Eotaxin Increases Monolayer Permeability of Human Coronary Artery Endothelial Cells

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Objective—The objective of this study was to determine the effects and molecular mechanisms of eotaxin, a newly discovered chemokine (CCL11), on endothelial permeability in the human coronary artery endothelial cells (HCAECs).

Methods and Results—Cells were treated with eotaxin, and the monolayer permeability was studied by using a costar transwell system with a Texas Red–labeled dextran tracer. Eotaxin significantly increased monolayer permeability in a concentration-dependent manner. In addition, eotaxin treatment significantly decreased the mRNA and protein levels of endothelial junction molecules including zonula occludens-1 (ZO-1), occludin, and claudin-1 in a concentration-dependent manner as determined by real-time RT-PCR and Western blot analysis, respectively. Increased oxidative stress was observed in eotaxin-treated HCAECs by analysis of cellular glutathione levels. Furthermore, eotaxin treatment substantially activated the phosphorylation of MAPK p38. HCAECs expressed CCR3. Consequently, antioxidants (ginkgolide B and MnTBAP), specific p38 inhibitor SB203580, and anti-CCR3 antibody effectively blocked the eotaxin-induced permeability increase in HCAECs. Eotaxin also increased the phosphorylation of Stat3 and nuclear translocation of NF-κB in HCAECs.

Conclusions—Eotaxin increases vascular permeability through CCR3, the downregulation of tight junction proteins, increase of oxidative stress, and activation of MAPK p38, Stat3, and NF-kB pathways in HCAECs. (Arterioscler Thromb Vasc Biol. 2009;29:2146-2152.)

Key Words: endothelial permeability ■ HCAEC ■ eotaxin ■ tight junction molecules ■ oxidative stress ■ ginkgolide B ■ MAPK p38

The vascular endothelium forms a barrier between the circulation and the interstitium. Aberration of endothelial barrier function leads to an abnormal extravasation of blood components and accumulation of fluid in the extravascular space, resulting in organ dysfunction. This injurious process has been implicated in inflammation, trauma, sepsis, ischemia-reperfusion, diabetes, atherosclerosis, and tumor development and metastasis. The endothelial barrier function is predominantly maintained by the interendothelial junction structures including tight junctions, adherens junctions, and gap junctions. Tight junctions include transmembrane proteins such as occludin, claudin, and junctional adhesion (JAM) molecules. These transmembrane molecules are linked intracellularly to the cytoskeleton via zonula occludens (ZO-1, ZO-2, and ZO-3). Adherens junctions are mainly composed of cadherins and β-catenin and provide strong mechanical connections between adjacent cells. Gap junctions are communication structures, which allow the passage of small molecular weight solutes between neighboring cells. Several inflammation cytokines such as tumor necrosis factor α (TNF-α) can significantly induce endothelial permeability by changing these junction structures.
cular system. However, the exact roles and mechanisms of eotaxin in the vascular system are largely unknown.

In the current study, we determined whether eotaxin could affect endothelial monolayer permeability. Human coronary artery endothelial cells (HCAECs) were treated with eotaxin, and monolayer permeability was investigated. In addition, potential molecular mechanisms such as the role of CCR3 receptors, endothelial junction molecules, oxidative stress, MAPK signal transduction, Stats, and NF-κB transcription factors were also studied. These experiments, for the first time, explore the molecular mechanisms of eotaxin-induced endothelial dysfunction, thereby having clinical relevance and therapeutic potential.

Methods
Endothelial Permeability
HCAECs were obtained from Gelantis (San Diego, Calif) and cultured in HCAEC Growth medium (Gelantis). Human recombinant eotaxin was obtained from Peprotech (Rocky Hill, NJ). Paracelluar permeability was studied in a Coaster Transwell system as previously described.18

Real-Time RT-PCR Analysis
HCAECs were treated with different concentrations of eotaxin (50, 100, and 200 ng/mL) for 24 hours. Total RNA extraction and cDNA reverse transcription were done as previously described.19 Primers for VE-cadherin, ZO-1, claudin-1, occludin, and JAM-1 were described in our previous publication.5,19–21

Western Blot Analysis
Equal amount of proteins (40 μg) was loaded onto 10% SDS-PAGE, fractionated by electrophoresis, and transferred to nitrocellulose membranes (BioRad). Primary antibodies against ZO-1, claudin-1, occludin, JAM-1, VE cadherin, CCR3, p38, NF-κB p65, Stat3, and β-actin were used.

Cellular Glutathione Assay
HCAECs were treated with either eotaxin (100 ng/mL) or pretreated with MnTBAP (2 μmol/L) for 30 minutes followed by eotaxin treatment for 45 minutes. Antimycin A (10 μg/mL) and TNF-α (2 ng/mL) were used for the positive controls. Cellular glutathione (GSH) was measured as per manufacturer’s instructions by following a GSH-Glo Glutathione assay kit (Promega).

Statistical Analysis
Data were expressed as the mean±SD. Comparisons were made using the Student t test. A probability value <0.05 was considered statistically significant.

Supplemental Materials
Supplemental materials include the background information about gap junction, detail Materials and Methods, and 4 supplemental figures (available online at http://atvb.ahajournals.org).

Results
Eotaxin Increases Endothelial Monolayer Permeability in HCAECs
To determine whether eotaxin could affect the paracellular permeability of the endothelial cell monolayer, HCAECs were treated with eotaxin in a concentration-dependent manner, and endothelial permeability was analyzed by a Costar transwell system with a Texas Red-labeled dextran tracer. Treatment of HCAEC monolayer with increasing concentra-

ions (100 and 200 ng/mL) of eotaxin for 24 hours significantly increased endothelial permeability by 43% and 59%, respectively, compared with untreated cells (n=3, P<0.05, Figure 1A). TNF-α (2 ng/mL) was used as a positive control because it can significantly increase monolayer permeability.18 Heat inactivated eotaxin did not increase the permeability through HCAECs indicating that the eotaxin effect is specific (Figure 1B). Interestingly, eotaxin-induced permeability increase in HCAECs was significantly blocked by the treatment with antioxidant ginkgolide B (Figure 1A). As a positive control, antimycin A (ROS generator) also increased endothelial permeability and this effect was effectively blocked by MnTBAP (cell permeable SOD mimetic; n=3, P<0.05, Figure 1C). Similar to HCAECs, eotaxin treatment also increased the paracellular permeability in human umbilical vein endothelial cells (HUVECs) in a concentration-dependent manner (supplemental Figure I). Eotaxin treatment (100 ng/mL) for 24 hours did not induce apoptosis when compared to untreated cells in HCAECs (supplemental Figure II).

CCR3 Receptors Are Involved in Eotaxin-Induced Endothelial Permeability Increase
CC chemokines such as eotaxin act predominantly via the CCR3 receptors, members of the family of G protein–coupled receptors. Experiments were designed to determine whether CCR3 receptors are expressed on HCAECs and whether CCR3 receptors are involved in the effects of eotaxin on HCAECs. Western blotting analysis showed that CCR3 receptors were expressed in HCAECs, and eotaxin treatment did not change CCR3 receptor expression levels compared with untreated cells (Figure 1D). To further determine the involvement of CCR3 receptors in eotaxin-mediated hyperpermeability, HCAECs were pretreated with anti-CCR3 antibody for 30 minutes followed by eotaxin treatment for another 24 hours. Interestingly, anti-CCR3 antibody effectively reduced the basal endothelial permeability and eotaxin-induced permeability increase in HCAECs (n=3, P<0.05, Figure 1B). Immunohistological analysis showed that CCR3 was detected in human atherosclerotic arteries (supplemental Figure III). These data demonstrate that CCR3 receptors play an important role in eotaxin-meditated endothelial permeability.

Eotaxin Decreases the Expression of Endothelial Tight Junction Proteins ZO-1, Claudin-1, and Occludin in HCAECs
To determine whether eotaxin could affect the expression of endothelial junction molecules, especially the tight junction proteins at both mRNA and protein levels, HCAECs were treated with eotaxin for 24 hours. Eotaxin treatment decreased the expression of tight junction proteins in a concentration-dependent manner. At 50, 100, and 200 ng/mL of eotaxin, ZO-1 mRNA was decreased by 8%, 25% (P<0.05), and 39% (P<0.05), respectively, compared with the control (n=3, Figure 2A); occludin mRNA was decreased by 19% (P<0.05), 33% (P<0.05), and 55% (P<0.05), respectively; and claudin-1 mRNA was decreased by 5%, 45% (P<0.05), and 48% (P<0.05), respectively, compared with the control. There were no significant differences of VE
cadherin and JAM-1 mRNA levels after eotaxin treatment compared with the control. Western blot analysis showed a parallel decrease in the protein levels of ZO-1, occludin, and claudin-1 in HCAECs when treated with eotaxin (100 ng/mL) compared with the control (Figure 2B). The commonly used antioxidant MnTBAP (2 μmol/L), a cell permeable SOD mimic, effectively blocked the eotaxin-induced decrease these molecules at the protein level. Consistent with the mRNA expression data, protein levels of VE cadherin and JAM-1 did not show any significant differences after eotaxin treatment (Figure 2B). To further confirm protein levels of tight junction proteins, flow cytometry analysis was used and the results showed that eotaxin treatment substantially decreased the protein levels of ZO-1, occludin and claudin-1 in HCAECs (Figure 2C).

We also performed a time course study of the expression of tight junction molecules ZO-1, occludin, and claudin-1 in HCAECs. HCAECs were treated with eotaxin (100 ng/mL) and the mRNA levels of ZO-1, occludin, and claudin-1 were determined at different time points (0, 45 minutes, 2 hours, 6 hours, 12 hours, and 24 hours, respectively) by real-time PCR. Results showed that mRNA levels of these tight junction molecules were significantly decreased by more than 40% at or after 2 hours of eotaxin treatment compared with untreated controls (n = 3, P < 0.05, Figure 3).

**Eotaxin Increases ROS Production in HCAECs**

To determine whether oxidative stress could be involved in the action of eotaxin, ROS levels were analyzed by GSH assay. Superoxide can be converted to H₂O₂ by superoxide dismutase (SOD), and then H₂O₂ can be rapidly removed by catalase or peroxidases such as the GSH peroxidases, which use reduced GSH as the electron donor. Thus, cellular GSH levels are negatively correlated to ROS levels. Eotaxin (100 ng/mL) treatment of HCAECs for 45 minutes significantly reduced cellular GSH levels (n = 3, P < 0.05, Figure 4), whereas MnTBAP effectively blocked eotaxin-induced reduction in GSH levels in HCAECs. Two positive controls (antimycin A and TNF-α) also significantly reduced cellular GSH levels in HCAECs (n = 3, P < 0.05, Figure 4), whereas MnTBAP partially blocked the effect of antimycin A. These data demonstrate that eotaxin increases oxidative stress in HCAECs.

**Activation of p38, Stat3, and NF-κB Is Involved in the Eotaxin-Induced Permeability Increase in HCAECs**

To determine the signal transduction pathways involved in the action of eotaxin on HCAECs, the activation status of MAPK p38 was investigated using Western blot analysis because p38 is sensitive to oxidative stress. Treatment with eotaxin (100 ng/mL) substantially increased the phosphorylation of p38 at 45 minutes of the treatment (Figure 5A), whereas MnTBAP effectively blocked eotaxin-induced phosphorylation of p38 in HCAECs. Two positive controls (antimycin A and TNF-α) also significantly reduced cellular GSH levels in HCAECs (n = 3, P < 0.05, Figure 4), whereas MnTBAP partially blocked the effect of antimycin A. These data demonstrate that eotaxin increases oxidative stress in HCAECs.

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** Effects of eotaxin, antimycin A, MnTBAP, ginkgolide B, and CCR3 on endothelial monolayer permeability in HCAECs. Endothelial monolayer permeability was analyzed with a costar transwell system and a Texas Red-labeled dextran tracer. A, Concentration-dependent study. HCAECs were treated with increasing concentrations of eotaxin (50, 100, and 200 ng/mL), TNF-α (2 mg/mL), or with ginkgolide B (5 μmol/L) for 24 hours. n = 3, *P < 0.05. B, Effects of CCR3 receptor blocking and heat-inactivation (HI) of eotaxin on endothelial permeability. HCAECs were treated with eotaxin (100 ng/mL) or HI-eotaxin (100 μg/mL) with or without anti-CCR3 antibody 24 hours. n = 3, *P < 0.05. C, Effect of antimycin A on endothelial permeability. HCAECs were treated with antimycin A (10 μg/mL) for 24 hours in the presence or absence of antioxidant MnTBAP (2 μmol/L). n = 3, *P < 0.05. D, Western blot analysis. HCAECs were treated with eotaxin (100 ng/mL) or MnTBAP (2 μmol/L) for 24 hours, and CCR3 protein levels were determined by Western blot. Loading efficiency was determined by reprobing the blot with β-actin antibody. Experiments were repeated twice. Statistical comparison is indicated by the arrow head. Error bars represent SD.
ity was analyzed using the Costar transwell system. Indeed, the specific p38 inhibitor SB203580 (10 μmol/L) completely blocked the eotaxin-induced permeability increase in HCAECs \( (n=3, P<0.05, \text{Figure}\ 5B) \).

We further determined whether transcriptional factors such Stat3 and NF-κB could be involved in the eotaxin-induced signal as pathways because these transcriptional factors are also sensitive to oxidative stress. Eotaxin or antimycin A

![Figure 2](image-url)

**Figure 2.** Effects of eotaxin on the expression of junctional molecules in HCAECs. HCAECs were treated with serial concentrations of eotaxin (50, 100, and 200 ng/mL) for 24 hours. A, The mRNA levels of ZO-1, claudin-1, occludin, JAM-1, and VE cadherin were analyzed by real-time PCR, and values were normalized to GAPDH \( [2^{\Delta\Delta CT} \text{GAPDH–CT gene of interest}] \) in each sample. \( n=3, P<0.05 \) (compared with controls). B, The protein levels of ZO-1, occludin, claudin-1, VE cadherin, and JAM-1 were analyzed by Western blot analysis. Loading efficiency was determined by reprobing the blot with β-actin antibody. Eotaxin and antioxidant MnTBAP were used. Experiments were repeated twice. C, Histogram of flow cytometry analysis showing protein levels of ZO-1, claudin-1, and occludin under the control and eotaxin-treated conditions. HCAECs were treated with 100 ng/mL eotaxin for 24 hours. Experiments were repeated twice.

![Figure 3](image-url)

**Figure 3.** Time course study of the effect of eotaxin on mRNA levels of tight junction molecules in HCAECs. Cells were treated with eotaxin (100 ng/mL) for different time points (0, 45 minutes, 2 hours, 6 hours, 18 hours, and 24 hours), and the mRNA levels of ZO-1 (A), occludin (B), and claudin-1 (C) were determined by real-time PCR and values were normalized to GAPDH \( [2^{\Delta\Delta CT} \text{GAPDH–CT gene of interest}] \) in each sample. \( n=3, P<0.05 \) (compared with controls). Error bars represent SD.
treatment for 45 minutes substantially increased the phosphorylation of Stat3 and nuclear translocation of NF-κB in HCAECs, whereas antioxidant the MnTBAP effectively blocked these effects (Figure 5A). Thus, MAPK p38 and the transcriptional factors Stat3 and NF-κB may be involved in the eotaxin-induced signal transduction pathways through the oxidative stress mechanism.

Discussion

Eotaxin (CCL11) is an eosinophil-specific chemo attractant which has been found to be highly expressed at sites of vascular pathology.13 Eotaxin selectively attracts eosinophils by activating CCR3 receptors.22 However, it is not clear whether eotaxin could contribute to the progression of atherosclerosis. The present study, for the first time, reports 3 novel findings: (1) eotaxin increases the paracellular permeability in HCAECs; (2) eotaxin decreases the expression of tight junction proteins involved in the regulation of endothelial barrier functions; and (3) eotaxin may mediate its effects through oxidative stress and activation of the p38 MAPK, Stat3, and NF-κB signaling pathways.

In this study, we used a Costar transwell permeability model system to study the effect of eotaxin on paracellular permeability in HCAECs. This model has been used successfully in this laboratory to analyze the effects of ritonavir,20 lysophosphatidylcholine,21 stanniocalcin-1,17 and secretoneurin19 on endothelial permeability in vitro. We selected eotaxin concentrations ranging from 50 to 200 ng/mL based on the plasma eotaxin levels in patients with coronary artery disease.22 Our results show that eotaxin treatment for 24 hours significantly increased endothelial permeability in HCAECs in a concentration-dependent manner. To rule out the possibility that the permeability increase is not attributable to the leakage through confluent HCAECs, the cell monolayer was grown to confluence, stained with Calcein AM, and checked under a fluorescent microscope to make sure that the monolayer was confluent (data not shown). Heat-inactivated (HI) eotaxin did not show any effects on endothelial permeability indicating its specific effect, but not potential contamination of endotoxin. The endothelial apoptotic cascade is an important underlying mechanism of capillary leakage.23,24 In this study, we have shown that eotaxin did not affect apoptosis of HCAECs in the experimental condition.

Chemokines and chemokine receptors have emerged as important factors involved in the mobilization and function of leukocytes. Chemokine receptors are expressed on a wide range of leukocytes, as well as on endothelial cells, neurons, and possibly other cell types where they are involved in signaling events that can lead to eosinophil and mast cell degranulation, T cell activation, lymphocyte homing, chemotaxis, and mitogenic effects as well as hematopoiesis.25 In addition, overexpression of eotaxin and its receptor, CCR3, in human atherosclerosis have also been reported.11 In our study, we found that the CCR3 receptor is constitutively expressed in HCAECs, whereas eotaxin treatment did not further increase the expression of CCR3 receptors. CCR3 expression was also observed in human atherosclerotic arterioles.

Figure 4. Effects of eotaxin, antimycin A, TNF-α, and MnTBAP on the cellular glutathione (GSH) levels in HCAECs. Cells were treated with eotaxin (100 ng/mL), antimycin A (10 μg/mL), TNF-α (2 ng/mL), or MnTBAP (2 μmol/L) for 45 minutes. Cellular GSH levels were determined by a GSH-Glo Glutathione assay kit. n=3, *P<0.05 (the comparison is indicated by the arrow head). Error bars represent SD.

Figure 5. Effects of eotaxin, antimycin A, and MnTBAP on the activation of p38, Stat3, and NF-κB in HCAECs. A, Phosphorylation of p38 and Stat3 as well as nuclear translocation of NF-κB p65 in HCAECs. Cells were treated with either eotaxin (100 ng/mL) or antimycin A (10 μg/mL) for 45 minutes or pretreated with MnTBAP (2 μmol/L) for 30 minutes followed by antimycin A treatment for 30 minutes. Cytoplasmic and nuclear extracts were isolated, respectively. Phosphorylated (p) and total p38 and Stat3 as well as NF-κB p65 proteins were detected by the Western blotting analysis. Loading efficiency was determined by reprobing the blot with β-actin antibody. The experiment was repeated twice. B, Endothelial permeability. HCAECs were treated with eotaxin (100 ng/mL) alone or pretreated with specific p38 inhibitor SB203580 followed by eotaxin treatment for 24 hours. Endothelial permeability was assayed. n=3, *P<0.05 (the comparison is indicated by the arrow head). Error bars represent SD.
ies. Pretreatment of HCAECs with anti-CCR3 antibody significantly reduced eotaxin-induced permeability increase. These findings suggest that CCR3 may play an important role in the eotaxin-mediated endothelial permeability increase in HCAECs.

Intercellular junctional structures mediate adhesion and communication between adjoining endothelial cells and comprise tight junction molecules including transmembrane proteins such as occludin, claudin, and JAM-1 and intracellular proteins such as ZO-1 and cingulin as well as adherens junction proteins including transmembrane protein VE-cadherin and intracellular protein β-catenin. Tight junctions serve the major functional purpose of providing a "barrier" and a "fence" within the membrane by regulating paracellular permeability and maintaining cell polarity. In this study, we show that eotaxin significantly decreases the expression of tight junction proteins ZO-1, occludin, and claudin-1 in a concentration-dependent manner at both mRNA and protein levels. However, eotaxin does not decrease the expression of VE-cadherin and JAM-1. These data indicate that downregulation of tight junction proteins may be the key mechanism involved in the paracellular permeability increase in eotaxin-treated HCAECs.

ROS including superoxide anion, hydrogen peroxide, hydroxyl radical, and peroxynitrite play critical roles in cardiovascular disease.26 They could directly cause vascular damages, and could also act as signaling molecules for the gene expression in response to proinflammatory stimuli.27–29 ROS cause endothelial barrier dysfunction through alterations in the cytoskeleton and extracellular matrix.30 ROS are known to quench NO.30 NO synthesis inhibition can potentiate agonist-induced increases in vascular permeability or increase basal microvascular permeability via an alteration of endothelial actin cytoskeleton.31 In the present study, we showed a significant decrease of cellular GSH levels in eotaxin-treated cells, indicating that eotaxin may increase ROS production in HCAECs. Cotreatment of HCAECs with eotaxin and antioxidant MnTBAP substantially increased GSH levels, suggesting that MnTBAP may inhibit eotaxin-induced oxidative stress in HCAECs. To further elucidate the role of ROS in eotaxin-mediated permeability increase, we treated the cells with antiinmycin A, an inhibitor of electron transport in mitochondria that has been used as a ROS generator in biological systems. We found that antiinmycin A can induce endothelial permeability and ROS production in HCAECs. To further confirm the involvement of ROS in eotaxin-mediated hyper-permeability, HCAECs were treated with either antioxidant ginkgolide B32 or commonly used antioxidant MnTBAP, a cell-permeable SOD mimetic, which effectively blocked the eotaxin-induced increase in vascular permeability and decrease in the expression of tight junction proteins, respectively. The natural antioxidant gingo kolide B, a traditional Chinese herb from the plant Gingko Biloba, has less toxicity and side effects. Antioxidants are believed to counteract ROS and reduce the incidence of coronary artery disease.33 Thus, the current study suggest that eotaxin-induced oxidative stress may be one of the molecular mechanisms involved in the damage of endothelial barrier function, and the use of antioxidants could be a novel strategy in the treatment of patients with high incidence of cardiovascular disease.

MAPKs play an important role in mediating cellular functions in response to many extracellular stimuli. There are three important MAPKs including extracellular signal regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 in the cell. In the present study, we show that eotaxin can activate p38 in HCAECs. MAPK p38 activation was observed at 45 minutes of eotaxin treatment, and there was no activation of p38 at 2 hours after eotaxin treatment. We previously reported that quick activation of MAPK p38 (within 5 to 10 minutes) was observed during lysosphatidylcholine-induced increase of monolayer cell permeability in HCAECs.21 Sidestream cigarette smoke could also activate p38 within 60 minutes and induce an endothelial permeability increase in human pulmonary endothelial cells.34 More importantly, the specific p38 inhibitor SB203580 effectively blocked eotaxin-induced permeability increase in HCAECs. Blocking ROS generation also inhibited eotaxin-induced phosphorylation of p38, which indicates ROS acts as an upstream effector of p38 under the stimulation of eotaxin. Possibly transient expression of p38 initiates the events leading to dysregulation of barrier function through the activation of downstream signal transduction pathways. Indeed, IL-6 can increase endothelial permeability through activation of the transcription factor Stat3.35 Activation of transcription factor NF-κB has also been indicated for the TNF-α-permeability increase in HCAECs.18 We found activation of Stat3 and NF-κB under the stimulation of eotaxin in HCAECs. It is possible that Stat3 and NF-κB may regulate the expression of tight junction proteins. However, a direct link between Stat3 and NF-κB activation and repression of tight junction proteins has not been determined in this study.

In summary, the present study demonstrates that eotaxin can increase vascular permeability in HCAECs. The underlying molecular mechanisms may involve downregulation of tight junction proteins, increase of oxidative stress, and activation of MAPK p38, Stat3, and NF-κB. This study provides a new understanding of the biological functions of eotaxin on the vascular system. Reducing oxidative stress or inhibiting p38 activation may be a new strategy for inhibiting the detrimental effects of eotaxin on the vascular system, thereby preventing cardiovascular disease.

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**Disclosures**

None.

**References**


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Additional Introduction

Gap junctions are channel-like assemblies of connexin (Cx) family proteins. The channels provide direct intercellular communication pathways allowing rapid exchange of ions and metabolites up to ~1.5 kDa in size. Vascular endothelial cells express Cx37, Cx40, and Cx43, and Cx32 (1-3). Gap-junction plaques are often observed with tight-junction strands of vascular endothelial cells. Gap-Junction proteins Cx40 and Cx43 are colocalized and coprecipitated with tight-junction molecules occludin, claudin-5, and ZO-1 in porcine blood-brain barrier endothelial cells. Gap junction blockers 18β-glycyrrhetinic acid (18β-GA) and oleamide inhibited the barrier function of tight junctions in cells as determined by measurement of transendothelial electrical resistance and paracellular flux of mannitol and inulin. 18β-GA also significantly reduced the barrier property in rat lung endothelial cells expressing doxycycline-induced claudin-1, while it did not change the interaction between Cx43 and either claudin-1 or ZO-1, nor their expression levels or subcellular distribution. These findings suggest that Cx40- and/or Cx43-based gap junctions might be required to maintain the endothelial barrier function (4).

Detail Materials and Methods

Chemicals and Reagents

Human recombinant eotaxin was obtained from Peprotech (Rocky Hill, NJ). Horseradish peroxidase-conjugated goat anti-rabbit IgG and anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Ginkgolide B was obtained from LKT laboratories (St. Paul, MN). MnTBAP [Mn(III)tetrakis(4-benzoic acid)porphyrin Chloride] was obtained from Calbiochem (La Jolla, CA). All other chemicals and reagents were obtained from Sigma (St Louis, Mo) unless stated.

Endothelial Permeability

HCAECs were obtained from Gelantis (San Diego, CA) and cultured in HCAEC Growth medium (Gelantis). Cells were seeded onto 100-mm plates at $5 \times 10^5$ cells per plate and the medium was replaced every 48 h. Paracellular permeability was studied in a Coaster Transwell system as previously described. Briefly, HCAECs at full confluence were treated with increasing concentrations of eotaxin (50, 100 or 150 ng/mL, respectively) for 24 h. In separate experiments, cells were treated with either TNF-α (2 ng/mL) or with eotaxin (100 ng/mL) in the presence or absence of ginkgolide B (5 µM) for 24 h. To check the involvement of MAPK, cells were pretreated with specific inhibitors ERK1/2 (PD098059 at 50 µM) and p38 (SB203580 at 10 µM) for 30 min and then treated with eotaxin (100 ng/mL) for 24 h. To check the effect of antimycin A (inducer of ROS production) and MnTBAP (SOD mimetic) on endothelial permeability, cells were treated with either antimycin A (10 µg/mL) or MnTBAP (2 µM) alone or both together for 24 h. Equal amount of Texas-Red-labeled dextran tracer was added to the upper chamber of the transwell system. The amount of tracer penetrating through the cell monolayer into the lower chamber was measured by a using fluorometer. The permeability index was calculated based on the tracer concentration in the lower chamber and the upper chamber.

Real-time RT-PCR Analysis

HCAECs were treated with different concentrations of eotaxin (50, 100 and 200 ng/mL) for 24 h. Total RNA extraction and cDNA reverse transcription were done as previously described. Primers for VE-cadherin, ZO-1, claudin-1, occludin, and JAM-1 were described in our previous publication. The iQ SYBR green Supermix Kit and iCycler iQ Real-time PCR detection system (Bio-Rad) were used for real-time PCR reaction. Sample cycle threshold (Ct) values were determined. Relative gene
expression for each sample was calculated as $2^{40-Ct}$. Expression for each target gene in each sample was normalized to GAPDH. Ct values were calculated as $\Delta Ct = 2^{(Ct_{\beta-GAPDH} - Ct\text{ gene of interest})}$.

**Western Blot Analysis**

HCAECs grown to confluence were treated with eotaxin (200 ng/mL) in the presence or absence of MnTBAP (2 µM) for 24 h. Proteins from HCAECs were extracted using cell lysis buffer [(Tris-HCl (20 mM, pH 7.5), NaCl (150 mM), Na2EDTA (1 mM), EGTA (1 mM), Triton X100 (1%), sodium pyrophosphate (2.5 mM), β- glycerophosphate (1 mM), Na3VO4 (1 mM), leupeptin (1 µg/ml), PMSF (1 mM) was added into the lysis buffer immediately before use). Catalog No. 9803, Cell Signaling Technology, Inc., Danvers, MA]. Equal amount of proteins (40 µg) was loaded onto 10% SDS-PAGE, fractionated by electrophoresis, and transferred to nitrocellulose membranes (BioRad). The membrane was incubated with the primary antibody at 4°C overnight. Primary antibodies against ZO-1, claudin-1 and occludin-1 were obtained from Zymed (South San Francisco CA) and used at a dilution of 1:2000. VE cadherin (Cell Signaling Technology Inc) and JAM-1 (BD Biosciences) antibodies were used at a dilution of 1:10000, while β-actin antibody was used at a dilution of 1:20000 (Milipore, Billerica, Mass). Monoclonal anti-human CCR3 antibody (R&D Systems, Cat No. MAB155) was used for western blot at 1:1000. For assay of nuclear translocation of NF-κB and Stat3, nuclear proteins were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Catalog No. 78833, Thermo Scientific) following manufacturer’s instructions. Equal amounts of nuclear proteins (10 µg) were loaded onto 10% SDS-PAGE, fractionated by electrophoresis, and transferred to nitrocellulose membrane. The membrane was incubated with either NF-κB p65 (Catalog No.sc8008, Santa Cruz Biotech), p38, p-p38, Stat3 or pStat3 (Cell Signaling Technology, Inc., Danvers, MA) antibody at a dilution of 1:1000 at 4°C overnight. The membrane was then incubated with secondary anti-rabbit (1:5000) or anti-mouse (1:10,000) horseradish peroxidase-labeled antibodies for 45 min at room temperature. Bands were visualized with Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL).

**Flow Cytometry**

Flow cytometry analysis was done as previously described. Briefly, cultured HCAECs were treated with eotaxin (100 ng/mL) for 24 h. In separate experiments, cells were treated with TNF-α (2 ng/mL), antimycin A (10 µM) or eotaxin (100 ng/mL) for 45 min. The treatment was halted by washing cells 3 times with PBS. Cells were fixed with 2% paraformaldehyde for 15 min and analyzed by FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). For detention of protein levels of
junctional molecules, the FITC-conjugated antibodies against ZO-1, and occluding, claudin-1 were used.

**Cellular Glutathione (GSH) Assay**

HCAECs were treated with either eotaxin (100 ng/mL) or pretreated with MnTBAP (2 μM) for 30 min followed by eotaxin treatment for 45 min. Antimycin A (10 μg/mL) and TNF-α (2 ng/mL) were used for the positive controls. Cellular GSH levels were measured as per manufacture’s instructions by following a GSH-Glo Glutathione assay kit (Promega, Madison, WI).

**Terminal Deoxynucleotidyl Transferase Mediated dUTP Nick End Labeling (TUNEL) Assay**

APO direct kit was used (Catalog No. 556381, BD Biosciences, San Diego, CA) to check the eotaxin-induced apoptosis. Cell apoptosis was assessed 24 h after treatment of HCAECs with eotaxin (100 ng/ml). Cells were trypsinized and fixed in 1% (wt/vol) paraformaldehyde in phosphate-buffered saline (pH 7.4) at the concentration of 1–2 × 10^6 cells/mL. After washing with phosphate-buffered saline and storing in 70% (vol/vol) ice cold ethanol at −20°C for 12 h, the cells were resuspended with 1.0 mL of wash buffer, and centrifuged. The supernatant was removed by aspiration, and cell pellets were resuspended in 50 μL of the staining solution and incubated for 60 min at 37°C. The APO-DIRECT cell samples were analyzed by flow cytometry. Two dyes were used including propidium iodide staining total DNA and fluorescein isothiocyanate (FITC)-dUTP labeling apoptotic cells. Propidium iodide fluoresces were detected at 623 nm and FITC at 520 nm. The gating display was set at the standard dual parameter DNA doublet with the DNA area signal on the y-axis and the DNA width on the x-axis.

**Immunohistochemistry**

Full-thickness arterial wall specimens of carotid arteries were obtained from patients with atherosclerosis undergoing autopsy (National Disease Research Interchange (NDRI), Philadelphia, PA). All samples were fixed in formalin and embedded in paraffin. Immunohistochemistry was done with anti-CCR3 antibody (1:50) (R&D systems), biotinylated secondary antibody, and avidin-biotin reaction using peroxidase enzyme (ABC kit; Vector Laboratories, Burlingham, CA). The protocol of use of human tissues obtained from NDRI was approved by the Institutional Review Board (IRB) at the Baylor College of Medicine. The investigation conformed to the principles outlined in the Declaration of Helsinki.
**Statistical Analysis**

Data were expressed as the mean ± SD. Comparisons were made using the Student’s t-test. A $p$ value < 0.05 was considered statistically significant.
**Figure S1.** Effects of eotaxin on endothelial monolayer permeability in HUVECs. Endothelial monolayer permeability was analyzed with a costar transwell system and a Texas Red-labeled dextran tracer. HUVECs were treated with increasing concentrations of eotaxin (50, 100 and 200 ng/mL) for 24 h. Endothelial permeability was significantly increased at 100 and 200 ng/mL concentrations of eotaxin compared to control. n=3, *p<0.05

**Figure S2.** Effects of eotaxin on apoptosis of HCAECs. HCAECs were untreated or treated with eotaxin (100 ng/mL) for 24 h, and cell apoptosis was assessed by TUNEL assay. Cells were fixed in 1% (wt/vol) paraformaldehyde and stained with FITC-dUTP for 60 min at 37°C. Two dyes were used including propidium iodide staining total DNA and fluorescein isothiocyanate (FITC)-dUTP labeling apoptotic cells. Propidium iodide fluoresces were detected at 623 nm and FITC at 520 nm. The gating display was set at the standard dual parameter DNA doublet with the DNA area signal on the y-axis and the DNA width on the x-axis. Eotaxin failed to induce apoptosis in HCAECs after 24 h.
**Figure S3.** CCR3 immunoreactivity of human atherosclerotic arteries. Human carotid arteries were fixed in formalin and embedded in paraffin. Immunostaining was performed by using the anti-human CCR3 antibody (1:50), biotinylated secondary antibody, and avidin-biotin reaction using peroxidase enzyme. Brown color represents positive staining of CCR3.

**Figure S4.** Effect of eotaxin and antimycin A on the phosphorylation of p38 after 2 h of treatment in HCAECs. HCAECs were treated with eotaxin (100 ng/mL) or antimycin A (10 µg/ml) for 2 h. Total proteins were isolated by using cell lysis buffer (Cell Signaling Technology, Inc, Cat No. 9803). phosphorylated (p) and total p38 proteins were detected by the western blotting analysis. Phosphorylation of p38 was peaked at 45 min following treatment with either eotaxin or antimycin A but there was no activation of p38 at 2 h treatment (Representative figures have shown).