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 cells¹–³ and adventitial mast cells⁴–⁵ in coronary arteries. Moreover, histamine can also derive from activated platelets.⁶ T lymphocytes, and monocytes/macrophages.⁷ By occupying H₁ receptors on endothelial cells, histamine activates endothelial nitric oxide synthase (eNOS).⁸ The nitric oxide (NO) produced by eNOS dilates all kinds of blood vessels, including human coronary arteries,⁹ and has protective effects against platelet and leukocyte adhesion and smooth muscle proliferation and, thus, probably atherosclerosis.¹⁰ 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,¹¹–¹³ an important onset event in atherogenesis. Histamine has also been shown to increase smooth muscle cell proliferation and migration,⁶ and it has been implicated in intimal thickening and atherogenesis.¹⁴,¹⁵ In animal models, antihistamines provided protection against intimal thickening and the development of atherosclerosis.¹⁴
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.

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 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.

Assay of Ca²⁺/Calmodulin-Dependent Kinase II Activity

Ca²⁺/calmodulin-dependent kinase II (CaMK II) activity was assayed using the SgnATect 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 CaMK 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.

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 H₂, but not H₃, 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-[2-(4-imidazolyl)ethylamine]-N-(4-trifluoromethylphenyl)-heptanecardoxamide 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...
Incubation with histamine (1 μmol/L) increased the promoter activity 2-fold. This promoter activation was completely prevented by mepyramine. Also in HUVECs, incubation with histamine (1 μmol/L) resulted in a marked increase in eNOS promoter activity (263.1±24.0% of control; P<0.001 versus control; 3 experiments).

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 Gö 6983 (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).

**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 Ca2+ 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,22 as well as HUVECs.8 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

Figure 5. Histamine increases eNOS protein expression. A, electron microscopic immunocytochemistry showing eNOS expression in EA.hy 926 cells (treated with or without 1 μmol/L histamine [His] for 24 hours). Post-embedding immunolabeling was performed using a rabbit polyclonal anti-eNOS antibody and a gold-labeled anti-rabbit secondary antibody. The ultrathin sections were then analyzed with an energy-filtering transmission electron microscope. B shows the quantified density of gold particles in 20 sections. Columns represent mean±SEM. ***P<0.001 vs untreated controls (Co).

Figure 6. Short-term and long-term effects of histamine on endothelial NO production. A, EA.hy 926 cells were left untreated (control, Co) or were treated with 1 μmol/L histamine. Histamine was present either for 24 hours (His 24h) or only during the last hour (His 1 hour, without changing the media). Supernatants were collected at the end of the 24-hour incubation, and NO2/NO3 (NOx) was measured with an NO Analyzer (upper left). Then, the cells were washed twice, kept in normal media for 2 hours, washed again, and stimulated with 10 μmol/L Ca2+ ionophore A23187 for 1 hour. Supernatants were collected again for NOx measurement (upper right). NOx amount was normalized to protein content of cell lysates. B, EA.hy 926 cells were left untreated or were treated with 1 μmol/L histamine for 24 hours. The cells were then washed, stimulated with 10 μmol/L Ca2+ ionophore A23187 for 2 minutes, and bioactive NO was determined by RFL-6 reporter cell assay. Columns represent mean±SEM of 3 experiments. **P<0.01; ***P<0.001.
vasculature endothelial cells. The histamine-induced eNOS expression was completely abolished by KN-93, a specific inhibitor of CaMK II (Figure 4A), whereas the inactive analog of KN-93, KN-92, was without effect (Figure 4A). Histamine treatment of EA.hy 926 cells led to activation of CaMK II, which was sensitive to KN-93 (Figure 4B). Also in HUVECs, histamine activated CaMK II. These data indicate that the histamine-induced eNOS expression involves CaMK II.

An upregulation of eNOS gene expression could result in a sustained enhancement of NO production in addition to the known short-term and transient effect of histamine on eNOS activity. Stimulation of EA.hy 926 cells with histamine for 1 hour resulted in an ≈2-fold increase in NOx (Figure 6A). This likely reflects the short-term activation of eNOS by histamine. If, however, the cells were treated with histamine for 24 hours, the increase of accumulative NOx was ≈4-fold. Agonist-stimulated eNOS enzyme activation is usually transient, and the enzyme activity returns to control levels within 30 minutes. Thus, the cumulative NO released from endothelial cells during 24 hours is likely to consist of 2 components: short-term NO release due to eNOS activation and a persistent NO release, probably resulting from increased eNOS expression. Indeed, when the cells were washed and then stimulated with Ca²⁺ ionophore, increased NO production was only seen in cells pretreated with histamine for 24 hours, not in those treated for 1 hour (Figure 6A). In agreement with this, bioactive NO, as measured by RFL-6 reporter cell assay, was increased after 24 hours of treatment with histamine (Figure 6B). Thus, under normal conditions and even at the early stages of vascular disease, the histamine-induced eNOS expression may represent a vaso-protective factor.

However, advanced stages of vascular diseases (atherosclerosis, diabetes, and hypertension) are associated with endothelial dysfunction and oxidative stress. In the present study, we tried to mimic these conditions by exposing 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. 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. Histamine-induced vasospastic constriction has also been observed in atherosclerotic coronary arteries and may contribute to the onset of an acute coronary syndrome. Interestingly, an increased histamine content has been found in atherosclerotic coronary segments. 

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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