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Histamine induces Toll-like receptor 2 and 4 expression in endothelial cells and enhances sensitivity to Gram-positive and Gram-negative bacterial cell wall components

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SUMMARY

Histamine is a major inflammatory molecule released from the mast cell, and is known to activate endothelial cells. However, its ability to modulate endothelial responses to bacterial products has not been evaluated. In this study we determined the ability of histamine to modulate inflammatory responses of endothelial cells to Gram-negative and Gram-positive bacterial cell wall components and assessed the role of Toll-like receptors (TLR) 2 and 4 in the co-operation between histamine and bacterial pathogens. Human umbilical vein endothelial cells (HUVEC) were incubated with lipopolysaccharide (LPS), lipoteichoic acid (LTA), or peptidoglycan (PGN) in the presence or absence of histamine, and the expression and release of interleukin-6 (IL-6), and NF-κB translocation were determined. The effect of histamine on the expression of mRNA and proteins for TLR2 and TLR4 was also evaluated. Incubation of HUVEC with LPS, LTA and PGN resulted in marked enhancement of IL-6 mRNA expression and IL-6 secretion. Histamine alone markedly enhanced IL-6 mRNA expression in HUVEC, but it did not stimulate proportional IL-6 release. When HUVEC were incubated with LPS, LTA, or PGN in the presence of histamine marked amplification of both IL-6 production and mRNA expression was noted. HUVEC constitutively expressed TLR2 and TLR4 mRNA and proteins, and these were further enhanced by histamine. The expression of mRNAs encoding MD-2 and MyD88, the accessory molecules associated with TLR signalling, were unchanged by histamine treatment. These results demonstrate that histamine up-regulates the expression of TLR2 and TLR4 and amplifies endothelial cell inflammatory responses to Gram-negative and Gram-positive bacterial components.

Keywords endothelial cells; histamine; interleukin-6; Toll-like receptor 2; Toll-like receptor 4

INTRODUCTION

Histamine is a major secretory product of mast cells, and is generally implicated in allergic and hypersensitivity reactions. This amine and other mast cell products are known to regulate vasodilatation and bronchoconstriction[1,2] and to modulate the functions of a variety of cell types, including monocytes/macrophages,[3,4] eosinophils,[5,6] T cells,[7] neutrophils[8] and endothelial cells.[9] A direct relationship between histamine and vascular inflammation is evident from the observation that the coronary arteries of patients with ischaemic heart disease contain more mast cells and histamine than normal vessels.[10] The possible role of histamine in vascular disease is further supported by the presence of elevated levels of histamine in the coronary circulation of patients with variant angina.[11] The ability of histamine to induce the production of cytokines such as interleukin-6 (IL-6) and IL-8 by endothelial cells[9,12,13] suggests that this mast cell mediator can act as an important inflammatory signal in addition to its well-recognized function as a vasoactive substance. The physiological

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processes exerted by histamine are mediated through a family of G-protein-coupled receptors, H1, H2, H3 and H4.[14] H1 receptors are highly expressed in smooth muscle cells and endothelial cells, and by signalling through these receptors histamine modulates inflammatory and hypersensitivity responses.[9,15] H2 receptors participate in the stimulation of gastric acid secretion in the gut and in the regulation of cytokine production by many cell types in cardiac and smooth muscle tissues, and the immune system.[3,16–18] H3 receptors are predominantly found in the brain, where they function as presynaptic autoreceptors on histamine-containing neurons.[19,20] H4 receptors, which share approximately 40% homology with H3 receptors, are highly expressed in the bone marrow and in leucocytes, and are moderately expressed in spleen, thymus, lung, small intestine, colon and heart.[21–24] Reports from our laboratory have demonstrated that histamine-induced IL-6 and IL-8 production by human vascular endothelial cells are mediated through H1 receptors,[9,25] which suggested a major role for this receptor subtype in endothelial cell functions.

The role of infection in atherosclerosis has been proposed and bacterial pathogens are widely recognized as inflammatory stimulants.[26] Lipopolysaccharide (LPS), a major cell wall component of Gram-negative bacteria, induces the production of pro-inflammatory cytokines and the expression of adhesion molecules on endothelial cells.[27] The Gram-positive cell wall components, peptidoglycan (PGN) and lipoteichoic acid (LTA) also induce endothelial activation.[28,29] The response to bacterial pathogens via pattern recognition receptors on endothelial cells is important for innate immune defence but their amplified activation may lead to persistent vascular inflammation. The innate immune system recognizes bacterial pathogens through a family of receptors called Toll-like receptors (TLRs). Mammalian cells express at least 10 TLRs, and among them, TLR4 is the major LPS receptor[30–32] and TLR2 recognizes both PGN and LTA.[33–35] Further evidence for the possible role of TLRs in coronary disease is the substantial increase in TLR2 and TLR4 message in the endothelium of human atherosclerotic lesions.[36–38]

A previous report from our laboratory has shown that LPS-induced production of IL-6 and IL-8 by human endothelial cells is greatly enhanced by the presence of histamine.[9] These results suggest that the co-operative action between histamine and Gram-negative bacterial components lead to amplified inflammatory responses in vascular endothelium. The mechanism by which histamine enhances sensitivity to the bacterial component is unknown. The objective of this study was to first test whether a similar co-operation also exists between Gram-positive bacterial cell wall components and histamine, and then to define the possible mechanisms of synergy between histamine and the bacterial pathogens in endothelial cell activation. The results presented in this report demonstrate that histamine amplifies endothelial cell responsiveness to both Gram-negative and Gram-positive cell wall components and the synergy between histamine and bacterial pathogens is associated with enhanced expression of TLR2 and TLR4.

MATERIALS AND METHODS

Materials

Human umbilical endothelial cells (HUVEC), endothelial cell growth medium (EGM-2 MV), trypsin–ethylenediaminetetraacetic acid (EDTA) and trypsin neutralizing solution were purchased from Cambrex (San Diego, CA). *Escherichia coli* (0111:B4) LPS, LTA, PGN, polymyxin B and protease inhibitors were supplied by Sigma Chemical Co. (St Louis, MO). Enzyme-linked immunosorbent assay (ELISA) kits for IL-6 were purchased from R & D Systems (Minneapolis, MN). TLR2 (N-17, H-175) and TLR4 (H-80) antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA), and Cy3 goat anti-rabbit and normal goat serum were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). TRIzol Reagent, RNAse-free DNAse1, Superscript^{TM} II RNase H^{-} Reverse Transcriptase System, and *Taq* polymerase were obtained from Life Technologies (Rockville, MD). The gene-specific primers used in reverse transcription–polymerase chain reaction (RT-PCR) were purchased from Invitrogen Life Technologies (Carlsbad, CA). Nuclear and cytoplasmic extraction reagents (NE-PER^{TM}) were product of Pierce-Endogen (Rockford, IL) and poly(dI-dC) was obtained from Pharmacia (Piscataway, NJ).

Culture of HUVEC

HUVEC were grown in EGM-2 MV 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-B, recombinant insulin-like growth factor-1 (R3I) growth factor, ascorbic acid and 5% fetal bovine serum. At confluence, the cells were detached from the culture flasks using trypsin–EDTA, washed twice, and resuspended in EGM-2 MV. The cells used in all experiments were between three and five passages.

Assay of IL-6 production

HUVEC (2×10^4) were plated on to each of the wells of a 96-well microtitre plate and allowed to adhere for 24 hr. Following adherence, selected concentrations of the activating stimuli or medium were added to the monolayers and incubated at 37° in 5% humidified CO_2 for 24 hr. After the incubation, culture supernatants were harvested and assayed for IL-6 levels by ELISA.

RT-PCR

Total RNA was isolated from HUVEC treated with medium or appropriate agonists, using TRIzol reagent and treated with RNAse-free DNAse I. For a reverse transcription reaction, Superscript^{TM}II RNase H^{-} Reverse Transcriptase system was employed. PCR amplification was performed with *Taq* polymerase for 32 cycles at 95° for 45 seconds, 54° for 45 seconds, and 72° for 1 min (for TLR2, TLR4 and GAPDH), 95° for 45 seconds, 60° for 45 seconds, and 72° for 1 min (for IL-6), 95° for 30 seconds, 52° for 45 seconds, and 72° for 45 seconds (for MD-2), 94° for 30 seconds, 60° for 40 seconds, and 70°

Table 1. Gene-specific primers used in RT-PCR

Gene	Primer sequence (5′ → 3′)	PCR product (size, bp)
IL-6	ATGAACTCCTTCTCCACAAGCGC	
	GAAGAGCCCTCAGGCTGGACTG	620
TLR2	GCCAAAGTCTTGATTGATTGG	347
	TTGAAGTTCTCCAGCTCCTG	
TLR4	TGGATACGTTTCCTTATAAG	
	GAAATGGAGGCACCCCTTC	548
MD-2	GAAGCTCAGAAGCAGTATTGGGTC	
	GGTTGGTGTAGGATGACAAACTCC	422
MyD88	TAAGAAGGACCAGCAGAGCC	
	CATGTAGTCCAGCAACAGCC	200
GAPDH	TGATGACATCAAGAAGGTGGTGAAG	
	TCCTTGGAGGCCATGTGGGCCAT	240

for 2 min (for MyD88). PCR products were electrophorosed on 2% agarose gel. The oligonucleotide primers used for RT-PCR are given in Table 1.

Immunofluorescent staining

HUVEC (20 000/well) grown on chamber slides were incubated either with medium or histamine (10 μM) for 4, 8, 16 and 24 hr. The cells were then fixed with 4% paraformaldehyde, washed and treated with blocking solution (1% bovine serum albumin, 5% normal goat serum and 0·3% Triton X-100 in phosphate-buffered saline) and stained with rabbit anti-human TLR2 (H-175) or TLR4 (H-80). After overnight staining, the cells were washed, and incubated for 1 hr with the secondary antibody (Cy3 goat anti-rabbit). The slides were then viewed under a fluorescence microscope and imaged.

Western blot

Confluent HUVEC monolayers were incubated with histamine (10 μM) for 16 hr at 37°. The cells were then lysed in buffer containing 20 mM Tris–HCl (pH 7·4), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 25 mM β-glycerolphosphate, 2 mM sodium pyrophosphate, 1% protease inhibitor cocktail and 0·5 mM dithiothreitol at 4° for 30 min. Cell debris was removed by centrifugation of the lysate at 13 000 ***g*** for 10 min. Aliquots of supernatants normalized for protein concentrations were mixed with equal volumes of 2 × sodium dodecyl sulphate sample buffer and heated to 100° for 5 min. Samples were resolved on 10% sodium dodecyl sulphate–polyacrylamide gel elcetrophoresis and transferred onto a nitrocellulose membrane. After blocking for 2 hr in TBST (20 mM Tris–HCl, 150 mM NaCl, 0·1% Tween-20) containing 5% non-fat milk, membranes were washed thrice in TBST and probed for 1 hr at 4° with anti-TLR4 (H-80) and for 18 hr at 4° for TLR2 using anti-TLR2 antibody (N-17). After washing thrice, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and washed five times and bands were detected using enhanced chemiluminescence reagents (Bio-Rad, Hercules, CA).

NF-κB activation

Confluent HUVEC monolayers were treated with LPS (100 ng/ml), PGN (100 μg/ml), or LTA (10 μg/ml) in the absence or presence of histamine (10 μM) for 2 hr at 37°. After the incubation, nuclear proteins were extracted using nuclear and cytoplasmic extraction reagents (NE-PER^{TM}) according to the manufacturer's protocol. An electrophoretic mobility shift assay (EMSA) of nuclear proteins was carried out utilizing the oligonucleotide probe, 5′-AGTT GAGGGGACTTTCCCAGGC-3′. The specific oligonucleotide and its complimentary strand were annealed and then end-labelled using γ-[^{32}P]ATP (6000 Ci/mmol; New England Nuclear, Boston, MA) and T4-polynucleotide kinase (Amersham, Piscataway, JS), as described.[39,40] Nuclear proteins (8 μg) were mixed with 3 μg poly(dI-dC) and ~2 ng (100 000–400 000 counts per minute) of end-labelled DNA in 30 μl of 50 mM Tris–HCl buffer (pH 7·5) containing 250 mM NaCl, 5 mM EDTA, 5 mM dithiothreitol and 25% glycerol, and incubated for 30 min at 25°. Following the initial binding reaction, 20 μl of the mixture were electrophoresed on a 6% native polyacrylamide gel, and then processed for autoradiography.

Statistical analysis

The data were analysed by one-way analysis of variance with subsequent Newman–Kuel test. All results were expressed as mean ± SD and $P < 0{\cdot}05$ was considered significant.

RESULTS

Histamine amplifies LPS, LTA and PGN-induced endothelial cell production of IL-6

To examine the direct effect of histamine on endothelial cell production of IL-6, HUVEC monolayers were incubated with different concentrations of histamine (0·1–100 μM) for 24 hr, and the levels of secreted IL-6 in the culture media were quantified. The results presented in Fig. 1(a) reveal that histamine is capable of inducing IL-6 production in a dose-dependent manner. The effect of histamine begins at a concentration of 1 μM and achieved a plateau at a concentration of 10 μM.

To evaluate the modulatory effect of histamine on endothelial cell responses to Gram-negative and Gram-positive cell wall components, HUVEC monolayers were incubated with different doses of LPS, LTA, or PGN in the presence or absence of histamine (10 μM) for 24 hr and the secreted IL-6 levels were quantified. Figure 1(b) demonstrates that LPS induced significant stimulation of IL-6 production in a dose-dependent fashion, which achieved a plateau at a dose of 100 ng/ml. Simultaneous presence of histamine markedly enhanced LPS-induced IL-6 production at all concentrations of LPS-tested.

Gram-positive bacterial cell wall components, LTA and PGN, also stimulated endothelial cells to generate IL-6 in a dose-dependent manner. When compared to the effect of LPS, the responses by LTA and PGN were of a lesser magnitude whether compared on a weight or molar basis.

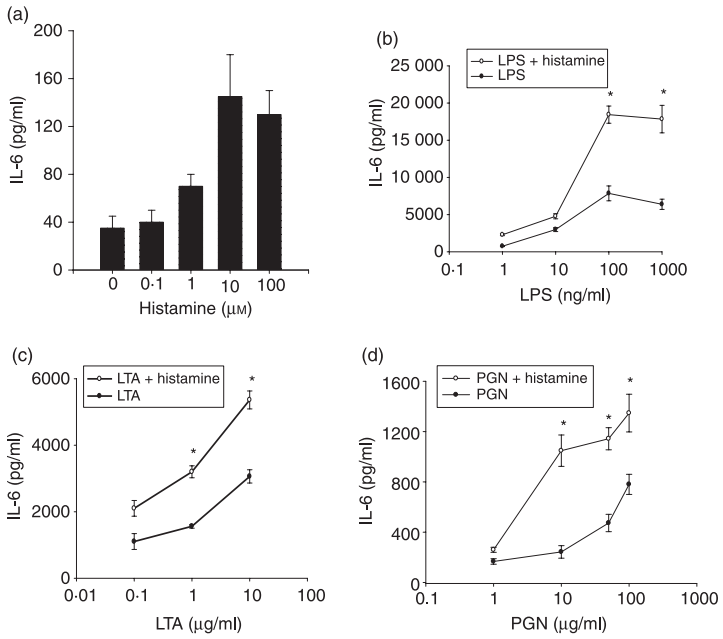

Figure 1. Amplification of LPS, LTA and PGN-induced IL-6 production by histamine. HUVEC (1×10^4 cells) were plated on to each of the wells of a 96-well culture plate and allowed to adhere for 18–20 hr. The monolayers were then incubated with the different doses of histamine (0·1–100 μM) (a), and histamine (10 μM) in the presence of LPS (1–1000 ng/ml) (b), LTA (0·1–10 μg/ml) (c), or PGN (1–100 μg/ml) (d). After a 24-hr incubation, IL-6 levels in the culture media were assayed by ELISA. Each value presented is the mean ± SD of quadruplicate determinations. The results presented are representatives of four independent experiments. An asterisk indicates $P < 0{\cdot}01$ when compared to the value for the TLR agonist alone.

As in the case of LPS, LTA- and PGN-induced IL-6 production was also amplified by histamine (Fig. 1c,d). The effects of LTA and PGN were resistant to polymyxin B treatment, demonstrating that the effects were not the result of LPS contamination in the LTA and PGN preparations (data not shown).

Increased IL-6 mRNA expression after stimulation with LPS, LTA, PGN and histamine

To examine whether the amplified release of IL-6 by combined effects of histamine and bacterial cell wall components reflects in enhanced gene expression, IL-6 mRNA expression was examined (Fig. 2). Unactivated HUVEC expressed relatively low levels of IL-6 mRNA. IL-6 mRNA expression was significantly increased when HUVEC were stimulated with LPS, LTA, PGN and histamine. LPS was found to be the most potent inducer of IL-6 mRNA expression. A further enhancement of LPS, LTA and PGN-induced IL-6 mRNA expression was noted in the presence of histamine.

H1 receptor antagonist inhibits histamine-mediated enhancement of IL-6 production

Diphenhydramine (10 μM), an H1 receptor antagonist, completely abrogated histamine-induced potentiation of IL-6 production by LTA- and PGN-stimulated HUVEC (Fig. 3). Famotidine, an H2 receptor antagonist, had no

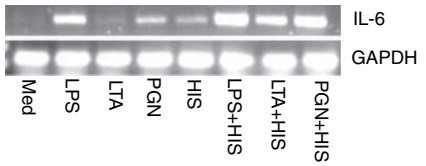

Figure 2. Enhancement of IL-6 mRNA expression by histamine and bacterial cell wall components. HUVEC (1×10^6 cells) were allowed to adhere in each of the wells of six-well culture plates for 18–20 hr. The monolayers were then incubated with LPS (100 ng/ml), LTA (10 μg/ml), PGN (100 μg/ml), or histamine (HIS, 10 μM), and in combinations of histamine + LPS, histamine + LTA, or histamine + PGN. After a 2-hr incubation, total RNA was isolated and subjected to RT-PCR using IL-6 primers (22 cycles) and the amplified products were electrophoresed on a 2% agarose gel. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression was used for normalization. The results presented are representatives of four independent experiments.

effect on histamine-induced potentiation of IL-6 production. A concentration of 10 μM diphenhydramine is sufficient to block the effect of histamine in endothelial cells whereas famotidine, even at 100 μM, did not have any effect.[9]

Increased NF-κB activation by histamine and bacterial cell wall components

The exposure of endothelial cells to LPS or LTA for 2 hr substantially increased translocation of NF-κB proteins to

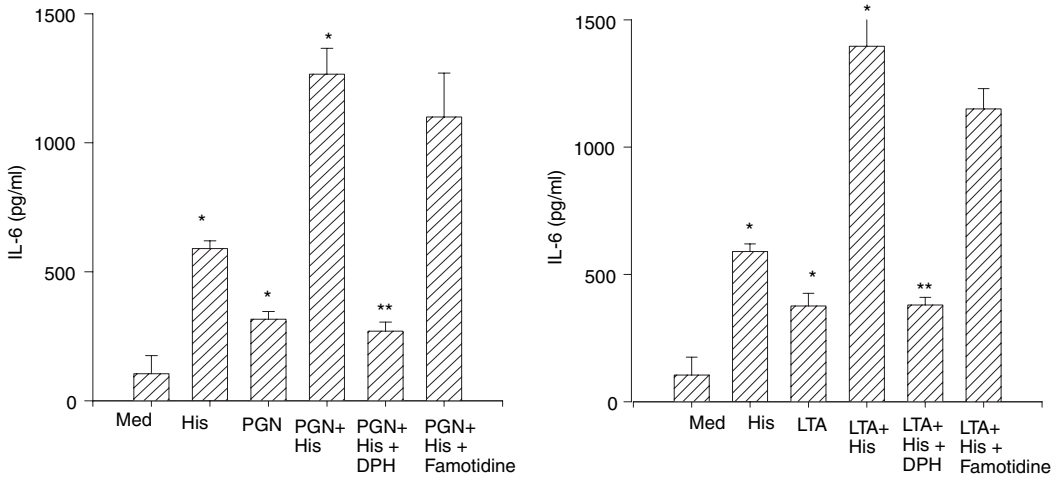

Figure 3. H1 receptor antagonist, but not H2 receptor antagonist, abrogates histamine-induced enhancement of PGN and LTA on IL-6 production. HUVEC (1×10^4 cells) were plated on to each of the wells of a 96-well culture plate and allowed to adhere for 18–20 hr. HUVEC monolayers were subsequently incubated with 100 μg/ml PGN (left panel) or 10 μg/ml LTA (right panel) in the presence and absence of histamine (10 μM). Wherever applicable, 10 μM diphenhydramine (DPH, H1R antagonist), or 10 μM famotidine (H2R antagonist) was added simultaneously with the agonists. After a 24-hr incubation, the culture media were assayed for IL-6 levels by ELISA. Each value presented is the mean ± SD of the quadruplicate determinations. The results presented are representative of four independent experiments. Asterisk indicates a statistically significant difference at $P < 0{\cdot}05$ when compared to HUVEC incubated with medium alone. Double asterisk indicates a statistically significant effect of DPH at $P < 0{\cdot}05$ when compared to the effect of histamine plus LTA or histamine + PGN.

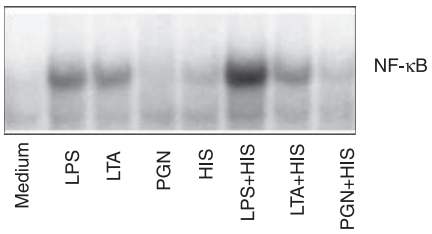

Figure 4. Increased nuclear NF-κB proteins in HUVEC treated with histamine and bacterial cell wall component**s.** HUVEC ($2{\cdot}5 \times 10^6$ cells) were plated in 60-mm culture dishes and allowed to adhere for 18–20 hr. The cell monolayers were then incubated with LPS (100 ng/ml), LTA (10 μg/ml), PGN (100 μg/ml) or histamine (HIS, 10 μM), and in combinations of histamine + LPS, histamine + LTA, or histamine + PGN. After a 2-hr incubation, the nuclear proteins extracted from the cells were incubated with ^{32}P-labelled oligonucleotide containing NF-κB binding sites. The samples were then subjected to electrophoresis on 6% native polyacrylamide gels and processed for autoradiography. The autoradiograph shown is the representative of three separate experiments.

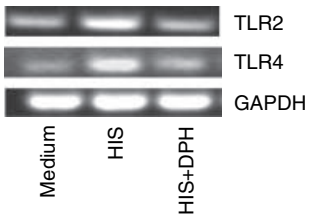

Figure 5. Histamine induces TLR2 and TLR4 mRNA expression. HUVEC (1×10^6 cells) were allowed to adhere in each of the wells of a six-well culture plates for 18–20 hr. Cell monolayers were then incubated with histamine (10 μM) alone or histamine + diphenhydramine (DPH, 10 μM) for 2 hr. After the incubation, total RNA was isolated and subjected to RT-PCR using TLR2 and TLR4 primers. The amplified products were electrophoresed on a 2% agarose gel. GAPDH mRNA expression was used for normalization. The results presented are representatives of four independent experiments.

the nuclei (Fig. 4). Incubation of HUVEC with LPS in the presence of histamine further enhanced nuclear translocation of NF-κB, although histamine alone was a weak stimulant of NF-κB translocation. In contrast to the combined effect of histamine and LPS, histamine did not amplify NF-κB activation in LTA or PGN-treated HUVEC.

Histamine induces TLR2 and TLR4 mRNA expression

Gram-positive and Gram-negative bacterial cell wall components are recognized by TLR2 and TLR4, respectively. To assess the role of these receptors in histamine-mediated amplification of the effects of LPS, LTA and PGN, the expression of TLR2 and TLR4 mRNA was assessed. The results demonstrate that histamine (10 μM) induced both TLR2 and TLR4 mRNA expression in HUVEC after 2 hr of stimulation (Fig. 5). The histamine-induced TLR2 and TLR4 mRNA expression was also blocked by the H1 receptor antagonist, diphenhydramine (Fig. 5).

Up-regulation of TLR2 and TLR4 proteins by histamine

To determine whether histamine-induced TLR2 and TLR4 mRNA expression is associated with increased expression

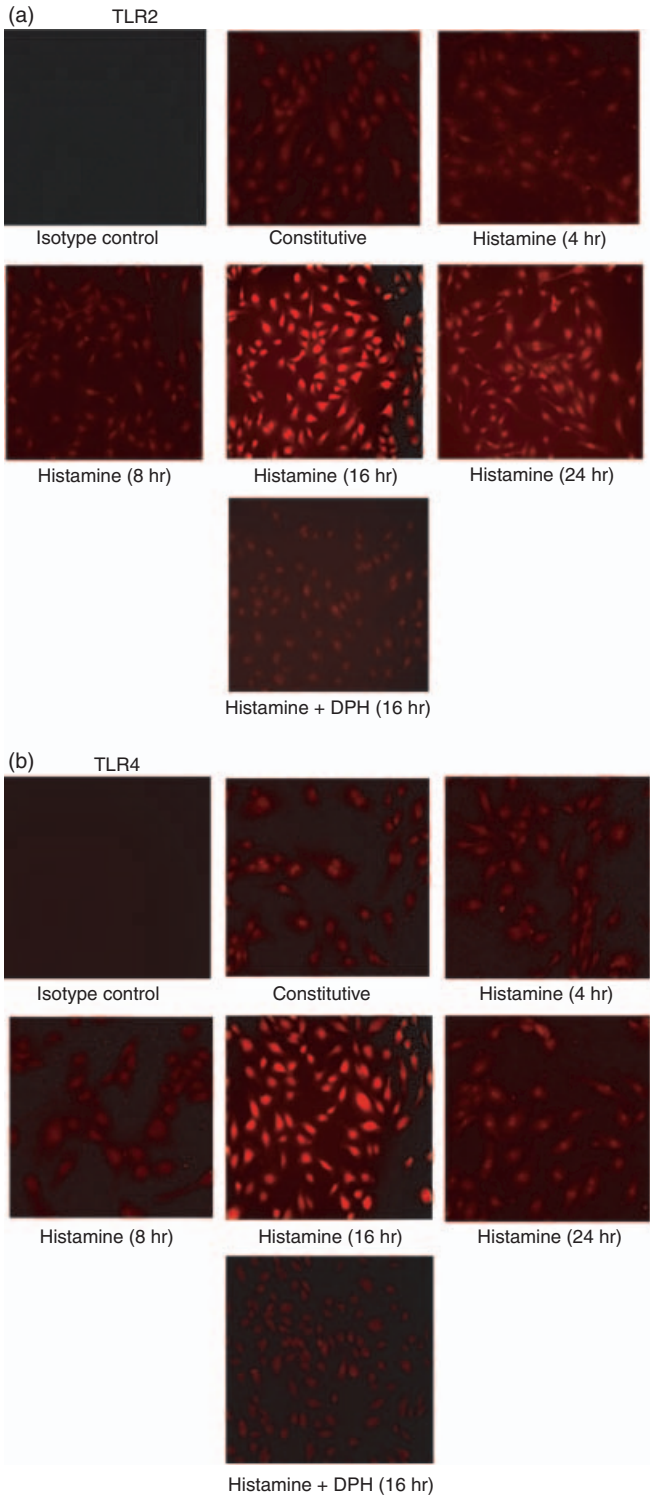

Figure 6. Histamine induces expression of TLR2 (a) and TLR4 (b) proteins as determined by immunofluorescence. HUVEC (2×10^4 cells) were plated on each of the wells of a chamber slide and allowed to adhere for 18–20 hr. The cells were incubated with medium (constitutive expression), histamine (10 μM) for 4, 8, 16 and 24 hr, and with histamine plus diphenhydramine (DPH, 10 μM) for 16 hr. After stimulation, the cells were fixed with paraformaldehyde and stained with the respective antibodies, or isotype immunoglobulin G (control). After extensive washing, the cells were incubated with rhodamine-conjugated secondary antibodies and viewed under a fluorescence microscope for imaging.

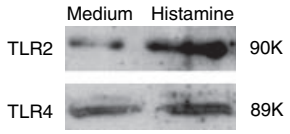

Figure 7. Histamine up-regulates the expression of TLR2 and TLR4 as determined by Western blot. HUVEC (2×10^6 cells) were plated in 60-mm culture dishes and allowed to adhere for 18–20 hr. Cell monolayers were then incubated with medium or histamine (10 μM) for 16 hr. After washing, total cell lysates were prepared as described in the Materials and methods section. Protein samples were separated by sodium dodecyl sulphate–polyscrylamide gel electrophoresis (10%), transferred to nitrocellulose membranes, and probed with anti-human TLR2 and TLR4 antibodies. The signals were detected by standard enhanced chemiluminescence technique after labelling with horseradish peroxidase-conjugated secondary antibodies.

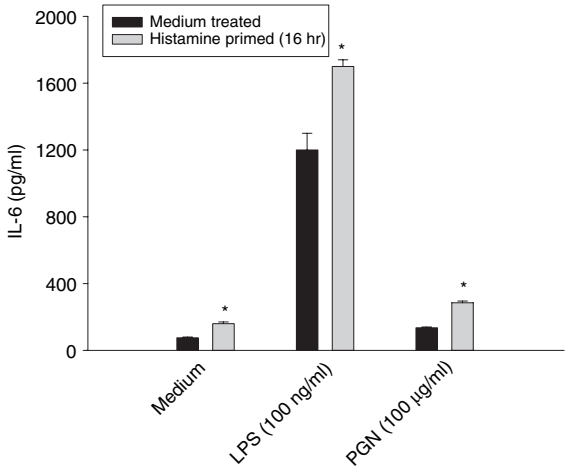

Figure 8. Histamine priming enhances the responsiveness of endothelial cells to TLR2 and 4 ligands. HUVEC (1×10^4 cells) were plated on to each of the wells of a 96-well culture plate and allowed to adhere for 18–20 hr. HUVEC monolayers were incubated with medium or histamine (10 μM) for 16 hr, and then activated with LPS (100 ng/ml), or PGN (100 μg/ml) for an additional 24 hr. After the incubation, IL-6 levels in the culture media were assayed. Each value presented is the mean ± SD of quadruplicate determinations. The results presented are representatives of three independent experiments. An asterisk indicates $P < 0{\cdot}01$ when compared to value for medium-treated cells.

of proteins, immunofluorescence and Western blot analyses were carried out. Immunofluorescence analyses revealed detectable levels of expression of TLR2 (Fig. 6a) and TLR4 (Fig. 6b) proteins which were markedly enhanced when HUVEC were incubated with histamine for 16 hr and was completely inhibited by the H1 receptor antagonist, diphenhydramine. Histamine-induced TLR2 and TLR4 expression was transient as it returned to constitutive levels by 24 hr even in the continued presence of histamine.

The constitutive expression of TLR2 and TLR4 proteins and their up-regulation by histamine were further verified by Western blot analysis. As evident from Fig. 7, treatment

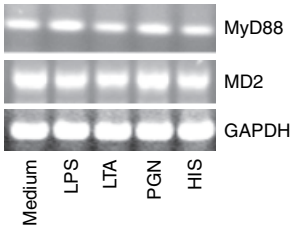

Figure 9. MD-2 and MyD88 mRNA expression. HUVEC (1×10^6 cells) were allowed to adhere in each of the wells of a six-well culture plate for 18–20 hr. HUVEC monolayers were then incubated with LPS (100 ng/ml), LTA (10 μg/ml), PGN (100 μg/ml) and histamine (HIS, 10 μM) for 2 hr. After the incubation, total RNA was isolated, and subjected to RT-PCR using MD-2 and MyD88 primers. The amplified products were electrophoresed on a 2% agarose gel. GAPDH mRNA expression was used for normalization. The results presented are representatives of four independent experiments.

of HUVEC with histamine for 16 hr resulted in enhanced expression of TLR2 and TLR4 proteins.

Priming with histamine enhances the responsiveness of HUVEC to TLR2 and TLR4 ligands

The results presented in Figs 6 and 7 demonstrated that exposure of HUVEC for 16 hr results in a maximum level of expression of TLR2 and TLR4. To relate the histamine-induced expression of TLRs with enhanced sensitivity to the bacterial cell wall components, HUVEC were primed with histamine for 16 hr and subsequently challenged with LPS and PGN, and IL-6 production was measured. As shown in Fig. 8, histamine priming significantly enhanced the responsiveness of cells to both LPS and PGN. These results further suggest that the newly expressed receptors are functionally active and contribute to the amplified inflammatory responses.

MD-2 and MyD88 mRNA expression is not altered by histamine or by the bacterial cell wall components

MD-2 is an accessory protein, which is required by TLR4 to respond to LPS.[31,41,42] Similarly, MyD88 is an adapter molecule that is required for TLR-mediated signal transduction and NF-κB activation.[43,44] To evaluate the role of MD-2 and MyD88 in histamine-mediated amplification, their mRNA expressions were determined by RT-PCR. The results demonstrate that treatment of HUVEC with LPS, LTA, PGN, or histamine for 2 hr did not affect MD-2 and MyD88 mRNA expression (Fig. 9).

DISCUSSION

The results of the present study demonstrate that histamine, a major secretory product of the mast cell, is not only able to stimulate endothelial cells to synthesize and secrete IL-6, but can also amplify the stimulatory effects of Gram-positive and Gram-negative bacterial cell wall components. The

ability of histamine to induce both IL-6 mRNA and protein expression suggests that the amplifying effect was not solely the result of histamine-induced secretion of pre-formed cytokine but is the result of enhanced gene expression and protein synthesis. The fact that histamine by itself is a strong stimulant of IL-6 mRNA expression suggests that the primary effect of histamine in the amplification cascade is induction of IL-6 gene expression, whereas the combined action of histamine and bacterial pathogens involves both transcriptional and post-transcriptional regulation.

Toll-like receptors are involved in innate immune recognition and cellular activation in response to microbial antigens.[34,35,45–48] The present results show that histamine-induced amplification of the effects of the bacterial components was associated with enhanced expression of TLR2 and TLR4 mRNA and proteins. Although TLR2 and TLR4 mRNA expression is seen in 2 hr, the maximum protein expression was noted at 16 hr after activation with histamine. Histamine-induced TLR2 and TLR4 expression returned to constitutive levels by 24 hr, which suggests that the up-regulation of receptors is transient. To our knowledge, this is the first study, which demonstrates the ability of histamine to up-regulate TLR2 and TLR4 expression and function, and modulate cytokine secretion in endothelial cells.

The modulation of the expression of the TLRs by histamine may provide a critical role for this amine in endothelial cell response to microbial pathogens and activation of inflammatory responses. The histamine-mediated enhancement of IL-6 production, as well as TLR2 and TLR4 expression, was inhibited by the H1 receptor antagonist diphenhydramine and not by famotidine, demonstrating that the effect is mediated via H1 receptors. It appears that the surface expression of TLR2 and TLR4 is relatively low when compared to the level of mRNA expression. The low level surface expression of TLR2 and TLR4 suggests the requirement of only a limited number of functional receptors and the stringent regulation of pathogen-mediated inflammatory responses in the vessel wall. The low level surface expression of TLRs in endothelial cells is consistent with the recent report by Zeuke *et al.*[49] who failed to detect TLR4 surface proteins in human coronary artery endothelial cells despite the presence of substantial amounts of mRNA. It is noteworthy that TLR4 remains functional even at extremely low receptor numbers in several cell types including immature dendritic cells and neutrophils.[50]

Since histamine induces TLR2 and TLR4 expression, it is reasonable to postulate that the amplified responsiveness of endothelial cells to bacterial products is the result of enhanced or sustained signalling via newly expressed TLR2 and TLR4. This contention is supported by the finding that endothelial cells primed with histamine for 16 hr, which induced maximum levels of TLR2 and TLR4, produced significantly higher amounts of IL-6 when challenged with LPS or PGN. It is noteworthy that although TLR2 and TLR4 are expressed at low levels on human endothelial cells[49] a substantial increase of their messages was noted in the endothelium covering human atherosclerotic lesions.[37] These findings, together with the presence of an increased number of mast cells in the atherosclerotic lesions,[51,52] suggest that the co-operative action of mast cell-derived histamine and bacterial products possibly derived from colonized or circulating pathogens cause persistent vascular inflammation. It is of interest that a TLR4 polymorphism, which diminishes the inflammatory response to Gram-negative pathogens, is associated with decreased risk of atherosclerosis in humans.[37]

Although TLRs are the primary signalling receptor for the bacterial cell wall components, TLR2 and TLR4 alone are not capable of sensing the presence of LPS. Another accessory molecule MD-2, which is physically associated with TLR4, is required for LPS recognition and signalling.[41,53] The present results reveal that histamine did not modulate the expression of MD-2 mRNA in endothelial cells. Based on the constitutive expression of MD-2 in naïve endothelial cells it is presumed that adequate amounts of MD-2 molecules are present for full response to the bacterial pathogens and histamine-mediated amplification does not involve newly expressed MD-2. Similarly, exposure of endothelial cells to histamine did not alter the expression of MyD88, an accessory molecule that is recruited to the intracellular domains of TLR2 and TLR4 after recognition of the pathogens.[43,44] It is possible that histamine may enhance the recruitment of both MD-2 and MyD88 from existing pools without enhancing their syntheses. Alternatively, histamine may enhance the release of MD-2 proteins or stabilize the LPS/MD-2 complex, as secreted MD-2 in the absence of LPS is labile.[54]

Activation of TLR2 and TLR4 involves NF-κB translocation.[55,56] Promoters of the gene for IL-6 contain recognition sites for the transcription factor NF-κB[57,58] and a number of studies have demonstrated the involvement of NF-κB in the regulation of IL-6 transcription.[59,60] In this study, activation of endothelial cells by histamine alone caused only a minimal level of NF-κB translocation although it caused a substantial amount of IL-6 mRNA expression. Interestingly, histamine-induced IL-6 mRNA expression did not reflect proportional levels of IL-6 protein synthesis. The exposure of endothelial cells to LPS for 2 hr substantially increased translocation of NF-κB proteins to the nuclei, which was further amplified by the presence of histamine. Incubation of HUVEC with LTA or PGN also caused nuclear translocation of NF-κB proteins but the effect was of lesser magnitude when compared to that of LPS, and was not enhanced by histamine. Although both LTA and PGN induced signal transduction via TLR2, LTA was more effective in inducing NF-κB activation. It is apparent that the low level of NF-κB translocation in PGN-treated cells is associated with a lesser degree of IL-6 production when compared to LTA.

The results presented here unravel one of the mechanisms by which histamine amplifies endothelial cell sensitivity to TLR2 and TLR4 ligands. Since both bacterial products and histamine were simultaneously present in the culture system, and such an environment could presumably be present *in vivo* under certain pathological conditions, the

modulation of histamine responsiveness by TLR2 and TLR4 ligands is also feasible. Thus, it is conceivable that the amplification of IL-6 production by the combined effects of histamine and bacterial products could also be the result of an enhanced endothelial cell response to histamine secondary to the action of bacterial components. To this effect, we have noted increased H1 receptor mRNA expression in endothelial cells after exposure to LPS, LTA, or PGN (Talreja *et al*. unpublished data). It remains to be tested whether endothelial cells, which are primed by the bacterial pathogens, express functionally active H1 receptors and whether the enhanced H1 receptor signalling amplifies the inflammatory responses in the endothelium.

In conclusion, the present study demonstrates that incubation of human endothelial cells with LPS, LTA, or PGN results in the stimulation of IL-6 mRNA expression and IL-6 release. The effects of LPS, LTA, or PGN on endothelial activation are further enhanced by the simultaneous presence of histamine. Both the direct and potentiating effects of histamine on endothelial cell activation are completely abolished by an H1 receptor antagonist, and not by an H2 receptor antagonist. The histamine-induced amplification of the effects of the bacterial cell wall components on endothelial cell activation is associated with the up-regulation of TLR2 and TLR4 expression. The results also suggest that histamine enhances cellular responses to LPS via amplified NF-κB translocation. This phenomenon was not observed with LTA and PGN. Studies on the effects of Gram-positive and Gram-negative bacterial pathogens on the modulation of H1 receptor expression and signalling, and their contribution to the synergy are currently underway. Collectively, the results suggest that individuals who have increased levels of circulating histamine may be at a risk for persistent and amplified inflammatory responses to bacterial pathogens.

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