100 ng/ml) or medium for 24 hr. After incubation the cells were harvested non-enzymatically and total membranes were isolated. Specific binding with H1R ligand, [^{3}H]pyrilamine, to the isolated membrane was measured. Data are the mean ± SD of triplicate determinations, and are representative of two independent assays performed with similar results.

LPS-mediated H1R mRNA expression (Fig. 4d), which further confirms that this effect is mediated by TLR4.

LPS pre-treatment increases the responsiveness of HCAEC to histamine

The LPS-activated HCAEC are potentially hyper-responsive to histamine because of the presence of increased H1R and may exhibit enhanced functional activity. To examine this possibility, HCAEC were pre-treated with LPS (10, 100 and 1000 ng/ml) for 24 hr, washed, and then challenged with histamine in the presence or absence of cetirizine, a specific H1R antagonist. The production of PGI_2, PGE_2 and IL-6 was determined as indices of cell activation. As shown in Fig. 5, HCAEC pre-treated with LPS and subsequently incubated with medium alone produced increasing amounts of prostanoids (Fig. 5a,b) and IL-6 (Fig. 5c), which was dependent on the concentrations of LPS used for pre-treatment. When LPS-primed cells were challenged with histamine, further enhancement in the production of prostanoids and IL-6 was noted when compared with LPS-primed cells incubated with medium alone. The histamine-induced enhancements in prostanoids and IL-6 production were completely blocked by the H1R antagonist cetirizine (Fig. 5a–c) indicating that LPS-mediated hyper-responsiveness of HCAEC was mediated by H1R. The addition of cetirizine to medium or LPS-treated HCAEC did not affect their functional activity. These results indicate that the newly formed H1R proteins induced by LPS were functionally active and transmit the signals from agonist.

Discussion

The results presented in this study demonstrate that LPS acting through TLR4 stimulates H1R gene and protein expression, suggesting cooperation between the innate immune mechanism and histamine signalling in HCAEC. The LPS/TLR4-induced H1Rs are functionally active, as shown by their increased binding of specific radioligand to the membrane proteins of LPS-primed HCAEC and the amplified production of prostanoids and IL-6 in response to histamine challenge. The fact that LPS did not induce the expression of H2R (Fig. 1a,c) or H4R (data not shown) and the H1R antagonist, cetirizine, completely abrogated histamine-induced production of PGI_2, PGE_2 and IL-6 (Fig. 5), demonstrate that LPS-induced amplification of histamine responsiveness is via activation of the newly expressed H1R. We have also confirmed that the priming effect of LPS on HCAEC is mediated through TLR4 because gene silencing by TLR4-siRNA, and blocking of TLR4 signalling by TAK-242 inhibited LPS-induced H1R expression. Our previous studies[9,23,24] to explore the mechanism of synergy between LPS and histamine showed that histamine is able to up-regulate the expression of TLR4 and enhances endothelial cell sensitivity to LPS.[23] The present findings demonstrating the ability of LPS to stimulate the expression of functionally active H1R suggests that a bi-directional regulation of TLR4 and H1R signalling contribute to the synergy between LPS and histamine in HCAEC. This concerted action of LPS and histamine on the endothelial cell inflammatory response underscores the clinical significance of infectious episodes and mast cell degranulation in allergy and vascular disease. Since both mast cells and infections have been implicated in cardiovascular disease, the up-regulation of H1R expression by LPS has important clinical significance.

Toll-like receptors are integral components of the innate immune system that recognize microbial pathogens and initiate the inflammatory response.[29] Among the TLRs expressed in mammalian cells, TLR4 mediates responses to LPS.[30] The LPS induces a powerful innate immune response in mammals, which can lead to septic shock, organ damage and death. Increasing evidence suggests that inflammatory responses mediated by TLR4 may contribute to atherosclerosis progression.[31–33] Although increased production of many cytokines, lipid mediators and other bioactive molecules are associated with vascular inflammation and development of atherosclerosis, the homeostasis of monocyte chemotactic protein 1, IL-6, IL-8, prostaglandins and thromboxane A_2 are considered to be important. In this regard, we have previously shown that synthesis of IL-6, IL-8, PGE_2 and PGI_2 by endothelial cells is synergistically enhanced by LPS and histamine.[9,23,24] This synergy between LPS and histamine was attributed, at least in part, to histamine-induced over-expression of functionally

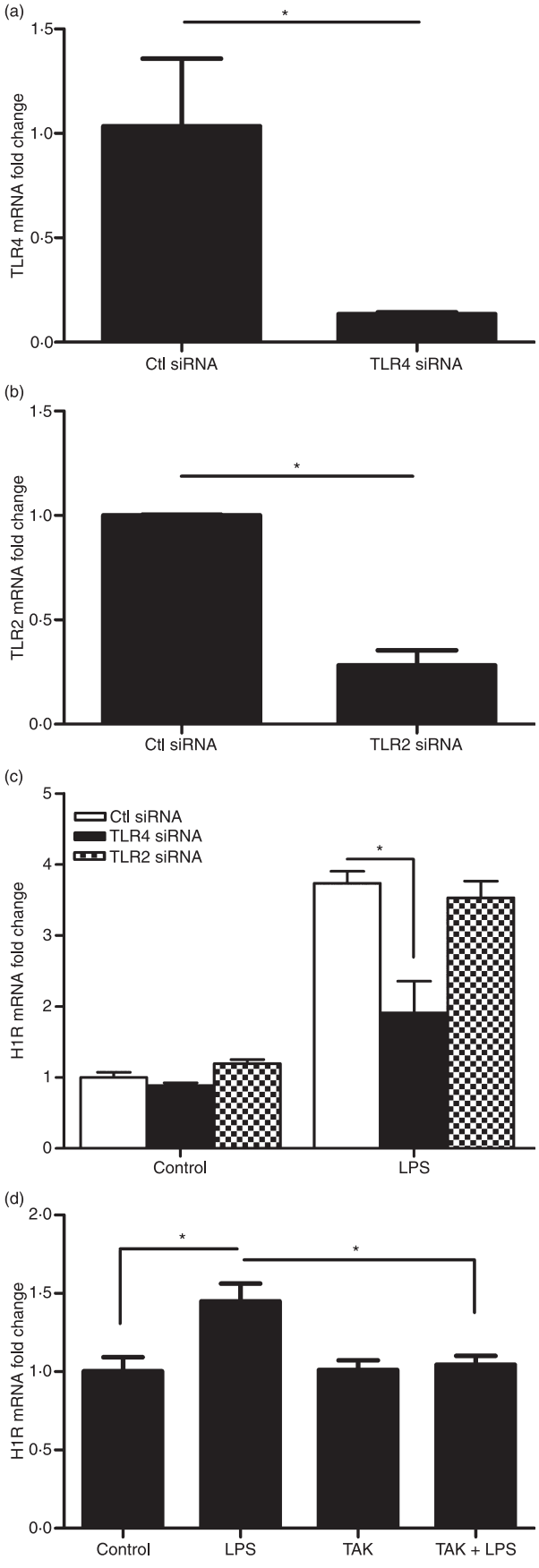

Figure 4. Lipopolysaccharide (LPS) -induced expression of histamine H1 receptor (H1R) in human coronary artery endothelial cells (HCAEC) is Toll-like receptor 4 (TLR4)-mediated. The HCAEC monolayers were transfected with a TLR4-specific or TLR2-specific small interfering RNA (siRNA) or negative control siRNA for 24 hr. The expression of TLR4 (a) or TLR2 (b) mRNA was determined to evaluate the transfection efficiency. The transiently transfected cells were treated with LPS (100 ng/ml) for 4 hr and H1R mRNA expression was determined by real-time PCR (c). Data presented in (a) and (b) were analysed by Student's *t*-test whereas those presented in (c) were analysed by two-way ANOVA followed by Bonferroni's multiple comparison test. The values presented are mean ± SD of triplicate determinations of a representative experiment out of three; $^*P < 0{\cdot}05$ (d) Inhibition of LPS-induced H1R expression by TLR4 inhibitor TAK-242. The HCAEC monolayer was pre-treated with 1 μM TAK-242 for 1 hr and stimulated with LPS (100 ng/ml) for 4 hr. After the incubation, total RNA was isolated, cDNA was synthesized and H1R gene expression was analysed by real-time PCR. Data were analysed by one-way ANOVA followed by Newman–Keuls multiple comparison test. The values presented here are the mean ± SD of triplicate determinations of a representative experiment out of three; $^*P < 0{\cdot}05$.

active TLR4 and resultant enhancement in nuclear factor-κB activation.[23] Therefore, the regulation of the expression of TLR4 by histamine was considered to be a critical link between the endothelial cell response to microbial pathogens and activation of inflammatory responses.[34,35] Here, we show that LPS up-regulates H1R expression at both the transcriptional and translational levels. This finding further enhances our understanding of the cooperative role of mast cell mediators and infectious agents in coronary artery diseases and atherosclerosis.

It is of interest that most of the newly expressed H1R are localized intracellularly and little or no increase in surface expression of H1R is noted as a result of LPS treatment. In spite of the low-level surface expression of H1R and no induction of H2R or H4R, LPS-primed HCAEC demonstrated markedly higher H1R-specific responsiveness to histamine. These results suggest that only limited numbers of functional H1R are required for the physiological functions and a stringent regulation may exist for the histamine-mediated inflammatory responses in the vessel wall. As most of the LPS-induced H1R protein was found to be intracellular, transient mobilization of the receptors to the cell surface is plausible. Alternately, LPS-priming may also up-regulate common or distinct signal transduction pathways, which act in concert to induce the synergy. The fact that the histamine effect on endothelial cells is mediated via H1R,[9,23,24] and that both H1R[36,37] and TLR4[31–33] are implicated in vascular inflammation and atherosclerosis, make it reasonable to postulate that such a bidirectional activation of inflammatory response would lead to persistent and sustaining vascular inflammation. Although TLR4 is considered to be the receptor for LPS, TLR2 activation has been implicated

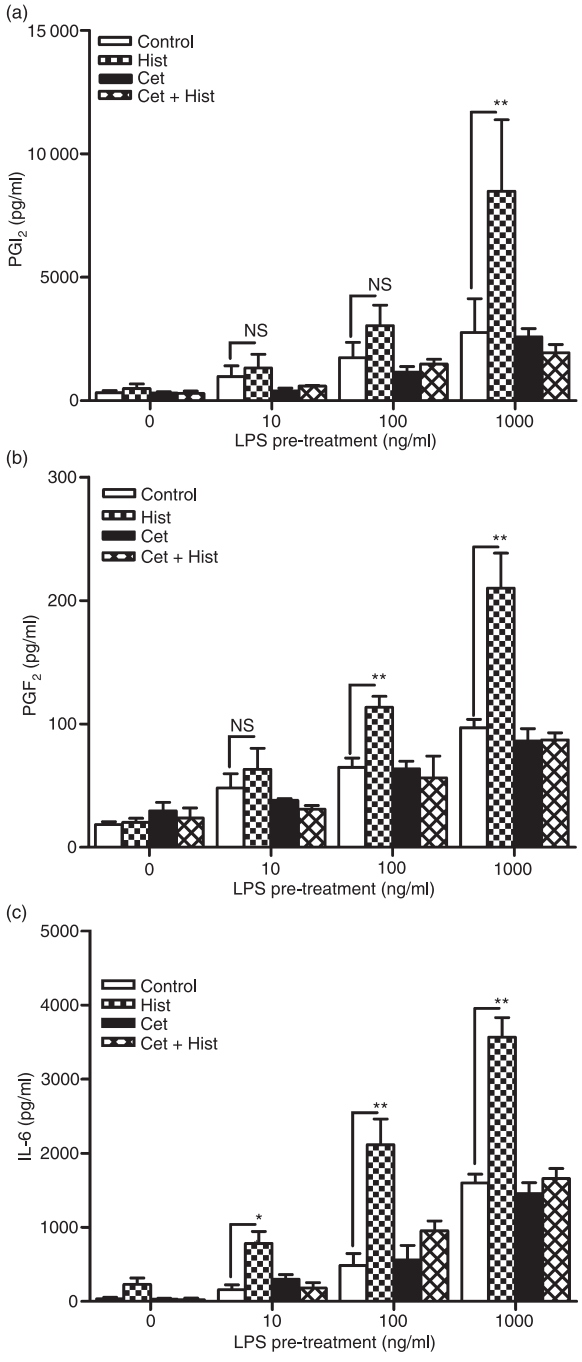

Figure 5. Lipopolysaccharide (LPS) pre-treatment enhances histamine H1 receptor (H1R)-mediated histamine responsiveness on the production of prostaglandin I_2 (PGI_2), PGE_2 and interleukin-6 (IL-6). Human coronary artery endothelial cell (HCAEC) monolayers were incubated for 24 hr with medium or 10, 100 or 1000 ng/ml LPS. The cells were then washed twice with Hanks' balanced salt solution, and subsequently incubated with medium of 10 μM histamine for 24 hr in the presence or absence of 10 μM cetirizine, a specific H1R antagonist. After the incubation, culture supernatants were collected and assayed for PGI_2, (a), PGE_2 (b), and IL-6 (c). Each value is the mean ± SD of quadruplicate determinations; $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}001$. ns, not significant.

under certain conditions.[28,29,38–41] Here, we present evidence that the LPS-induced H1R expression is mediated via TLR4 and not via TLR2, as gene silencing by TLR4-specific siRNA, and not TLR2 siRNA, abrogated LPS-mediated H1R expression. These findings also rule out the possibility of any contaminating TLR2 ligand in the LPS preparation. The importance of the mast cell and its secretory products in vascular inflammation is well recognized.[1,42,43] A direct clinical significance of mast cell-derived histamine in coronary artery disease is attributed to the elevated levels of histamine in the coronary circulation of patients with angina[6] as well as the increased synthesis of histamine[44,45] and high level expression of H1R[37] in atherosclerotic lesions. Increasing interest is now focused on the role of cyclo-oxygenase 2 (COX2) and prostanoid homeostasis in the pathogenesis of atherosclerosis.[46] Previous reports from our laboratory have documented the ability of mast cell proteases and histamine to interact with endothelial cells and enhance inflammatory responses as determined by the production of IL-6 and IL-8[47] and expression of TLR2 and TLR4.[48] We have also demonstrated that histamine, acting through H1R, induces the expression of COX2, and not COX1, and stimulates the production of PGI_2 and PGE_2 in HCAEC; this effect was greatly amplified by the presence of LPS.[24] In the present study, we demonstrated that when LPS-primed HCAEC were stimulated with histamine, marked increases in the production of PGE_2 and PGI_2 were noted. Although, the synergy between LPS and histamine on the expression of PGE_2 synthase and PGI_2 synthase was not determined in this study, based on our previous results[24], we speculate that over-expression of COX2 together with PGE_2 synthase and PGI_2 synthase might contribute to the amplified synthesis of PGE_2 and PGI_2 by LPS-primed HCAEC challenged with histamine. In regard to histamine-stimulated amplification of IL-6 production by LPS-primed HCAEC, we have reported markedly elevated levels of IL-6 mRNA expression in endothelial cells after treating with LPS and histamine.[23] It is also noteworthy that LPS is capable of up-regulating the expression of histidine decarboxylase, the enzyme responsible for the *de novo* synthesis of histamine[49–52], as well as enhancing antigen-induced release of histamine from basophils.[53] Hence, the ability of LPS not only to induce the expression of H1R but also to stimulate the synthesis and release of histamine further emphasizes the importance of infectious agents in amplifying vascular inflammation.

In conclusion, the present study demonstrates that incubation of HCAEC with LPS leads to up-regulation of H1R gene and protein expression. This is associated with an increase in H1R ligand binding and amplified responsiveness to histamine as documented by generation of PGI_2, PGE_2 and IL-6. The LPS-induced hyper-responsiveness to histamine challenge was found to be mediated via H1R as

the amplified functional response was abrogated by the H1R antagonist, cetirizine. Although HCAEC express both TLR2 and TLR4, the LPS-induced H1R expression is mediated through TLR4. These results underscore the importance of infection-related increases in susceptibility of the vascular tissues against inflammatory mediators like histamine that may lead to atherogenesis.

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Disclosures

The authors have no financial conflict of interest.

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