Histamine Induces Tissue Factor Expression
Implications for Acute Coronary Syndromes

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Background—Histamine can induce coronary vasospasm, leading to variant angina and acute myocardial infarction. However, the role of histamine in thrombus formation is ill defined. Hence, this study investigates whether histamine induces tissue factor (TF) expression in vascular cells.

Methods and Results—Histamine (10⁻⁸ to 10⁻⁵ mol/L) induced TF expression in a concentration-dependent manner in human aortic endothelial and vascular smooth muscle cells, whereas TF pathway inhibitor expression remained unaffected. RT-PCR and Northern blotting revealed that histamine stimulated TF mRNA transcription, peaking at 1 hour. Protein expression increased 18-fold (P<0.02) with a maximum at 5 hours, which was paralleled by a 4-fold augmentation in surface activity (P<0.01). These effects were completely prevented by pretreatment with the H₁ receptor antagonists mepyramine (P<0.0001), chlorpheniramine, and diphenhydramine but not the H₂ receptor antagonist cimetidine (P=NS). Histamine induced a time-dependent, H₁ receptor–mediated activation of p38 MAP kinase (p38), p44/42 MAP kinase (ERK), and c-jun terminal NH₂ kinase (JNK). Blocking of p38, ERK, or JNK with SB203580 (P<0.0001), PD98059 (P<0.0001), or SP600125 (P<0.0001), respectively, impaired histamine-induced TF expression in a concentration-dependent manner. In contrast, histamine-stimulated TF expression was increased by phosphatidylinositol 3-kinase inhibition with LY294002 or wortmannin, whereas it was not affected by Rho-kinase inhibition with Y-27632 or hydroxyfasudil.

Conclusions—Histamine induces expression of TF, but not TF pathway inhibitor, in vascular cells via activation of the H₁ receptor. This effect is mediated by the MAP kinases p38, ERK, and JNK. This observation may open novel perspectives in the treatment of variant angina and acute coronary syndromes. (Circulation. 2005;112:341-349.)

Key Words: coagulation • coronary disease • endothelium • signal transduction • thrombosis
patients with unstable angina.\textsuperscript{15} Thus, TF is involved in the initiation and propagation of acute coronary syndromes.

In the present study, we show that histamine induces TF expression and activity in human aortic endothelial cells (HAECs) and human vascular smooth muscle cells (HAVSMCs). This effect, mediated through activation of the MAP kinase pathway, is completely abolished by H\textsubscript{1} receptor antagonists.

**Methods**

**Cell Culture**

HAECs and HAVSMCs were cultured as described.\textsuperscript{16} Cells were grown to confluence in 6-cm culture dishes and rendered quiescent for 24 hours before stimulation with histamine (Sigma). Mepiparmine, chlorpheniramine, diphenhydramine, and cimetidine (all from Sigma) were added to the dishes 30 minutes before stimulation. Y-27632, hydroxyfasudil, wortmannin, SB203580 (all from Sigma), LY294002, PD98059 (both from Cell Signaling), and SP600125 (Calbiochem) were added 60 minutes before stimulation. To assess cytotoxicity, a colorimetric assay for detection of lactate dehydrogenase was used according to the manufacturer’s recommendations. A commercially available limulus amoebocyte lysate assay was used according to the manufacturer’s recommendations (Cambrex) to assess for potential contamination with endotoxin. For some experiments, histamine and/or lipopolysaccharide (LPS) solutions were heated in a boiling water bath for 1 hour before being added to the cells.

**Western Blot Analysis**

Protein expression was determined by Western blot analysis. Cells were lysed in 50 mmol/L TRIS buffer as described.\textsuperscript{17,18} Then, 40-μg samples were loaded and separated by 10% SDS-PAGE. Proteins were transferred to a PVDF membrane (Millipore) by semidry transfer. Equal loading was confirmed by Ponceau S staining. Antibodies to human TF and TFPI (both from American Diagnostics) were used at 1:2000 and 1:1000 dilution, respectively; antibodies against phosphorylated p38 MAP kinase (p38), phosphorylated p44/42 MAP kinase (ERK), and phosphorylated c-jun terminal NH\textsubscript{2} kinase (JNK; all from Cell Signaling) were used at 1:1000, 1:5000, and 1:1000 dilution, respectively. Antibodies against total p38, total ERK, and total JNK (all from Cell Signaling) were used at 1:2000, 1:10 000, and 1:1000 dilution, respectively. Blots were normalized to α-tubulin expression (1:10 000 dilution; Sigma).

**RT-PCR and Northern Blot Analysis**

Total RNA was extracted from HAECs (1.5×10\textsuperscript{6} cells) with 1 mL TRIzol Reagent (Invitrogen) according to the manufacturer’s recommendations. Conversion of total cellular RNA to cDNA was carried out with Moloney murine leukemia virus reverse transcriptase and random hexamer primers (Amersham Biosciences) in a final volume of 35 μL, using 4 μg of RNA. The total cDNA pool obtained served as a template for subsequent PCR amplification with TF (F3)\textsuperscript{-specific primers (508 to 529 bp, 913 to 933 of F3 cDNA; NCBI No. NM 001993).18 The PCR conditions were as follows: 25 μL final reaction containing 2.5 mmol/L MgCl\textsubscript{2}, 0.2 mmol/L dNTP, 10 μmol/L of each primer, 2.5 μL of dimethyl sulfoxide, 0.5 U of TaKaRa Taq DNA polymerase (TaKaRa Biomedicals), and 2 μL of cDNA template by using the following cycling parameters: 94\textdegree C for 3 minutes for 1 cycle, 94\textdegree C for 1 minute, 60\textdegree C for 30 seconds, 65\textdegree C for 45 seconds, for a total of 35 cycles, followed by a final elongation step at 65\textdegree C for 5 minutes. S15 primers served as loading controls for PCR. Products were separated by electrophoresis on a 1% agarose gel and visualized with ethidium bromide.

Total RNA (15 to 20 μg) per sample was analyzed by Northern blotting. A 405-bp human TF cDNA (508 to 913 bp) fragment was labeled with [\textsuperscript{32}P]dCTP (Amersham Biosciences) using the Prime-It Kit (Stratagene) according to the manufacturer’s recommendations. Afterward, the membrane was stripped and rehybridized with a control probe against the housekeeping gene human β-actin (Clontech). Bands were visualized by autoradiography.

**TF Surface Activity**

TF surface activity was analyzed with a colorimetric assay (American Diagnostica) according to the manufacturer’s recommendations with some modifications as described.\textsuperscript{19} Cells were grown in 6-well plates; after stimulation, cells were washed twice with PBS, followed by incubation with human FVIIa and FX at 37\textdegree C, allowing the formation of a TF/FVIIa complex at the cell surface. The TF/FVIIa complex converted human FX to activated FX, which was measured by its ability to metabolize a chromogenic substrate. Lipidated human TF was used as a positive control to confirm that the results obtained were in the linear range of detection (data not shown).

**Statistical Analysis**

Data are presented as mean±SEM. An unpaired Student t test was applied to compare 2 groups; ANOVA with Bonferroni’s correction was used for ≥3 groups. A value of P<0.05 was considered significant.

**Results**

**Histamine Stimulates TF Expression and Activity in HAECs and HAVSMCs**

Stimulation of HAECs with histamine (10\textsuperscript{-5} to 10\textsuperscript{-3} mol/L) led to a concentration- and time-dependent induction of TF expression up to 18-fold the basal level; a maximal effect was observed with 10\textsuperscript{-3} mol/L histamine (Figure 1A) and after 5 hours (Figure 1B). Under such conditions, the level of TF expression corresponded to more than one third of that observed with 10 ng/mL tumor necrosis factor (TNF)-α, a known potent inducer of TF expression in vascular cells (Figure 1D, left). Histamine also enhanced TF expression in HAVSMCs; the maximal increase was 3-fold compared with control conditions (Figure 1C), again corresponding to more than one third of the effect of 10 ng/mL TFN-α (Figure 1D, right).

TF expression is very sensitive to LPS. Therefore, a limulus amoebocyte lysate assay was performed that did not detect endotoxin in water or histamine samples (detection limit of the test, 0.125 EU/mL; n=3; data not shown). Moreover, the influence of heat treatment on histamine-induced TF expression was examined. Histamine is very heat stable,\textsuperscript{20} whereas LPS is inactivated at least in part by boiling for 1 hour.\textsuperscript{21} Boiling of histamine (10\textsuperscript{-3} mol/L) for 1 hour did not affect its ability to induce TF (P=NS; Figure 1E). In contrast, boiling of LPS (100 ng/mL) for 1 hour reduced its ability to induce TF expression by 81% (P<0.0001). LPS potentiated histamine-induced TF expression, whereas boiling of LPS (100 ng/mL) plus histamine for 1 hour resulted in a decrease in TF induction by 80% compared with unheated control (n=3; P<0.0001; Figure 1E). When a lower concentration of LPS (2 ng/mL) was added with histamine (10\textsuperscript{-5} mol/L), TF was reduced to the level observed after stimulation with histamine alone (n=3; data not shown), consistent with the observation that lower concentrations of LPS are more readily inactivated by heat treatment.\textsuperscript{21} Thus, in contrast to histamine alone, boiling of LPS both alone and together with histamine resulted in a significantly reduced TF induction. These results demonstrate that no significant contamination of histamine solutions with LPS occurred and confirm
that the observed induction of TF is due to the action of histamine.

RT-PCR (Figure 2A) and Northern blot analysis (Figure 2B) revealed that TF mRNA was induced in a time-dependent manner. Maximal upregulation was observed after 1 hour and then declined within 5 hours.

The increase in endothelial TF protein expression was reflected by a 4-fold increase in TF surface activity after stimulation with $10^{-5}$ mol/L histamine for 5 hours (Figure 3A). TF surface activity of HAVSMCs was increased to 1.4-fold the basal level under these conditions (Figure 3B). Omission of either FVIIa or FX was used as negative control for the specificity of the enzymatic reaction. No measurable increase in TF activity over background levels was observed under such conditions ($n=3$; $P=NS$; data not shown).

Histamine Does Not Affect Expression of TFPI

TFPI is the direct physiological inhibitor of the TF/FVIIa complex. Interestingly, stimulation with histamine did not affect TFPI expression in HAECs (Figure 4A). As a control, the same lysates were blotted for TF expression (Figure 4B), revealing the typical pattern of TF induction in response to histamine (Figure 1A).

Histamine-Induced TF Expression Is Mediated by Activation of the $H_1$ Receptor

Histamine is known to exert most of its actions on cells through the $H_1$ or $H_2$ receptor. To determine the receptor involved in histamine-induced TF expression, cells were pretreated with the $H_1$ receptor antagonists mepyramine ($10^{-8}$ to $10^{-5}$ mol/L), chlorpheniramine ($10^{-8}$ to $10^{-5}$ mol/L), or diphenhydramine ($10^{-8}$ to $10^{-5}$ mol/L) or with the $H_2$ receptor antagonist cimetidine ($10^{-7}$ to $10^{-5}$ mol/L). Preincubation with mepyramine completely abolished histamine-induced TF expression in HAECs (Figure 5A, top); the same effect was observed with chlorpheniramine and diphenhydramine (Figure 5A, middle and lower, respectively). In contrast, TF expression in response to histamine remained unaffected by...
preincubation with cimetidine (Figure 5B). Similarly, pretreatment of histamine-stimulated HAVSMCs with mepyramine (10^{-5} mol/L; Figure 5C) significantly reduced TF expression, whereas cimetidine (10^{-5} mol/L) did not (Figure 5D). Consistent with these observations, the histamine-induced increase in TF surface activity was inhibited by mepyramine (10^{-5} mol/L) in both HAECs (Figure 3A) and HAVSMCs (Figure 3B). Basal expression of TF was detected in HAVSMCs but not HAECs. A small increase in basal TF expression in HAVSMCs occurred after addition of both mepyramine (10^{-5} mol/L) and cimetidine (10^{-5} mol/L) in the absence of histamine; this effect was not observed in HAECs. Lactate dehydrogenase release did not reveal any cytotoxic effect of histamine, mepyramine, chlorpheniramine, diphenhydramine, or cimetidine (n=4; P=NS; data not shown).

H_{1}-Receptor Activation Regulates TF Expression via the MAP Kinase Pathway

MAP kinase activation regulates TF expression in response to several mediators. To assess whether histamine can activate MAP kinases, HAECs were examined at different time points after stimulation (Figure 6A). p38, ERK, and JNK were transiently activated after stimulation with histamine (10^{-5} mol/L). Maximal phosphorylation of p38 and ERK occurred after 5 minutes and returned to basal levels within 30 and 60 minutes, respectively. Maximal phosphorylation of JNK was observed after 15 minutes and declined to basal levels within 60 minutes. No change in total expression of p38, ERK, or JNK was observed at any time point. Blocking of the H_{1} receptor with mepyramine (10^{-5} mol/L) completely abolished activation of p38, ERK, and JNK (Figure 6B). Again, total expression of the MAP kinases remained unchanged.

To analyze the involvement of MAP kinases in histamine-induced TF expression, we examined the effect of MAP kinase inhibitors on TF induction. SB203580 (10^{-6} to 10^{-5} mol/L), a specific inhibitor of p38, reduced histamine-induced TF expression in a concentration-dependent manner to 17% of control (Figure 7A). Similarly, preincubation with PD98059 (3×10^{-7} to 3×10^{-6} mol/L), a specific inhibitor of ERK phosphorylation, decreased TF expression in a
concentration-dependent manner to 38\% of control (Figure 7B). Finally, SP600125 (10^{-7} to 10^{-6} mol/L), a specific inhibitor of JNK, diminished histamine-induced TF expression to 44\% of control (Figure 7C). No cytotoxic effect of either of these drugs was observed at the concentrations used \( (n=3; P=NS; \text{data not shown}). \)

The Rho pathway is involved in the regulation of thrombin-induced TF expression. Therefore, the effect of Rho-kinase inhibitors on histamine-induced TF expression was examined. Pretreatment with Y-27632 (10^{-5} mol/L) or hydroxyfasudil (10^{-5} mol/L) did not result in a significant alteration of TF expression after stimulation with histamine (10^{-2} mol/L; Figure 8A). Activation of the phosphatidylinositol 3-kinase (PI3-kinase) pathway has been described in thrombin-induced TF expression.\textsuperscript{22,24} Therefore, the effect of PI3-kinase inhibitors on histamine-induced TF expression was assessed. Pretreatment with LY294002 (5\times10^{-6} mol/L) or wortmannin (10^{-7} mol/L) enhanced histamine-induced TF expression (Figure 8B).

**Discussion**

The present study demonstrates that histamine induces a concentration- and time-dependent increase in TF expression in vascular cells. Biologically active TF is located at the cell surface; indeed, stimulation of TF expression by histamine was paralleled by enhanced TF surface activity. The increase in the latter was less pronounced than that in TF protein expression; the distribution of TF in several cellular compartments or the expression of encrypted TF at the cell surface may account for this difference.\textsuperscript{25,26} Unlike endothelial cells, unstimulated vascular smooth muscle cells displayed a relatively pronounced basal expression of TF, consistent with the rapid formation of a localized nidus of coagulation after endothelial denudation in vivo. This higher basal expression...
in HAVSMCs may account for the observation that a maximal increase in both TF expression and activity after stimulation with histamine was lower than in HAECs. The small but significant increase in TF expression in HAVSMCs after the addition of mepyramine or cimetidine compared with control conditions was probably due to partial agonistic activity of the respective antagonist.

TF expression is known to be regulated at the transcriptional level in response to many stimuli. Consistent with these observations, histamine induced TF mRNA transcription in a time-dependent manner. Indeed, the kinetics of mRNA induction in response to histamine were similar to those observed with other mediators. Moreover, the sequence of intracellular events after stimulation with histamine is consistent with a logical order: The maximum MAP kinase activation occurred after 5 to 15 minutes, RNA expression peaked after 1 hour, and protein expression reached its maximum after 5 hours.

Histamine-induced TF expression was compared with that in response to TNF-α, a known potent inducer of TF. In both HAECs and HAVSMCs, histamine induced TF to more than one third of the level observed after stimulation with 10 ng/mL TNF-α. Because this concentration of TNF-α is very high, the effect of histamine compares well with that of the classic inducer of TF expression.

At the cellular level, TF expression is counterbalanced by TFPI. Several inducers of TF expression have been investigated with respect to their ability to affect TFPI expression; indeed, an increase, a decrease, or no alteration in TFPI expression has been observed after endothelial stimulation with TNF-α, LPS, and 5-hydroxytryptamine. Stimulation of HAECs with histamine did not affect TFPI expression; thus, histamine-induced TF expression may result in particularly pronounced procoagulative changes because it is not paralleled by an increase in TFPI.

TF expression in HAECs and HAVSMCs is mediated by activation of the H1 receptor. Indeed, activation of this receptor plays an important role in the pathogenesis of cardiovascular disease. Vascular contractions to histamine are mediated through activation of the H1 receptor. Histamine-induced P-selectin expression on endothelial cells, a key step in leukocyte transmigration to the subendothelium, also is mediated by this receptor. Chlorpheniramine, a specific H1 receptor blocker used in our study, prevents lesion formation in a rabbit model of atherosclerosis, and the H1 receptor blocker diphenhydramine, but not the H2-receptor blocker cimetidine, prevents intimal thickening after vascular injury. Consistent with these observations, expression of the H1 receptor is increased in endothelial and vascular smooth muscle cells from atherosclerotic vessels. Hence, the effect of histamine on TF expression adds to the evidence that histamine-induced vascular dysfunction is mediated through activation of the H1 receptor.

Histamine leads to activation of p38, ERK, and JNK via the H1 receptor. Consistent with our observation, activation of MAP kinases has been demonstrated in vascular cells in response to histamine. Indeed, activation of p38 and ERK is mediated through the H1 receptor in a smooth muscle cell line. Other inflammatory agents such as TNF-α are known to activate all 3 MAP kinases in vascular cells. With respect to TF expression, the pattern of MAP kinase activation in vascular cells is largely dependent on the specific stimulus. p38 is involved in thrombin-induced TF expression in endothelial cells, and p38 and ERK mediate TNF-α- and vascular endothelial growth factor–induced TF expression in endothelial cells. Furthermore, JNK mediates TF expression in endothelial cells stimulated with TNF-α. In contrast, ERK, but not p38, regulates lysophosphatic acid–induced TF expression in vascular smooth muscle cells. Interestingly, in both LPS-stimulated monocytes and thrombin-stimulated en-
dothelial cells, TF expression occurs through activation of all 3 MAP kinase pathways.\textsuperscript{27,39} We show here that specific inhibition of any of the 3 MAP kinases leads to a marked reduction in histamine-induced TF expression in HAECs even at low concentrations of the respective inhibitor. Thus, similar to LPS-stimulated monocytes,\textsuperscript{27} activation of p38, ERK, and JNK cooperatively regulates histamine-induced TF expression in HAECs. Inhibition of p38 has beneficial effects on cardiac ischemia, reperfusion, endothelial function, and hypertensive end-organ damage.\textsuperscript{40,41} Hence, our study implicates that inhibiting MAP kinases might represent a potential target for the treatment of thrombotic vascular disease. Nevertheless, the use of MAP kinase inhibitors in vivo is still controversial and requires further study.\textsuperscript{41,42}

RhoA activation plays a major role in thrombin-induced TF expression in HAECs.\textsuperscript{22,24} Indeed, histamine exerts some of its effects on endothelial cells through activation of the Rho pathway.\textsuperscript{43,44} Surprisingly, Rho-kinase inhibition did not alter TF expression in response to histamine. Hence, the Rho pathway does not seem to be involved in histamine-induced TF expression. Indeed, RhoA activation may be affected by the cell type and/or the specific stimulus involved; alternatively, cell culture conditions may play a role, as has been implied in the regulation of cell permeability by histamine.\textsuperscript{43,44} PI3-kinase inhibition enhances thrombin- and TNF-α-stimulated TF expression in endothelial cells and monocytes, respectively.\textsuperscript{22,24,27} Consistent with these observations, histamine-induced TF expression was further increased after inhibition of PI3-kinase. Hence, similar to TF expression stimulated by other mediators, the PI-3 kinase pathway also plays a negative regulatory role in histamine-induced TF expression.

Histamine is released from mast cells, endothelial cells, and activated platelets and elicits potent arterial contractions.\textsuperscript{1–3,11,32,45} Vasospasm mainly occurs in coronary arteries exhibiting some atherosclerotic changes and is promoted by the presence of mast cells and dysfunctional endothelial cells. Coronary artery vasospasm is involved in variant angina and has been implicated in coronary artery thrombus formation.\textsuperscript{12–14} The latter is indeed crucial for the pathogenesis of arterial occlusion, and TF is a key enzyme for its initiation. As histamine induces TF expression, the release of the amine from mast cells or endothelial cells may represent an explanation for the frequent simultaneous occurrence of atherosclerosis, vasospasm, and thrombus formation. Moreover, the release of histamine from aggregating platelets may function as a positive feedback mechanism, further enhancing TF expression and thus thrombus formation. Interestingly, histamine levels are increased in platelets from patients with peripheral artery disease.\textsuperscript{46}

Augmented TF expression in HAECs and HAVSMCs after stimulation with histamine is consistent with atherosclerosis as an inflammatory disease.\textsuperscript{9,15} Indeed, elevated levels of TF have been observed in patients with hypertension, dyslipidemia, diabetic vasculopathy, and peripheral artery disease.\textsuperscript{15,47–50} Not surprisingly, TF has also been implicated in coronary artery disease and acute myocardial infarction.\textsuperscript{15} Here, we show that H\textsubscript{1} receptor antagonists completely abolish histamine-induced TF expression in HAECs. Hence,
our findings suggest a therapeutic potential of $H_1$ receptor antagonists in the treatment of acute coronary syndromes.

In summary, histamine-induced TF expression may mediate thrombus formation in atherogenesis, especially in inflammation and vasospasm, implicating interesting novel perspectives for the treatment of vascular occlusive diseases such as acute coronary syndromes.

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