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
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 TCTCCTTTTCTGTGGTATCC (sense) and CAGCTTAATTTCTGAGAAGGA (antisense) for H1; ATACACCTCTAAGTGCAAAGTCC (sense) and GATGCGTTCTAACACCTCTATTGAT (antisense) for H2; and CTGACTACTGTACGAAACCTCCT (sense) and CTCCCTGAGCAATTTTGTCTCTCTT (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 Pharmin- gen, 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 (CaMK II) activity was assayed using the SigmaTect 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.

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

Oxidation products of NO (NOx, i.e., NO²⁻ 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-6)-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 μU/mL), 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); e.g., 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 H₃ 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 H₃ agonist HTMT (6-[(4-imidazolyl)ethylamine]-N-(4-trifluoromethylphenyl)-heptane-carboxamide 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
cells (Figure 3). 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) did not prevent, but slightly increased, histamine-induced eNOS expression (3 experiments each; data not shown). Inhibitors of tyrosine kinases (10 μmol/L AG 490, 10 μmol/L genistein, 10 μmol/L erbstatin A, or 1 μmol/L herbimycin), mitogen-activated protein kinase kinase (10 μmol/L PD 98059), p38 mitogen-activated protein kinase (10 μmol/L SB 203580), and phosphatidylinositol-3-kinase (10 μmol/L LY 294002) had no effect on histamine-induced eNOS expression (3 experiments each; data not shown). A previous study demonstrated that CaMK II is also involved in H2O2-induced eNOS expression, a pathway that also requires the tyrosine kinase JAK2. However, the JAK2 inhibitor, AG 490, and other tyrosine kinase inhibitors did not affect histamine-induced eNOS expression, thus indicating that JAK2 is not involved.

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).
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, H1 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.

The 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...
vascular 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 vasoprotective 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 vascular 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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References


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