Abstract—RhoA and Rac1 regulate formation of stress fibers and intercellular junctions, thus modulating endothelial monolayer permeability. Posttranslational modifications of RhoA and Rac1 regulate enzyme activity and subcellular localization, resulting in altered cellular function. The role of RhoA and Rac1 carboxyl methylation in modulating endothelial monolayer permeability is not known. In this study, we found that inhibition of isoprenylcysteine-O-carboxyl methyltransferase (ICMT) with adenosine plus homocysteine or N-acetyl-S-geranylgeranyl-L-cysteine decreased RhoA carboxyl methylation, RhoA activity, and endothelial monolayer permeability, suggesting that RhoA carboxyl methylation may play a role in the ICMT-modulated monolayer permeability. Similar studies showed no effect of ICMT inhibition on Rac1 carboxyl methylation or localization. Bovine pulmonary artery endothelial cells (PAECs) stably overexpressing ICMT-GFP cDNA were established to determine if increased ICMT expression could alter RhoA or Rac1 carboxyl methylation, activation, and endothelial monolayer permeability. PAECs stably overexpressing ICMT demonstrated increased RhoA carboxyl methylation, membrane-bound RhoA, and RhoA activity. Additionally, PAECs stably overexpressing ICMT had diminished VE-cadherin and β-catenin at intercellular junctions, with resultant intercellular gap formation, as well as enhanced monolayer permeability. These effects were blunted by adenosine plus homocysteine and by inhibition of RhoA, but not by inhibition of Rac1. These results indicate that ICMT modulates endothelial monolayer permeability by altering RhoA carboxyl methylation and activation, thus changing the organization of intercellular junctions. Therefore, carboxyl methylation of RhoA may modulate endothelial barrier function. (Circ Res. 2004;94:306-315.)

Key Words: RhoA ■ Rac1 ■ adenosine ■ carboxyl methylation ■ endothelial monolayer permeability

Endothelial cells regulate the flow of nutrient substances, water, protein, biologically active molecules, and cells between blood and tissues. Increased vascular permeability is a hallmark of pathological conditions, such as acute respiratory distress syndrome (ARDS). Enhanced endothelial permeability is accompanied by reorganization of actin cytoskeleton and weakening of intercellular junctions.1 Rho GTPases modulate endothelial permeability by affecting assembly of stress fibers2–4 and by altering integrity of intercellular junctions.3,4

Rho proteins cycle between inactive GDP-bound and active GTP-bound states, a process regulated by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins, and guanine nucleotide dissociation inhibitors (GDI).5 Rho proteins translocate to membranes from cytosol on activation, and this translocation is critical for their biological functions.6–10

In addition to GDP/GTP cycling, the function of Rho GTPases is also dependent on posttranslational modifications. Rho proteins possess a preserved C-terminal CAAX motif. This is sequentially modified by prenylation of the cysteine residue by geranylgeranyltransferase or farnesyltransferase, cleavage of the AAX residues by prenyl-CAAX protease, carboxyl methylation of the prenylcysteine by isoprenylcysteine-O-carboxyl methyltransferase (ICMT), and palmitoylation of the CXXC domain in the hypervariable region immediately upstream of the CAAX motif by palmitoyltransferase.11 Both prenyltransferases

© 2004 American Heart Association, Inc.
Circulation Research is available at http://www.circresha.org DOI: 10.1161/01.RES.0000113923.85084.C1
are soluble, whereas prenyl-CAAX protease and ICMT localize in the endoplasmic reticulum (ER), and thus it is likely that prenylation occurs in the cytosol and that this modification specifically targets the prenylated Rho proteins to the ER, where they encounter the protease and ICMT. Prenylation of small GTPases modulates several vascular cell functions.

The importance of carboxyl methylation of small GTPases has not been extensively evaluated. Our previous studies have shown that Ras carboxyl methylation by ICMT modulates its activation and subcellular location and regulates endothelial apoptosis. Because prenylation of small GTPases regulates cell functions, we postulated that RhoA or Rac1 carboxyl methylation would also affect endothelial cell functions. In this study, using cultured bovine pulmonary artery endothelial cells (PAECs), we assessed the effects of both inhibition of ICMT activity and overexpression of ICMT on endothelial monolayer permeability. We demonstrated that RhoA was carboxyl methylated in PAECs, and factors that enhanced RhoA carboxyl methylation increased its activation and promoted endothelial monolayer permeability. Inhibition of RhoA prevented effects of ICMT overexpression on monolayer permeability, whereas inhibition of Rac1 did not. These studies suggest that changes in carboxyl methylation of RhoA, but not Rac1, may modulate endothelial monolayer permeability.

Materials and Methods

Materials

Bovine PAECs were obtained as described. Adenosine and DL-homocysteine were purchased from Sigma. N-acetyl-S-geranylgeranyl-L-cysteine (AGGC) and N-acetyl-S-geranyl-L-cysteine (AGC) were obtained from Biomol. Antibodies directed against RhoA, Rac1, VE-cadherin, and β-catenin were purchased from Santa Cruz Biotechnologies (Santa Cruz, Calif).

The pGST-C21 construct, pCMT-GFP construct (referred to as pCMT-GFP), and rabbit polyclonal antibody against amphibian ICMT were generous gifts from Drs J. Collard (Netherlands Cancer Institute, Amsterdam), M. Philips (New York University School of Medicine, New York, NY), and J. Stockand (University of Texas Health Science Center at San Antonio, Tex), respectively. The recombinant adenoviruses containing CDNA of dominant-negative RhoA were generously provided by Drs T. Hirase and A. Ridley (University College London, UK), respectively.

Immunoblot and Immunofluorescence Analysis

Proteins were resolved using 15% SDS-PAGE or 2-dimensional PAGE (pH 5.0 to 7.0), and immunoblotting was performed as previously described. Immunofluorescence studies were performed as described.

Endothelial Monolayer Permeability Assay

Changes in endothelial monolayer permeability were assessed using the electrical cell impedance sensor technique (Applied Biophysics) or the two-compartment Transwell system (Costar, Inc). Using the electrical cell impedance sensor technique, equivalent numbers of endothelial cells (2.0×10^6 cells per well) were plated on collagen-coated gold electrodes and permitted to adhere overnight in MEM containing 10% FBS. Once the resistance across monolayers has reached 500 ohms, the experiments were performed and the electrical resistance was recorded. Using the Transwell system, changes in endothelial monolayer permeability were assessed as previously described.

Results

ICMT Inhibition Decreased Endothelial Monolayer Permeability

In previous studies, we demonstrated that adenosine increases intracellular S-adenosylhomocysteine (SAH) in endothelial cells because of the reversible action of SAH hydrolase. The enhanced intracellular SAH, in turn, inhibits S-adenosylmethionine-dependent methyltransferase activity (Figure 1). We have previously shown that adenosine plus homocysteine (Ado/HC) or AGGC, a prenylcysteine analog which competes with endogenous prenylated proteins for methylation, blunts carboxyl methylation of Ras by inhibiting ICMT activity. In this study, we examined the effects of ICMT inhibition by Ado/HC or AGGC on endothelial monolayer permeability.

Electrical resistance across confluent PAEC monolayers was measured in the absence or presence of Ado/HC, AGGC, or inactive prenylcysteine analog, AGC (Figure 2). We found that both Ado/HC and AGGC dose-dependently increased the electrical resistance (Figures 2A and 2B), corresponding with diminished endothelial monolayer permeability. Maximal effects on endothelial barrier function were seen with 50 μmol/L Ado/HC or 1 μmol/L AGGC. The inactive analog, AGC, did not affect endothelial monolayer permeability (Figure 2C). There were no significant differences in the

Transfection and Infection

PAECs stably overexpressing ICMT-GFP or GFP were established by calcium phosphate transfection with pICMT-GFP or pGFP-C1 cDNAs, as previously described. PAEC clonal populations stably overexpressing ICMT-GFP or GFP were selected using neomycin resistance and amplified. ICMT or GFP protein overexpression was confirmed by immunoblot and immunofluorescence analyses and by ICMT enzyme activity assays, as previously described.

Confluent PAEC monolayers stably overexpressing ICMT-GFP or GFP were infected with Ad RhoA dn or Ad Rac1 dn at 40,000 optical forming units per cell. Parallel experiments were also performed using Ad GFP as control. After a 6-hour incubation at 37°C, the medium was replaced with complete MEM and cells were incubated overnight before performing experiments.

Rho GTPase Activation and Carboxyl Methylation Assays

Rho GTPase activity was measured using pull-down assay as described and by measuring subcellular relocation from the cytosol to the membrane fraction as previously described. Rho GTPase carboxyl methylation was assayed as previously described.

Materials and Methods

Materials

Bovine PAECs were obtained as described.

Adenosine and DL-homocysteine were purchased from Sigma. N-acetyl-S-geranylgeranyl-L-cysteine (AGGC) and N-acetyl-S-geranyl-L-cysteine (AGC) were obtained from Biomol. Antibodies directed against RhoA, Rac1, VE-cadherin, and β-catenin were purchased from Santa Cruz Biotechnologies (Santa Cruz, Calif).

The pGST-C21 construct, pCMT-GFP construct (referred to as pCMT-GFP), and rabbit polyclonal antibody against amphibian ICMT were generously provided by Drs J. Collard (Netherlands Cancer Institute, Amsterdam), M. Philips (New York University School of Medicine, New York, NY), and J. Stockand (University of Texas Health Science Center at San Antonio, Tex), respectively. The recombinant adenoviruses containing CDNA of dominant-negative RhoA were generously provided by Drs T. Hirase and A. Ridley (University College London, UK), respectively.

Immunoblot and Immunofluorescence Analysis

Proteins were resolved using 15% SDS-PAGE or 2-dimensional PAGE (pH 5.0 to 7.0), and immunoblotting was performed as previously described. Immunofluorescence studies were performed as described. Images were viewed either at ×630 magnification using a laser-scanning confocal microscope or ×100 magnification using a Nikon Eclipse E400 fluorescence microscope and recorded.

Electrode Resistance

PAECs stably overexpressing ICMT-GFP or GFP were established by calcium phosphate transfection with pICMT-GFP or pGFP-C1 cDNAs, as previously described. PAEC clonal populations stably overexpressing ICMT-GFP or GFP were selected using neomycin resistance and amplified. ICMT or GFP protein overexpression was confirmed by immunoblot and immunofluorescence analyses and by ICMT enzyme activity assays, as previously described.

Confluent PAEC monolayers stably overexpressing ICMT-GFP or GFP were infected with Ad RhoA dn or Ad Rac1 dn at 40,000 optical forming units per cell. Parallel experiments were also performed using Ad GFP as control. After a 6-hour incubation at 37°C, the medium was replaced with complete MEM and cells were incubated overnight before performing experiments.

Rho GTPase Activation and Carboxyl Methylation Assays

Rho GTPase activity was measured using pull-down assay as described and by measuring subcellular relocation from the cytosol to the membrane fraction as previously described. Rho GTPase carboxyl methylation was assayed as previously described.

Results

ICMT Inhibition Decreased Endothelial Monolayer Permeability

In previous studies, we demonstrated that adenosine increases intracellular S-adenosylhomocysteine (SAH) in endothelial cells because of the reversible action of SAH hydrolase. The enhanced intracellular SAH, in turn, inhibits S-adenosylmethionine-dependent methyltransferase activity (Figure 1). We have previously shown that adenosine plus homocysteine (Ado/HC) or AGGC, a prenylcysteine analog which competes with endogenous prenylated proteins for methylation, blunts carboxyl methylation of Ras by inhibiting ICMT activity. In this study, we examined the effects of ICMT inhibition by Ado/HC or AGGC on endothelial monolayer permeability.

Electrical resistance across confluent PAEC monolayers was measured in the absence or presence of Ado/HC, AGGC, or inactive prenylcysteine analog, AGC (Figure 2). We found that both Ado/HC and AGGC dose-dependently increased the electrical resistance (Figures 2A and 2B), corresponding with diminished endothelial monolayer permeability. Maximal effects on endothelial barrier function were seen with 50 μmol/L Ado/HC or 1 μmol/L AGGC. The inactive analog, AGC, did not affect endothelial monolayer permeability (Figure 2C). There were no significant differences in the
baseline resistance of vehicle and inhibitor-treated monolayers. Similar changes in endothelial monolayer permeability were noted when measuring the rate of horseradish peroxidase (HRP) diffusion through monolayers by using the Transwell system. The electrical resistance across the monolayers was measured over time in the absence (vehicle) or presence of indicated concentrations of Ado/HC (A), AGGC (B), or AGC (C). Arrow indicates the time of addition of vehicle or inhibitors. Data are presented as the mean±SE; n=3; *P<0.05 vs vehicle.

Figure 2. Effect of ICMT inhibition on endothelial monolayer permeability. Equivalent numbers of PAECs were grown to confluence in MEM containing 10% serum on collagen-coated gold electrodes. The electrical resistance across the monolayers was measured over time in the absence (vehicle) or presence of indicated concentrations of Ado/HC (A), AGGC (B), or AGC (C). Arrow indicates the time of addition of vehicle or inhibitors. Data are presented as the mean±SE; n=3; *P<0.05 vs vehicle.

ICMT Inhibition Decreased RhoA Carboxyl Methylation and Activation

We assessed whether ICMT inhibition altered RhoA methylation or activation. Changes in RhoA carboxyl methylation on exposure to Ado/HC or AGGC were assessed by 2-dimensional PAGE and immunoblot analysis and by assaying the incorporation of radiolabeled methyl groups into immunoprecipitated RhoA. The level of methylated RhoA was significantly diminished in endothelial monolayers exposed to 50 μmol/L Ado/HC compared with control cells (Figure 3A). Incubation of PAECs with MEM containing 1 μmol/L AGGC for 10 minutes decreased the level of methylated RhoA, as measured by 2-dimensional PAGE (methylated RhoA/total RhoA was 0.725±0.012 in vehicle-treated cells versus 0.640±0.006 in AGGC-treated cells, n=3, P=0.002). Similar results were also found by measuring [3H]methyl incorporation in PAECs treated with 1 μmol/L AGGC for 4 hours (Figure 3B). Affinity precipitation assays of GTP-bound RhoA demonstrated reduced RhoA activity in endothelial cells exposed to 50 μmol/L Ado/HC or 1 μmol/L AGGC (Figure 3C). These data indicate that ICMT may modulate RhoA carboxyl methylation and subsequent activation.

ICMT Overexpression Enhanced Endothelial Monolayer Permeability

To additionally address the role of ICMT in endothelial barrier function and RhoA methylation, we established PAEC cell lines stably overexpressing GFP or ICMT-GFP cDNAs. Clonal cell lines were isolated, and overexpression of GFP or ICMT-GFP protein was confirmed by fluorescence microscopy and immunoblot analysis. In each clone of the GFP cell line, GFP protein was present diffusely, with a pronounced signal in the nucleus (Figure 4A). Consistent with previous reports,15,17 ICMT-GFP fusion protein was localized primarily in the perinuclear region of each clone of the ICMT-GFP cell line (Figure 4A). Immunoblot analysis identified de novo ICMT in both GFP and ICMT-GFP cell lines, as well as a higher-molecular-weight species corresponding to GFP-conjugated ICMT protein in the ICMT-GFP cell line (Figure 4B). We found that ICMT overexpression increased ICMT enzymatic activity in each clone of the ICMT-GFP cell line compared with the GFP cell line or untransfected PAECs. The representative clone of the ICMT-GFP cell line demonstrated a 17-fold increase in ICMT activity compared with the representative clone of the GFP cell line (Figure 4C).

We next examined the effect of ICMT overexpression on endothelial monolayer permeability. PAECs stably overexpressing ICMT-GFP demonstrated significantly diminished electrical resistance compared with PAECs stably overexpressing GFP, indicating enhanced barrier dysfunction. ICMT overexpression was seen in each clone of ICMT-GFP cell line and decreased monolayer resistance. The electrical resistance of representative clones of GFP and ICMT-GFP cell lines is shown in Figure 4D. Again, these data suggest that ICMT modulates endothelial monolayer permeability. After these initial analyses, representative clones of each cell line were chosen for additional studies.
ICMT Overexpression Enhanced RhoA Methylation and Activation in Endothelial Cells

Two-dimensional PAGE and densitometric quantitation of the immunoblot signals demonstrated that PAECs stably overexpressing ICMT-GFP had a significantly greater amount of methylated RhoA compared with PAECs stably overexpressing GFP or untransfected PAECs (Figure 5A). To ensure that enhanced RhoA carboxyl methylation correlated with elevated activity, we determined the level of membrane-associated RhoA and GTP-bound RhoA. We demonstrated an enhanced level of membrane-associated RhoA in ICMT-GFP-overexpressing PAECs compared with GFP-overexpressing PAECs (Figure 5B). Affinity precipitation assays of GTP-bound RhoA demonstrated increased RhoA activity in lysates from ICMT-GFP–overexpressing PAECs (Figure 5C). Taken together, these data indicate that ICMT overexpression increased RhoA carboxyl methylation and promoted RhoA activation, suggesting that carboxyl methylation of RhoA by ICMT may regulate its subcellular localization and enzyme activity.

ICMT Overexpression Disrupted Adherens Junctions in Endothelial Cells

RhoA activation and intercellular junction disruption are implicated in thrombin and histamine-enhanced endothelial monolayer permeability.4 We found that VE-cadherin and β-catenin, major components of endothelial adherens junctions,30 were localized along the cell margins of PAECs stably overexpressing GFP (Figure 6A, left), similar to previous reports.4,30 However, PAECs stably overexpressing ICMT-GFP displayed intercellular gaps and diminished staining of VE-cadherin and β-catenin at cell-cell junctions (Figure 6A, right). Immunoblot analyses demonstrated no significant differences in the total protein content of either VE-cadherin or β-catenin between ICMT-GFP–overexpressing and GFP-overexpressing PAECs (data not shown). These results suggest that enhanced level of basal monolayer permeability noted in PAECs stably overexpressing ICMT-GFP was attributable to diminished adherens junctions and enhanced intercellular gaps. Incubation of PAECs stably overexpressing ICMT-GFP with Ado/HC enhanced the amounts of VE-cadherin and β-catenin at intercellular junctions (Figure 6B), indicating that disruption of adherens junction by ICMT overexpression was blunted by inhibition of ICMT activity. These data suggest that ICMT activity modulates adherens junction organization or stability.

Figure 3. Effect of ICMT inhibition on RhoA carboxyl methylation and activation. A, PAECs were incubated in MEM in the absence (vehicle) or presence of 50 μmol/L Ado/HC for 10 minutes. Cells were harvested, and equivalent volumes of lysates were resolved by 2-dimensional PAGE and immunoblotted for RhoA. Methylated RhoA (indicated by m) and unmethylated RhoA (indicated by u) are shown in the blots. The immunoblot signals were quantified by densitometry, and the data are presented as mean±SE of the ratio of methylated RhoA to total RhoA. n=3. B, PAECs were incubated in MEM in the absence (vehicle) or presence of 1 μmol/L AGGC or AGC for 4 hours. RhoA carboxyl methylation was detected by measuring the level of [3H]methyl incorporation into immunoprecipitated RhoA, as described in Materials and Methods. The level of RhoA carboxyl methylation is presented as the mean±SE relative to vehicle. n=3 for vehicle and AGGC; n=2 for AGC. C, PAECs were incubated in MEM in the absence (vehicle) or presence of 50 μmol/L Ado/HC or 1 μmol/L AGGC for 30 minutes. Cell lysates were collected, and active RhoA GTPase (GTP-bound RhoA) was purified and quantitated as described in Materials and Methods. The level of activated RhoA is presented as the mean±SE of the ratio of GTP-bound RhoA to total RhoA relative to vehicle, n=3. Immunoblots in A and C are representatives of 3 individual experiments. *P<0.05 vs vehicle.
RhoA Inhibition Attenuated Effects of ICMT on Endothelial Monolayer Permeability and Adherens Junction Formation

We speculated that ICMT augmented endothelial monolayer permeability by increasing RhoA carboxyl methylation, thus elevating RhoA activity. To test this, we transiently overexpressed dominant-negative RhoA in PAECs stably overexpressing GFP or ICMT-GFP by infection of Ad RhoA dn and assessed effects on endothelial monolayer permeability and adherens junction formation. Parallel experiments were also performed using Ad GFP as a control. Expression of GFP and RhoA proteins was confirmed by immunofluorescence microscopy (data not shown) and by immunoblot analysis (Figure 7A). Similar to findings in Figure 4D, PAECs stably overexpressing ICMT-GFP and transiently infected with Ad GFP had enhanced monolayer permeability compared with PAECs stably overexpressing GFP and infected with Ad GFP (Figure 7B). Inhibition of RhoA by infection of Ad RhoA dn significantly diminished endothelial monolayer permeability in PAECs stably overexpressing ICMT-GFP to a level not significantly different from PAECs stably overexpressing GFP and infected with Ad GFP (Figure 7B). Additionally, ICMT-GFP–overexpressing PAECs infected with Ad GFP displayed intercellular gaps and less VE-cadherin and β-catenin localized at the cell periphery (Figure 7C, left), which is similar to the findings in the noninfected ICMT-GFP–overexpressing PAECs (Figure 6A, right). Inhibition of RhoA attenuated the formation of intercellular gaps between PAECs stably overexpressing ICMT-GFP, with concomitant increase in VE-cadherin and β-catenin relocalization to the cell margins (Figure 7C, right). These data suggest that changes in monolayer permeability in ICMT-GFP–overexpressing PAECs are attributable to the effect of RhoA on adherens junctions.

Role of Rac1 in ICMT-Mediated Endothelial Monolayer Permeability

Rac1 also regulates endothelial monolayer permeability.4,31,32 To test whether Rac1 was also involved in ICMT-mediated endothelial monolayer permeability, PAECs stably overexpressing GFP or ICMT-GFP were infected with Ad Rac1 dn or Ad GFP. Rac1 overexpression was confirmed by immunoblot analysis (Figure 8A). Unlike RhoA inhibition, Rac1 inhibition significantly increased endothelial monolayer permeability to the same extent in both ICMT-GFP–overexpressing and GFP–overexpressing PAECs (Figure 8B), suggesting that Rac1 is not involved in ICMT-regulated endothelial permeability changes. Additionally, Rac1 subcellular localization was not significantly altered in PAECs exposed to 50 μmol/L Ado/HC or 1 μmol/L AGGC (Figure 8C) or in the ICMT-GFP–overexpressing PAECs (data not shown), suggesting that alterations in ICMT activity may not modulate Rac1 activity. Furthermore, we were unable to detect incorporation of radiolabeled methyl groups in immunoprecipitated Rac1 protein isolated from vehicle or 50 μmol/L Ado/HC-treated, 1 μmol/L AGGC–treated, or 1 μmol/L AGC-treated PAECs, suggesting that methylation may not be a prominent posttranslational modification of Rac1 or that the half-life of Rac1 is too long to detect a change in methylation after a 4-hour or overnight labeling period.

---

**Figure 4.** Effect of ICMT overexpression on endothelial monolayer permeability. PAECs stably overexpressing GFP or ICMT-GFP were established, and protein overexpression was confirmed by fluorescence microscopy (A), immunoblot analysis using an antibody directed against amphibian ICMT (B), and an in vitro methyltransferase assay (C). Equivalent numbers of GFP or ICMT-GFP overexpressing PAECs were grown to confluence on gold electrodes, and the electrical resistance across the monolayers was measured (D). Data are presented as mean±SE; n=3 (C), n=4 (D); *P<0.05 vs controls.
Discussion

We found that inhibition of ICMT activity with either Ado/HC or AGGC dose-dependently diminished endothelial monolayer permeability, as assessed by electrical resistance and HRP diffusion across monolayers. Increased ICMT enzyme activity by stable overexpression promoted endothelial monolayer permeability. In addition, RhoA inhibition prevented the effects of ICMT overexpression on monolayer permeability and adherens junctions. Thus, it appears that carboxyl methylation of RhoA by ICMT modulates endothelial monolayer permeability.

Figure 5. Effects of ICMT overexpression on RhoA methylation, localization, and activation. GFP-overexpressing or ICMT-GFP-overexpressing PAEC lysates were assessed for RhoA methylation by 2-dimensional PAGE and quantified by densitometry. Methylated RhoA (indicated by m) and unmethylated RhoA (indicated by u) are shown in the blots. RhoA methylation is presented as the mean±SE of the ratio of methylated RhoA to total RhoA (A). B, Membrane and cytosolic proteins from equivalent amounts of GFP-overexpressing or ICMT-GFP-overexpressing PAEC lysates were resolved by SDS-PAGE and immunoblotted for RhoA. Immunoblot signals were quantified and are presented as mean±SE of the ratio of membrane-bound RhoA to total RhoA. C, Both GTP-bound and total RhoA in lysates of PAECs stably overexpressing GFP or ICMT-GFP were assessed; activated RhoA is presented as mean±SE of the ratio of GTP-bound RhoA to total RhoA. n=3; *P<0.05 vs control.

Figure 6. Effects of ICMT overexpression on assembly of adherens junctions. VE-cadherin and β-catenin localization in confluent PAEC monolayers stably overexpressing GFP or ICMT-GFP (A) or in confluent ICMT-GFP-overexpressing PAEC monolayers, which were exposed to vehicle or 50 μmol/L Ado/HC for 30 minutes (B), were assessed by immunofluorescence and visualized by fluorescence confocal microscopy. Arrowheads indicate intercellular gaps. Representative images are shown, n=3.
ICMT is a 32-kDa protein localized to the ER, catalyzing carboxyl methylation of several C-terminal isoprenylated proteins, such as Ras and RhoA. ICMT-deficient mice die by midgestation, suggesting that protein carboxyl methylation is critical to embryonic development and that no redundant pathway exists for methylation of these proteins. Backlund et al demonstrated that most of RhoA is methylated shortly after synthesis in exponentially growing RAW264 macrophage cells. Choy et al demonstrated that Ras carboxyl methylation is required for efficient plasma membrane association. We have previously shown that inhibition of ICMT by Ado/HC or AGGC decreased Ras carboxyl methylation and caused apoptosis, whereas ICMT overexpression inhibited apoptosis in endothelial cells. Stockand et al have reported that aldosterone-induced sodium reabsorption by Xenopus laevis distal tubule epithelial cells is modulated by ICMT activity. Ahmad et al have reported that ICMT modulates tumor necrosis factor α-induced expression of vascular cell adhesion molecule-1 in human aortic endothelial cells. In the present study, we demon-
strated that RhoA methylation is altered on changes in ICMT enzyme activity, correlating with modulation of endothelial monolayer permeability. These results additionally demonstrate an important role in cell function for ICMT-mediated posttranslational modification of Rho-family GTPases.

Rho GTPases are synthesized as cytosolic proteins. To function properly, the Rho GTPases undergo a series of posttranslational modifications at the carboxyl-terminal CAAX motif, of which prenylation and carboxyl methylation have been best characterized. These modifications generate a hydrophobic carboxyl terminus, thus targeting the proteins to internal membranes and plasma membrane. It has been suggested that prenylation specifically targets Rho proteins to the ER, where they are then methylated by ICMT. Inhibition of carboxyl methyltransferase activity by homocysteine and the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)-adenine decreased plasma membrane localization of v-H-Ras in vascular endothelial cells. We have previously shown that Ado/HC, AGGC, and N-acetyl-S-farnesyl-L-cysteine significantly reduced membrane-associated Ras in PAECs. In addition, the absence of ICMT caused mislocalization of K-Ras from plasma membrane to the cytoplasmic compartment in cells derived from ICMT knockout embryos. Carboxyl methylation of Gγ1 protein is necessary for plasma membrane targeting of the Gγ1/β, heterodimeric protein complex. In the present study, we found that overexpression of ICMT increased membrane localization of RhoA. This result supports the idea that, like Ras, carboxyl methylation of RhoA enhances membrane localization. The mechanism of methylation-induced translocation of RhoA is not clear. It is possible that methylation enhances hydrophobicity, thus facilitating binding to membrane.

Subcellular translocation of Rho GTPases is also observed on activation by GTP binding. We speculated that there is a direct link between methylation, translocation, and activation of RhoA. In support of this idea, we found ICMT inhibitors diminished both RhoA methylation and RhoA activity. Conversely, overexpression of ICMT enhanced RhoA methylation, membrane translocation, and RhoA activity. The mechanism by which RhoA methylation enhances activation is not addressed by our studies. It is possible that methylation changes its interaction with other proteins, such as GEFs or RhoGDIα. Mizuno et al demonstrated that RhoGEF interacted only with posttranslationally processed RhoA but not unprocessed RhoA. Because RhoGDIα inhibits the exchange of bound GDP for GTP and inhibits Rho activation, it is possible that RhoA methylation releases bound RhoGDIα, thereby enhancing GTP binding and RhoA activation. We cannot exclude the possibility that RhoA was activated by some other substrates of ICMT and that activation of RhoA results in its methylation and translocation.

Endothelial monolayer permeability is regulated by actin stress fibers and intercellular junctions. RhoA increases endothelial monolayer permeability by enhancing stress fiber formation and by disrupting intercellular junctions. In this study, we found that ICMT inhibition reduced RhoA carboxyl methylation and activation and decreased monolayer permeability. Conversely, ICMT overexpression increased RhoA carboxyl methylation and activation and disrupted endothelial adherens junctions, inducing intercellular gaps and increasing monolayer permeability. These data are consistent with findings that thrombin and histamine disassemble intercellular junctions by activation of RhoA. Additionally, these findings provide strong evidence to support the idea that ICMT-mediated RhoA activation regulates endothelial monolayer permeability by affecting intercellular junctions. The effects of ICMT overexpression on disruption of adherens junctions and enhanced monolayer permeability were prevented by Ado/HC and by RhoA inhibition. These data strongly suggest that ICMT-mediated RhoA carboxyl methylation and activation are critical for organization of adherens junctions and modulation of endothelial monolayer permeability.

Although Cdc42 has been shown not to be involved in the regulation of endothelial permeability, four studies have suggested a role for Rac1. Overexpression of constitutively active Rac1 increased basal endothelial monolayer permeability with concomitant diminished adherens junctions and tight junctions. Wójciak-Stothard et al also showed that overexpression of dominant-negative Rac1 also augmented basal and agonist-stimulated endothelial monolayer permeability. In vivo studies suggest that vascular endothelial growth factor–mediated increase in endothelial permeability may occur via a Rac1-dependent pathway. Consistent with observations of Wójciak-Stothard et al, we also found that inhibition of Rac1 increased endothelial monolayer permeability to the same extent in both GFP-overexpressing and ICMT-GFP–overexpressing PAECs. These results suggest that Rac1 methylation may not be involved in regulation of endothelial monolayer permeability. As noted earlier, activation of Rho GTPases correlates with membrane translocation. We found that alteration of ICMT activity did not alter Rac1 subcellular localization, suggesting that ICMT may not regulate Rac1 activity.

Several studies have suggested an interdependence of the activities of RhoA and Rac1 in modulating cellular stress fiber, focal contact, tight junction, and adherens junction formation or stabilization. Rac1 activity has been suggested to be dependent on RhoA in modulating stress fiber and focal contact formation in unstimulated endothelial cells. Conversely, Rac1 seems to be independent of RhoA in thrombin- or histamine-induced changes in endothelial monolayer permeability. Also, the studies of Tsuji et al suggest that Rac1 activation is downstream from RhoA in inducing LPA-stimulated fibroblast ruffling but also parallel to RhoA-stimulated ROCK and mDia1 pathways in modulating the fibroblast cytoskeleton. Thus, it seems that RhoA can modulate Rac1 activity under several conditions and in several cell types. We showed that inhibition of RhoA blunted ICMT-mediated increase in endothelial permeability. Yet Rac1 inhibition enhanced endothelial barrier dysfunction independent of ICMT overexpression. Our findings suggest that RhoA and Rac1 regulate endothelial monolayer permeability independently.
In summary, ICMT modulated endothelial monolayer permeability. Alteration of ICMT activity affected RhoA carboxyl methylation, activation, and subcellular translocation, as well as organization of adherens junctions. In addition, the effects of ICMT on monolayer permeability and adherens junction formation were blunted by ICMT inhibitors and by RhoA inhibition. Thus, carboxyl methylation of RhoA may modulate endothelial barrier function. Additional elucidation of the mechanism by which carboxyl methylation of RhoA regulates endothelial barrier function may be useful in establishing therapeutic strategies against diseases characterized by increased vascular permeability.

Acknowledgments
This material is based on work supported in part by the Office of Research and Development, Department of Veterans Affairs, VA Merit Review, VA/Department of Defense Collaborative Research Awards and NIH HL 64936 to S.R., and VA Merit Review Type II Awards and NIH HL 64936 to S.R., and VA Merit Review Type II.

References


Isoprenylcysteine Carboxyl Methyltransferase Modulates Endothelial Monolayer Permeability: Involvement of RhoA Carboxyl Methylation
Qing Lu, Elizabeth O. Harrington, Chi-Ming Hai, Julie Newton, Megan Garber, Tetsuaki Hirase and Sharon Rounds

Circ Res. 2004;94:306-315; originally published online December 29, 2003;
doi: 10.1161/01.RES.0000113923.85084.C1
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/94/3/306

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/