Histamine Upregulates Gene Expression of Endothelial Nitric Oxide Synthase in Human Vascular Endothelial Cells

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Background—Histamine has a short-term, transient, stimulating effect on endothelial nitric oxide synthase (eNOS) activity; however, long-term effects on eNOS have not been described yet. In addition, the vascular effect of histamine seems to depend critically on eNOS functionality. Therefore, we studied the effects of histamine on eNOS gene expression and function.

Methods and Results—In human umbilical vein endothelial cells (HUVECs) and HUVEC-derived EA.hy 926 cells, histamine upregulated eNOS mRNA (RNase protection assay) and protein (electron microscopic immunocytochemistry) expression. The upregulation of eNOS could be prevented by mepyramine, a selective antagonist at the H₁ receptor, but not by H₂ and H₃ receptor antagonists. Incubation of EA.hy 926 cells with histamine led to the activation of calcium/calmodulin-dependent protein kinase II (CaMK II; in vitro phosphorylation assay). The histamine-induced eNOS expression was completely prevented by KN-93, an inhibitor of CaMK II. Histamine increased the activity of a 1.6-kb human eNOS promoter fragment (luciferase reporter gene assay), an effect that was also blocked by mepyramine. Under normal conditions, eNOS upregulation by histamine resulted in increased nitric oxide production (measured by nitric oxide chemiluminescence and RFL-6 reporter cell assay). Under conditions of oxidative stress, however, the eNOS upregulated by histamine produced reactive oxygen species (CM-H₂DCFDA oxidation-based fluorescence assay).

Conclusions—Stimulation of the H₁ receptor increases eNOS transcription in endothelial cells by a signaling pathway involving CaMK II. This eNOS upregulation may be protective under normal conditions, but it may become harmful under conditions of oxidative stress when eNOS produces reactive oxygen species at the expense of nitric oxide. ((Circulation. 2003;107:2348-2354.)

Key Words: endothelium-derived factors | signal transduction | oxidative stress | atherosclerosis | coronary disease

Histamine is an established endothelium-dependent vasodilator. There are numerous sources of histamine in the vasculature. In addition to histamine from circulating blood and vascular endothelial cells, histamine can be released locally from subendothelial mast cells and adventitial mast cells in coronary arteries. Moreover, histamine can also derive from activated platelets. T lymphocytes, and monocytes/macrophages. By occupying H₁ receptors on endothelial cells, histamine activates endothelial nitric oxide synthase (eNOS). The nitric oxide (NO) produced by eNOS dilates all kinds of blood vessels, including human coronary arteries, and has protective effects against platelet and leukocyte adhesion and smooth muscle proliferation and, thus, probably atherosclerosis. However, the direct activation of eNOS by histamine represents a short-term and probably transient action of the amine. Therefore, the present study was designed to investigate the potential long-term effects of histamine on eNOS gene expression and the ensuing changes in enzyme activity. The results show that under normal, healthy conditions, histamine can upregulate eNOS gene expression, resulting in increased NO production. This would suggest an indirect vasoprotective role for histamine.

However, there is evidence for pro-atherogenic effects of histamine. For example, histamine enhances the expression of adhesion molecules in vascular endothelial cells, thereby augmenting leukocyte-endothelial cell interactions and migration, and it has been implicated in intimal thickening and atherogenesis. In animal models, antihistamines provided protection against intimal thickening and the development of atherosclerosis.
In conditions of oxidative stress and atherosclerosis, eNOS can “uncouple” and become dysfunctional. A relative lack of (6R)-5,6,7,8-tetrahydro-L-biopterin due to oxidation seems to play a crucial pathophysiological role for eNOS dysfunction. The dysfunctional eNOS generates superoxide from its oxygenase domain instead of NO by dissociation of the ferrous-dioxygen complex. Therefore, in the present study, we also tested the functional consequences of a histamine-induced upregulation of eNOS under conditions of exogenous oxidative stress.

**Methods**

**Cell Culture**

Human umbilical vein endothelial cells (HUVECs, isolated by collagenase digestion) and HUVEC-derived EA.hy 926 cells (kindly provided by Dr Cora-Jean Edgell, Chapel Hill, NC) were cultured as previously described. HUVECs from passages 3 to 5 were used.

**RNase Protection Assay for eNOS mRNA Analyses**

Confluent HUVECs and EA.hy 926 cells were incubated with histamine, and total RNA was isolated. The expression of eNOS mRNA was analyzed by an RNase protection assay, as described previously.

**Reverse-Transcriptase Polymerase Chain Reaction for Analysis of Histamine Receptor Expression**

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using total RNA from HUVECs, EA.hy 926 cells, and human heart, brain, and ileum tissues as templates. Primers were designed to distinguish human H₁, H₂, and H₃ subtypes. The primers were TCTCTCTTTTCTGTGGGTATTCC (sense) and GATGCGTTCTAACACCTCATGGAT (antisense) for H₂, and CTGACTCTGTTACGAAACCTCT (sense) and TCTCTCAGCAATTTTGCTCTCTTT (antisense) for H₃. The sizes of the 3 RT-PCR products were 259 bp, 311 bp, and 244 bp, respectively.

**Electron Microscopic Immunocytochemistry**

After treatment with histamine, EA.hy 926 cells were collected by trypsin digestion. The cells were then concentrated by centrifugation, fixed, and embedded in London Resin White. Ultrathin sections were prepared, and post-embedding immunolabeling was performed using a rabbit polyclonal anti-eNOS antibody (BD Biosciences Pharmingen, San Diego, Calif) and a gold-labeled anti-rabbit secondary antibody (10-nm particles, Sigma, St Louis, Mo). The ultrathin sections were then analyzed with an energy-filtering transmission electron microscope, as previously described. The density of gold particles was determined in 20 sections and in 20 frames of 1 μm² in each section.

**Transient Transfection and Reporter Gene Assay for the Analysis of eNOS Promoter Activity**

Promoter activity was analyzed by reporter gene assay using the plasmid pGL₃-eNOS-Hu-1600 transiently transfected into EA.hy 926 cells. The plasmid contained a 1.6-kb human eNOS promoter fragment (~1600 to 23) cloned before the luciferase gene of pGL₃-Basic, as described elsewhere. The density of gold particles was determined in 20 sections and in 20 frames of 1 μm² in each section.

**Assay of Ca²⁺/Calmodulin-Dependent Kinase II Activity**

Ca²⁺/calmodulin-dependent kinase II (CaM II) activity was assayed using the SnaTect system (Promega). Briefly, 2.5 μg of total protein samples from EA.hy 926 cells were incubated at 30°C for 2 minutes in the presence of [γ-³²P]ATP with a biotinylated peptide, which is a selective substrate for CaM II. The reaction was then spotted onto a streptavidin-coated SAM-Biotin-Capture membrane. The membrane was washed, dried, and exposed to x-ray film.

**Determination of Total NO Synthesis as Nitrite/Nitrate and Measurement of Bioactive NO**

Oxidation products of NO (NOₓ, ie, NO⁻ and NO₃⁻) were assayed in the supernatant of EA.hy 926 cells as a measure of total NO synthesis using a NOA 280 NO Analyzer. Bioactive NO produced by EA.hy 926 cells was assayed using rat fetal lung fibroblast (RFL-6) as reporter cells.

**Measurement of Intracellular Reactive Oxygen Species**

The determination of intracellular oxidative formation was based on the oxidation of 5-((and)-chloromethyl)-2,7′-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) to yield an intracellular-trapped fluorescent compound. Fluorescence was measured in a FluoroCount Reader (Packard Bioscience). After 24 hours of pretreatment with 1 μmol/L histamine, the cells were washed, and intracellular reactive oxygen species (ROS) formation was assayed in the presence of 10 μmol/L A23187, because superoxide/ROS generation from eNOS is a Ca²⁺-dependent process. Other cells were exposed to oxidative stress (after the 24-hour histamine pretreatment) by a 1-hour incubation with xanthine (200 μmol/L)/xanthine oxidase (5 μmol/L), either alone or in combination with 1 mmol/L N⁶-nitro-L-arginine methyl ester (L-NAME). Then, the cells were washed several times to remove the xanthine/xanthine oxidase, and ROS production was assayed in the presence of 10 μmol/L A23187 for 1 hour.

**Statistics**

Statistical differences between mean values were determined by ANOVA followed by Fisher’s protected least-significant-difference test for comparison of different means.

**Results**

**Histamine Upregulates eNOS mRNA Expression in Human Endothelial Cells in a Concentration- and Time-Dependent Manner**

Treatment of EA.hy 926 human endothelial cells with histamine increased eNOS mRNA expression (Figure 1); eg, incubation with 1 μmol/L histamine for 24 hours increased eNOS expression to 189.5 ± 16.8% of control. Inducible nitric oxide synthase was not expressed in EA.926 cells, nor was it induced by histamine (3 experiments, data not shown). Also, in HUVECs, incubation with 1 μmol/L histamine for 24 hours increased eNOS expression to 230.3 ± 29.4% (mean ± SEM of 3 experiments, P < 0.001 compared with untreated cells).

**The Histamine-Induced eNOS Upregulation Is Mediated by the H₁ Receptor**

mRNAs for H₁ and H₃, but not H₂, were found in HUVECs and EA.hy 926 cells by RT-PCR (Figure 2A). To characterize the receptor subtype functionally responsible for the histamine-induced eNOS upregulation, selective antagonists to H₁ (mepyramine), H₂ (cimetidine), and H₃ (clobenpropit) were used. Mepyramine, but not cimetidine or clobenpropit, completely abolished the eNOS induction by histamine (Figure 2B). The selective H₁ agonist HTMT [6-[(4-imidazolyl)ethylamine]-N-(4-trifluormethylphenyl)-heptanecarboxamide dimaleate] also increased eNOS mRNA in EA.hy 926 cells in a concentration-dependent fashion (3 experiments, data not shown).

**Histamine Enhances the Activity of the Human eNOS Promoter**

A 1.6-kb human eNOS promoter fragment showed significant basal activity when transiently transfected into EA.hy...
Histamine-Induced eNOS Upregulation Is Prevented by an Inhibitor of CaMK II, But Not by Inhibitors of Protein Kinase C, Janus Kinase 2 (JAK2), Mitogen-Activated Protein Kinases, or Phosphatidylinositol-3-Kinase

The specific inhibitor of CaMK II, KN-93, completely abolished the eNOS induction by histamine (Figure 4A). The
inactive analog of KN-93, KN-92, did not have any effect on eNOS expression, thus confirming the specificity of KN-93 (Figure 4A).

It has been reported that H1 stimulation can also activate protein kinase C, tyrosine kinases, mitogen-activated protein kinases, and phosphatidylinositol-3-kinase. The protein kinase C inhibitors Ro-31-8220 (1 μmol/L) and Gö6983 (1 μmol/L), the cells were analyzed for luciferase activity. Columns represent the mean±SEM of 3 experiments. ***P<0.001 compared with control (Co); ###P<0.001 compared with cells treated with histamine alone.

Figure 3. Histamine enhances eNOS promoter activity in EA.hy 926 cells. The cells were transfected with either the vector pGL3-Basic (containing a promoterless luciferase gene) or pGL3-eNOS-Hu-1600 (containing a 1.6-kb human eNOS promoter fragment cloned before the luciferase reporter gene). After 24 hours of treatment with either histamine alone (His; 1 μmol/L) or in combination with the H1 receptor antagonist mepyramine (Mepy; 1 μmol/L), the cells were analyzed for luciferase activity. Columns represent the mean±SEM of 3 experiments. ***P<0.001 compared with control (Co); ###P<0.001 compared with cells treated with histamine alone.

Histamine Activates CaMK II in EA.hy 926 Cells
Incubation of EA.hy 926 cells with histamine led to the activation of CaMK II. The CaMK II-specific phosphorylation was increased after histamine treatment (Figure 4B). This activation was maintained between 5 minutes and 3 hours (Figure 4B). Preincubation with KN-93 (3 μmol/L, 1 hour) reduced the basal activity of CaMK II and completely abolished the activating effect of histamine (Figure 4B).

Histamine Upregulates eNOS Protein Expression in EA.hy 926 Cells
In addition, eNOS protein expression was increased in EA.hy 926 cells by histamine, as determined by electron microscopic immunocytochemistry. The histamine-treated cells showed an increased intracellular eNOS staining (Figure 5).

Figure 4. Activation of Ca2+/calmodulin-dependent-kinase II (CaMK II) is required for histamine-induced eNOS expression in human EA.hy 926 endothelial cells. A, the cells were treated for 24 hours with 1 μmol/L histamine (His), alone or in combination with KN-93 (a specific CaMK II inhibitor; 3 μmol/L) or KN-92 (an inactive analog of KN-93; 3 μmol/L), and eNOS mRNA expression was analyzed with an RNase protection assay. Columns represent the mean±SEM of 3 experiments. ***P<0.001 compared with control; ###P<0.001 compared with histamine-treated cells. B, histamine activates CaMK II in EA.hy 926 cells. Cell lysates were incubated with a biotinylated peptide (a selective substrate for CaMK II) in the presence of [γ-32P]ATP. The phosphorylated peptides were captured by a streptavidin membrane and visualized by autoradiography.

Short- and Long-Term Effects of Histamine on NO Production in EA.hy 926 Cells Under Normal Conditions
After 24 hours of treatment of EA.hy 926 cells with 1 μmol/L histamine, the cumulative NO production (determined as NOx in the culture supernatant) increased almost 4-fold (Figure 6A). When histamine was present only during the last hour of the 24-hour incubation, the increase in NOx was only 2-fold. After washout, EA.hy 926 cells were stimulated with 10 μmol/L Ca++ ionophore A23187 for 1 hour. An increase in NOx was only seen in those cells that had been pretreated with histamine for 24 hours (Figure 6A). These results were confirmed with a different method, the RFL-6 reporter cell assay. This assay demonstrated that EA.hy 926 cells whose eNOS had been upregulated by histamine (24 hours of pretreatment) showed an increased production of bioactive NO on stimulation with the Ca2+ ionophore A23187 (Figure 6B).
Histamine-Induced eNOS Produces ROS Under Conditions of Oxidative Stress

The treatment of EA.hy 926 cells with 1 μmol/L histamine for 24 hours under normal cell culture conditions did not increase ROS generation (Figure 7A). However, when the cells were preexposed to oxidative stress (1-hour treatment with xanthine/xanthine oxidase), a significant increase in ROS formation was observed in cells pretreated with histamine (Figure 7B). This increase was prevented by the NOS inhibitor L-NAME, indicating that eNOS contributed to ROS production under these conditions.

Discussion

The present study demonstrates that histamine upregulated eNOS mRNA and protein expression in HUVECs and HUVEC-derived EA.hy 926 cells (Figures 1 and 5).

Results from RT-PCR using subtype-specific primers indicated that H1 and H2 were expressed in HUVECs and EA.hy 926 cells (Figure 2A). However, the selective H2 antagonist cimetidine had no effect on histamine-induced eNOS expression, indicating that the H2 receptor is not involved in eNOS upregulation. Moreover, H2 receptors signal through adenylyl cyclase and cAMP, and our previous work has demonstrated that cAMP analogs do not affect eNOS expression in EA.hy 926 cells. Thus, the histamine-induced eNOS expression in human endothelial cells is probably mediated by the H1 receptor, because (1) eNOS induction was prevented by the selective H1-antagonist mepyramine (Figure 2B) and (2) the selective H1 agonist HTMT upregulated eNOS expression.

Histamine-induced eNOS expression is likely a transcriptional event. Histamine increased the activity of the 1.6-kb human eNOS promoter fragment in HUVECs and EA.hy 926 cells (Figure 3). In good agreement with mRNA expression data, activation of the eNOS promoter was blocked by mepyramine, indicating that the transcriptional activation is mediated through the H1 receptor.

H1 receptor stimulation results in an activation of phospholipase C and the production of inositol-1,4,5-trisphosphate, which in turn releases Ca2+ from inositol-1,4,5-trisphosphate–sensitive stores. Increases in intracellular free Ca2+, [Ca2+]i, in response to histamine, have been demonstrated for EA.hy 926 cells, as well as HUVECs. In human endothelial cells, the Ca2+–sensitive target responsible for the histamine-induced eNOS expression seems to be CaMK II, a ubiquitous enzyme that is also present in...
then stimulated with Ca2+ and eNOS expression. Indeed, when the cells were washed and persistent NO release, probably resulting from increased production of ROS after exposure of endothelial cells to xanthine/xanthine oxidase. Under these conditions, the eNOS upregulated by histamine indeed contributed to ROS production (Figure 7) and, thus, may enhance the preexisting oxidative stress. We have previously reported similar phenomena in animals with experimental diabetes and hypertension.16,24,25 In all of these cases, the NO bioavailability decreased despite an upregulation of eNOS.

Decreased NO bioavailability can also contribute to histamine-induced coronary vasospasm. Under conditions of dysfunctional eNOS, the histamine-induced vasorelaxation is converted into a contraction, which is mediated via H1 receptors on smooth muscle cells.9–26 Histamine-induced vasospastic coronary vasospasm has also been observed in atherosclerotic coronary arteries26–28 and may contribute to the onset of an acute coronary syndrome. Interestingly, an increased histamine content has been found in atherosclerotic coronary segments.5,27,29

In conclusion, the present study demonstrates a novel effect of histamine on endothelial NO production. In addition to its short-term and transient action on eNOS activity, histamine also possesses a long-term effect on eNOS expression. The effect is H1 receptor–dependent and CaMK II–mediated. This eNOS upregulation may be protective under normal conditions by increasing NO production, but it may become harmful under conditions of oxidative stress when eNOS produces ROS at the expense of NO.

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