Hi$tama$e is a classical inflammatory mediator mainly
produced from mast cell and exerts acute responses
ingcluding hypersensitivity, vasodilation, and vasoconstric-
tion. With regards to coronary artery, especially in athero-
sclerotic artery accompanying endothelial dysfunction, hista-
mine is a potent vasoconstrictor, and accumulation of
activated mast cells in adventitia and ruptured plaques in the
cases of acute coronary syndrome has been reported.1–3 On
the other hand, monocytes/macrophages are present in all
stage of atherosclerosis and also express a rate-limiting
histamine-producing enzyme: histidine decarboxylase
(HDC).4 Besides mast cells, monocytes/macrophages are
potential sources of histamine in the atherosclerotic lesions
and histamine from these cells would chronically participate
in the pathogenesis of atherosclerosis by regulating athero-
sclerosis-related gene expression.5,6

Histamine effects are mediated through its specific recep-
tors, which are classified into H1 to H4 types.7 Histamine
receptors are expressed in the atherosclerotic intima, and H1
receptors are predominantly expressed in the intimal smooth
muscle cells (SMCs) of human atheromatous plaques.8 In
relation to atherogenesis, histamine stimulates intimal SMCs
to proliferate and to express matrix metalloproteinase-1
(MMP-1).9 Moreover, monocylic expression of tumor necro-
sis factor (TNF)-α, monocyte chemoattractant protein-1
(MCP-1), and lectin-like oxidized low-density lipoprotein
receptor-1 (LOX-1) is regulated by histamine through histo-
mine H1 (HH1R) or H2 receptor (HH2R).5,10,11 These find-
ings indicate that locally produced histamine in the intima
might participate in regulation of atherosclerosis-related gene
expression in monocytes and SMCs, and in proliferation of
SMCs.6

In contrast, nitric oxide (NO) is a powerful vasodilator and
inhibits the proliferation of SMCs.12 The expression of
inducible NO synthase (iNOS) is induced by inflammatory
cytokines such as interferon (IFN)-γ, TNF-α, and interleukin
(IL)-1β and by bacterial lipopolysaccharide (LPS) through
NF-κB activation.13,14 In turn, NO inhibits NF-κB by induc-
ing IκBα.15 Because histamine is one of the major inflam-
matory mediators and known to activate NF-κB pathway
through HH1R stimulation, we speculated that histamine would upregulate the iNOS expression in the intimal SMCs. In addition, a recent report has demonstrated an induction of endothelial NO synthase (eNOS) expression by histamine mediated through HH1R in human umbilical vein endothelial cells, suggesting a presence of functional linkage of histamine, NO, and NF-κB in the vascular microenvironment.

In this study we have reported a molecular mechanism by which histamine induces the iNOS gene expression in cultured human intimal SMCs. Histamine upregulated the iNOS but not eNOS expression through activation of HH1R and NF-κB signaling pathway. Also, we examined histamine content in the neointima, media, and adventitia of the human atherosclerotic aortas from adults and neonates.

**Materials and Methods**

**Chemicals**

Histamine was purchased from Wako Pure Chemical (Tokyo, Japan). Histamine blocker (H1 blocker, pyrilamine and chlorphenilamine; H2 blocker, cimetidine and famotidine), NF-κB inhibitor (pyrrolidine dithiocarbamate [PDTC]), and iNOS inhibitor (aminoguanidine [AG]), nNOS inhibitor (7-nitroindazole [7-Ni]), and non-specific NOS inhibitor (N′-nitro-L-arginine methyl ester [L-NAME]) were purchased from Sigma. IL-1β and IFN-γ were obtained from PeproTech.

**Cell Culture**

Immortalized ISS10 cells derived from human aortic intimal SMCs were maintained in Dulbecco modified eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C. Each experiment was performed after 24-hour starvation in DMEM with 0.5% FCS. The cell viability was monitored by viable cell count using trypan blue exclusion test and by apoptotic cell count using annexin V detection (Annexin V fluorescein isothiocyanate [FITC] kit, Immunotech). No increased cell death was observed after histamine treatment (data not shown).

**NO Production**

The cells cultured on a slide glass and incubated with DMEM containing 10μmol/L 4-amino-5-methylamino-2′, 7′-difluoro-4-difluoromethyl fluorescein diacetate (DAF-DA; Molecular Probe), and each NOS inhibitor for 1 hour. Then the cells were stimulated with histamine for 5 minutes. For longer histamine stimulation, the cells were first incubated with histamine for 24 hours, and then DAF-DA and each NOS inhibitor were loaded to the cells. Nitrite content in the culture supernatant was measured by a fluorometric assay kit according to the manufacturer’s instruction (Cayman Chemical).

**Nuclear Localization of NF-κB**

The cells cultured on a slide glass were fixed in ice-cold 95% acetone for 2 minutes and air-dried. The slide glasses were incubated with mouse monoclonal anti-NF-κB (anti-p65 subunit; Chemicon) antibody, followed by FITC-conjugated anti-mouse IgG antibody (Zymed Laboratories).

**Western Blotting**

Protein extracts (50 μg/lane) prepared from SMCs and rabbit polyclonal anti-human iNOS antibody (Signal Transduction Laboratory) were used for Western blot analysis as described previously.

**Northern Blotting**

Northern blot analysis was performed as described previously. A 32P-labeled probe including 435-bp fragment of human iNOS cDNA was generated by RT-PCR with the following primer pairs: 5′-TGGAATTCATCAGCTGTGC/5′-CCATGATGGTCACATCTGCG.

**Plasmid Construction**

The luciferase reporter plasmid, including 8.5-kp fragment of the promoter region of human iNOS (hiNOS) gene, was provided by Dr Joel Moss (National Institute of Health, Bethesda, Md). The 5′-serial deletion constructs were generated by appropriate restriction enzyme digestion. Mutation of the NF-κB motif (kb4 located at −3922o to −3914) was generated from the Ncol construct (see the motif map in Figure 3) by a substitution of CC to AA (−3915 and −3914). Expression vectors for dominant negative IκBα or IκBβ were provided by Dr Anning Linn (Chicago University, Chicago, Ill).

**Luciferase Assay**

The SMCs cultured in 12-well plates were transfected with the plasmids (1.6 μg/well) by lipofectamine (Invitrogen) for 4 hours, and then incubated with histamine, histamine H1 and H2 blocker, and PDTC for 16 hours. The plasmids expressing dominant negative IκBα or IκBβ kinase β (IKKβ) was used for cotransfection (0.4 μg each/well). The luciferase activity was normalized by β-galactosidase activity obtained by cotransfection of 0.1 μg of β-gal-expressing plasmid.

**Electrophoretic Mobility Shift Assay**

Nuclear extracts were prepared as previously described. The NCol construct includes 4 potential NF-κB sites (named kβ1 to kβ4 located at −4663 to −4654, −4366 to −4357, −4310 to −4299, and −3922o to −3914, respectively). Thus 4 double-stranded oligonucleotide probes including the NF-κβ sites were used (kβ1, 5′-CAACCCCGGTAATCTCATATTCTTA-3′; kβ2, 5′-TAAATTAGGGAAAGCCAGGGCCTGG-3′; kβ3, 5′-TGGCATCAGGTTGTA-3′; and kβ4; 5′-GAGGAGGCATTCTCCACAGGAG-3′; see the motif map in Figure 3). These probes were biotin-labeled at their 3′-end by terminal deoxyxynucleotidyl transferase (Pierce). Nuclear extract (5 μg) and labeled probe (20 fmol) were incubated and applied to electrophoretic mobility shift assay (EMS) according to the manufacturer’s instruction (Lightshift Chiluminescent EMSA Kit, Pierce). For antibody supershift experiments, 2 μg of rabbit polyclonal antibodies specific for NF-κB (p65 subunit; Upstate Biotechnology) were incubated with the nuclear extract at 4°C for 1 hour before incubation with the labeled probes.

**Measurement of Histamine Content in Human Aortic Tissues**

The human aortic tissues obtained from autopsy cases were separated into the intima, media, and adventitia according to the guideline of Japanese Society of Pathology and approved by the Kenwakai Ohtemaci Hospital, Kokurakita-ku, Kitakyushu, Japan, where the autopsy was performed.

**Statistical Analysis**

Data are presented as mean±SD at least obtained from triplicate studies. ANOVA was applied and differences at P<0.05 were considered significant.

**Results**

**NO Production From SMCs**

Because histamine stimulates the SMCs to activate HH1R and Ca2+ signaling pathway, enhancement of NOS enzymatic activity and NO production were measured by DAF-DA method. Histamine treatment (5 minutes) increased NO production, which was partially inhibited by nNOS inhibitor 7-Ni and completely by nonspecific NOS inhibitor.
L-NAME, but not by iNOS inhibitor AG (Figure 1A, upper row). On the other hand, NO production after 24-hour stimulation with histamine was inhibited by AG (Figure 1A, lower row). This suggested that histamine increased NO production mediated through the induction of iNOS expression after the longer histamine treatment. The production of NO measured by the concentration of nitrite in the medium reached maximum at 24 to 36 hours after histamine stimulation (Figure 1B). During 24-hour incubation, histamine increased nitrite production in a dose dependent manner. The histamine-induced nitrite production was inhibited by NF-κB inhibitor PDTC or AG (Figure 1C).

**NF-κB Nuclear Translocation by Histamine**

When the SMCs were stimulated with histamine, NF-κB was translocated into the nuclei. This nuclear localization was inhibited by histamine H1 blocker pyrilamine and PDTC, but not by H2 blocker cimetidine (Figure 1D).

**RESULTS**

Enhanced NO production in intimal SMCs and NF-κB nuclear translocation by histamine. A, Upper row: DAF-fluorescent images in the SMCs showing immediate effects of histamine (50 μmol/L for 5 minutes) on NO production. Histamine-induced NO production was inhibited by nNOS inhibitor 7-Ni (100 μmol/L) and nonspecific NOS inhibitor L-NAME (300 μmol/L) but not by iNOS inhibitor AG (3 mmol/L). Lower row: DAF-fluorescent images after induction of iNOS by 24-hour incubation with histamine (50 μmol/L). Histamine-induced NO production was inhibited by AG (3 mmol/L). B, Time course of NO production by histamine stimulation (50 μmol/L). Maximal concentration of nitrite in the medium was observed at 24 to 36 hours after histamine stimulation (n = 6, *P < 0.05, **P < 0.01). C, The NO production was dose-dependently increased (10 to 50 μmol/L histamine for 24 hours; n = 6, *P < 0.05, **P < 0.01) and reduced by treatment with PDTC (25 μmol/L) and AG (3 mmol/L) (n = 6; ##P < 0.01, ###P < 0.001 vs histamine stimulation). D, Immuno-fluorescent demonstration of NF-κB in the SMCs. The NF-κB was translocated into the nuclei by histamine stimulation (50 μmol/L, 20 minutes), which was inhibited by 1-hour pretreatment with H1 blocker (pyrilamine; 10 μmol/L) and NF-κB inhibitor PDTC (25 μmol/L) but not H2 blocker (cimetidine; 10 μmol/L). As a positive control, the cells were incubated with IL-1β (20 ng/mL) for 20 minutes. Pyr indicates pyrilamine; cim, cimetidine; fam, famotidine.

**iNOS Expression by Histamine**

As well as cytokine mixture (CM: 0.5 ng/mL of IL-1β and 5 ng/mL of IFN-γ) induced the iNOS expression, the iNOS protein and mRNA expression were induced by histamine in a dose-dependent manner (Figure 2A). Furthermore, the histamine-induced iNOS mRNA expression was downregulated by pyrilamine and PDTC but not by cimetidine (Figure 2B). Endothelial NOS mRNA was constitutively expressed and showed no histamine response (data not shown).

**Luciferase Assay by Using Human iNOS Promoter Constructs**

The luciferase constructs harboring hiNOS promoter region were subjected to luciferase assay by using 5’-deletion constructs (Figure 3). The Xmal and Eco47III constructs showed no upregulation of the promoter activities by histamine. When the hiNOS promoter construct was deleted up to NcoI site (~4.8kb), the activity was enhanced by histamine (dashed bar). Further deletion up to EcoRV site (~1.3kb) resulted in low basal activity and no more response to histamine. In contrast, CM induced the hiNOS promoter activities (closed bar) in Xmal, Eco47III, and EcoRV constructs. The NcoI construct, which was only histamine responsive construct, showed no increased promoter activity by CM.

The promoter activity from the NcoI construct showed a dose dependent upregulation by histamine. This histamine-induced promoter activity was decreased by histamine H1 blocker pyrilamine and chlorphenilamine, and PDTC but not by H2 blocker cimetidine and famotidine (Figure 4A). Co-transfection of dominant negative IkBα or IκBβ, which inhibits NF-κB signaling pathway by interfering the dissociation of NF-κB/IκB complexes, downregulated the histamine-induced hiNOS promoter activity from the NcoI constructs (Figure 4B).
Involvement of kB4 Site for Histamine-Induced iNOS Gene Regulation

Because the luciferase assay indicated that histamine regulates the iNOS gene transcription through NF-κB sites, EMSA and mutation assay were performed to determine the NF-κB site necessary for the iNOS gene regulation. In the EMSA, the kB4 probe, but not kB1, kB2, and kB3 probes (data not shown), detected a specific DNA–protein complex (Figure 5A). Nonstimulated control extract showed a DNA–protein complex (lane 3, arrow), which were increased after histamine stimulation (lane 4). When cold competitor was coincubated in 50× excess, the complex was abolished (lane 8). The specific complex was supershifted by a coincubation with anti-p65 subunit antibody (arrowhead in lane 7) and decreased after the SMCs were incubated with PDTC and pyrilamine (lane 4 and 6, respectively), but not cimetidine (lane 5). Therefore, these results indicate that the histamine-induced NF-κB (kB4 located at −3922 to −3914) binding activity included p65 subunit of NF-κB and was mediated through histamine HH1R.

The kB4 mutation, which disrupts the NF-κB binding, introduced in the Ncol construct of the luciferase reporter

![Figure 3](http://atvb.ahajournals.org/)

**Figure 3.** Luciferase reporter analysis using 5′-deletion constructs. 5′-serial deletion constructs were transfected into the SMCs and incubated with 100 μmol/L histamine for 16 hours. Note that the deletion up to Ncol site is necessary for the histamine inducible activity (dashed bar). Further deletion up to the EcoRV site results in no more basal activity and histamine response. The region between Ncol and EcoRV sites include 4 potential NF-κB sites. In contrast, CM enhanced the promoter activities in Xma1, Eco47III, and EcoRV constructs (closed bar). The luciferase activity was normalized by β-gal activity and expressed as fold activation over the activity of the Xma1 construct without stimulation. Three different experiments were performed in triplicate (n = 6, **P < 0.01, ***P < 0.001). pyr indicates pyrilamine; chl, chlorphenilamine; cim, cimetidine; fam, famotidine

![Figure 4](http://atvb.ahajournals.org/)

**Figure 4.** Involvement of HH1R and NF-κB pathway in histamine-induced iNOS gene regulation. A. Histamine upregulates the transcriptional activity from Ncol construct in a dose-dependent manner (10 and 50 μmol/L). Histamine H1 blocker (pyr and chl, 10 μmol/L each) and NF-κB inhibitor (PDTC, 25 μmol/L), but not H2 blocker (cim and fam, 10 μmol/L each) downregulated the histamine-induced promoter activity (n = 6, **P < 0.01). #P < 0.01 vs histamine stimulation). B. Coexpression of dominant negative IκBα or IκBδ (dn-IκB and dn-IKK) showed reduced histamine-induced (50 μmol/L) activity but still preserved the basal activity. The luciferase activity was normalized by β-gal activity and expressed as fold activation over the activity of the Ncol construct without stimulation. Two different experiments were performed in triplicate (n = 6, **P < 0.01, ***P < 0.001). pyr indicates pyrilamine; chl, chlorphenilamine; cim, cimetidine; fam, famotidine

![Figure 5](http://atvb.ahajournals.org/)

**Figure 5.** Involvement of NF-κB of kb4 site in histamine-induced iNOS gene regulation. A. Nuclear extracts were prepared from SMCs treated with histamine (50 μmol/L, 15 minutes). NF-κB including kb4 probe detected the specific complexes (arrow), which were competed by cold competitor (comp. in lane 8). Histamine-induced binding activity (compare lane 2 with lane 3) was inhibited by PDTC (lane 4) and pyrilamine (pyr in lane 6) but not by cimetidine (cim in lane 5). After coinubcation with anti-p65 subunit of NF-κB, the DNA–protein complex was shifted (lane 7). B. Introduction of mutation in the NF-κB site (kb4) reduced the histamine-induced (50 μmol/L) promoter activities. The luciferase activity was normalized by β-gal activity and expressed as fold activation over the activity of the Ncol construct without stimulation. Two different experiments were performed in triplicate (n = 6, **P < 0.001).
plasmid, resulted in decreased histamine-induced promoter activity (Figure 5B). Therefore, the NF-κB motif located at −3922 to −3914 (kB4) would be necessary for maintenance of histamine-inducible promoter activity.

**Histamine Content in Human Aortic Tissues**

To address whether histamine is accumulated in the human aortic tissues, histamine content in each part of the aortic wall was measured. The histamine content in the adventitia from adult aortas was nearly 1000 pmol/mg protein (Figure 6). The histamine content in the adult intima tissues with fatty streaks was higher than that from the adult and neonatal media. The histamine content was estimated as pmol/mg protein. Med indicates media; Int, intima; Adv, adventitia; #P<0.05 vs neonatal media, ***P<0.001.

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Discussion

Most recently histamine has been implicated in smooth muscle proliferation in the lesions of stent restenosis in pig coronary artery and the histamine content in the restenotic neointima was determined to be 30 to 140 μmol/L. In the present study, we showed that the human atherosclerotic intima included 310 pmol/mg protein (estimated as 16 μmol/L) of histamine, which was significantly higher than that in the adult and neonatal media. Therefore, the histamine concentration (10 to 100 μmol/L) used in the present and previous studies was considered to be reasonable range for the in vitro study.

Cytokine regulation of iNOS gene expression has been well studied in various types of cells, including macrophages, SMCs, and other cells. LPS and inflammatory cytokines such as IL-1β, IFN-γ, and TNF-α stimulate the cells to upregulate iNOS expression in NF-κB-dependent manner. Most of these studies were, however, performed using non-SMCs or using non-human vascular SMCs. Thus, the control of iNOS gene regulation by histamine in human intimal SMCs is particular interest of this study. The results obtained from the luciferase reporter assay showed that −4.8 to −1.3kb 5′-flanking region is required for both basal and histamine-induced promoter activity and that the NF-κB inhibitor and dominant negative IκBα or IKKβ downregulated the histamine-induced promoter activity. Furthermore, the results from EMSA and mutational analysis showed that the most downstream NF-κB (kB4 located at −3922 to −3914) is potentially the responsible site for histamine-induced promoter activity. These results indicated that NF-κB pathway is involved in the HH1R-mediated iNOS regulation in the human SMCs.

Requirement of NF-κB elements for the regulation of cytokine induced iNOS expression was well established by two other groups using hiNOS promoter construct and human lung adenocarcinoma cell line. They have pointed out the importance of NF-κB sites located at −5.8kb and −8.3kb/−115bp for cytokine (mixture of TNF-α, IL-1β, and INF-γ) induction of iNOS. According to their reports, cytokine mixture did not enhance the promoter activity of the constructs including proximal sequence from NcoI site or <3.7kb of the iNOS promoter, which include the sequence (−4.8 to −1.3kb) required for histamine induction of iNOS promoter. On the other hand, a negative regulatory element (NRE), which is an active silencer for NF-κB–dependent gene expression such as IFN-β and IL-8, is present in the hiNOS promoter at −6.7kb upstream. Unlike IFN-β and IL-8, the NRE site does not overlap the NF-κB sites in the hiNOS but the NRE-dependent mechanism represses the basal activity of the hiNOS promoter. In the present study, however, the basal promoter activity was not affected by the NRE and deletion up to NcoI (−4.8kb) site was necessary for the histamine-induced activity. These results indicate that an inhibitory element(s) between −8.5 to −4.8kb would repress the histamine-induced promoter activity independently on the NRE. Although we could not explain the molecular mechanism of the histamine-specific repression, the inhibitory element(s) and NF-κB sites necessary for iNOS transcriptional regulation might be quite different depending on cell type and stimulation.

Histamine and NO have a contradictory effect on the proliferation of vascular SMCs; histamine is proliferative whereas NO is antiproliferative. In contrast with the present study, proliferation and cell death of the intimal SMCs was not affected by the inhibition of NO production during 96-hour histamine stimulation (data not shown). This situation is very similar to that reported in cultured rat aortic intimal SMCs. In comparison with the medial SMCs, higher production of NO by the intimal SMCs is attributable to higher transcriptional activity and overexpression of iNOS when the cells are stimulated by inflammatory cytokines (INF-γ and TNF-α). The proliferation inhibiting effect of NO is, however, much less pronounced in the intimal than medial SMCs. As primary human intimal SMCs are highly reactive to platelet-derived growth factor to proliferate than medial SMCs, the intimal SMCs may have higher capacity to proliferate even in the environment including iNOS–inducing cytokines and histamine. Major targets of intima-derived NO are not the intimal SMCs.

Our previous studies demonstrated that histamine regulates the gene expression of monocyteic TNF-α, MCP-1, and LOX-1 expression as well as MMP-1 expression in the intimal SMCs. Here, we demonstrated that iNOS gene regulation is potentially another target of histamine in the atherosclerotic neointima. In addition, we previously showed...
that histamine-producing enzyme, HDC, is expressed in the macrophages of human atherosclerotic lesions as a source of histamine in the neointima and that HDC-knockout mice showed less pronounced arteriosclerosis. Taken together, these suggest an involvement of histamine metabolism, histamine production, and response in the atherosclerotic vascular wall.

In conclusion, the present study demonstrated histamine induction of iNOS gene in intimal SMCs mediated through HH1R and NF-κB activation. Histamine would be one of NO-regulating factors in histamine-rich atherosclerotic environment and related to the pathogenesis of atherosclerosis.

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Disclosures

This study was partly done in the Department of Pathology, Toranomon Hospital and Okinaka Memorial Institute for Medical Research, 2-2 Toranomon, Minato-ku 105-8470, Tokyo Japan by A.T.

References


Histamine Upregulates the Expression of Inducible Nitric Oxide Synthase in Human Intimal Smooth Muscle Cells via Histamine H1 Receptor and NF-κB Signaling Pathway

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