Isoprenylcysteine Carboxyl Methyltransferase Activity Modulates Endothelial Cell Apoptosis

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Extracellular ATP, adenosine (Ado), and adenosine plus homocysteine (Ado/HC) cause apoptosis of cultured pulmonary artery endothelial cells through the enhanced formation of intracellular S-adenosylhomocysteine and disruption of focal adhesion complexes. Because an increased intracellular ratio of S-adenosylhomocysteine/S-adenosylmethionine favors inhibition of methylation, we hypothesized that Ado/HC might act by inhibition of isoprenylcysteine-O-carboxyl methyltransferase (ICMT). We found that N-acetyl-S-geranylgeranyl-L-cysteine (AGGC) and N-acetyl-S-farnesyl-L-cysteine (AFC), which inhibit ICMT by competing with endogenous substrates for methylation, caused apoptosis. Transient overexpression of ICMT inhibited apoptosis caused by Ado/HC, UV light exposure, or tumor necrosis factor- α . Because the small GTPase, Ras, is a substrate for ICMT and may modulate apoptosis, we also hypothesized that inhibition of ICMT with Ado/HC or AGGC might cause endothelial apoptosis by altering Ras activation. We found that ICMT inhibition decreased Ras methylation and activity and the activation of the downstream signaling molecules Akt, ERK-1, and ERK-2. Furthermore, overexpression of wild-type or dominant active H-Ras blocked Ado/HC-induced apoptosis. These findings suggest that inhibition of ICMT causes endothelial cell apoptosis by attenuation of Ras GTPase methylation and activation and its downstream antiapoptotic signaling pathway.

INTRODUCTION

Vascular injury has been implicated in the pathogenesis of disorders such as sepsis and acute respiratory distress syndrome (ARDS). Endothelial cell apoptosis, or programmed cell death, may be important in vascular injury and repair. Apoptotic cells have been identified in increased quantities in the lungs of patients with ARDS, indicating that apoptosis occurs in this syndrome (Polunovsky *et al.*, 1993). Apoptosis can be triggered by disruption of cell–extracellular matrix communication (anoikis) (Frisch and Francis, 1994) or by extracellular factors, such as lipopolysaccharide (Han and Wyche, 1994; Hoyt *et al.*, 1995; Mebmer *et al.*, 1999), tumor necrosis factor (TNF)- α (Polunovsky *et al.*, 1994), or UV light (Chatterjee and Wu, 2001). We have previously demonstrated that increased extracellular ATP causes endothelial cell apoptosis after conversion to adenosine and uptake into

cells. Moreover, apoptosis caused by intracellular adenosine was enhanced by homocysteine (Dawicki *et al.*, 1997). In subsequent work, we found that increased concentrations of adenosine and homocysteine or inhibition of S-adenosylhomocysteine hydrolase resulted in enhanced levels of S-adenosylhomocysteine (SAH) (Rounds *et al.*, 1998). Because enhanced intracellular concentrations of SAH may result in product inhibition of S-adenosylmethionine (SAM)-dependent methyltransferases (Perna *et al.*, 1997) (Figure 1), we postulated that methyltransferase activity is important in the modulation of endothelial cell apoptosis.

Among the methyltransferases is isoprenylcysteine-O-carboxyl methyltransferase (ICMT), the substrates of which are proteins containing the CAAX motif (C = cysteine, A = aliphatic amino acid, and X = any amino acid) at the C terminus. Such proteins first undergo prenylation of the cysteine residue, followed by proteolytic cleavage of the AAX sequence. ICMT subsequently catalyzes reversible carboxyl methyl esterification of the prenylated cysteine, using SAM as the methyl donor (Clarke *et al.*, 1988; Philips *et al.*, 1993; Philips and Pillinger, 1995). This posttranslational modification may be important in maintaining normal function of signaling proteins, such as the Ras superfamily of GTPases (Boivin *et al.*, 1996).

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Figure 1. Schematic representation of the intracellular pathways involved in adenosine metabolism. Dashed line represents product inhibition of SAM-dependent methyltransferases.

In the present experiments, we assessed the role of ICMT in endothelial cell apoptosis by inhibiting the enzyme or by increasing expression of the enzyme through transient transfection. We now report that inhibition of ICMT causes apoptosis of pulmonary artery endothelial cells and that overexpression of ICMT protects against apoptosis induced by Ado/HC or UV light. We also show that inhibition of ICMT with Ado/HC enhances TNF- α -induced apoptosis. We demonstrate that inhibition of ICMT decreased Ras GTPase carboxyl methylation and activity, resulting in diminished activation of the downstream signaling molecules Akt, ERK-1, and ERK-2. Finally, we demonstrate that overexpression of dominant active and wild-type H-Ras prevented apoptosis caused by ICMT inhibition. Hence, ICMT activity modulates apoptosis induced by various conditions, possibly through effects on the activity of small GTPases.

MATERIALS AND METHODS

Cell Lines and Reagents

Endothelial cells were obtained from bovine main pulmonary arteries as previously described (Dawicki *et al.*, 1997).

Adenosine, DL-homocysteine, 7-amino-4-methylcoumarin (AMC), and Hoechst 33342 were purchased from Sigma (St. Louis, MO). TNF- α was obtained from Calbiochem (La Jolla, CA). N-Acetyl-S-geranylgeranyl-L-cysteine (AGGC), N-acetyl-S-geranyl-L-cysteine (AGC), N-acetyl-S-farnesyl-L-cysteine (AFC), S-farnesylthioacetic acid (FTA), and S-farnesylthiosalicylic acid (FTS) were purchased from Biomol (Plymouth Meeting, PA). The fluorogenic peptide N-acetyl-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) was purchased from PharMingen (San Diego, CA). S-[Methyl- 3 H]-adenosyl-L-methionine (3 H-SAM) was obtained from NEN Life Science Products, Inc (Boston, MA). Glutathione agarose beads were obtained from Amer-sham Pharmacia Biotech (Piscataway, NJ).

The eukaryotic expression vector encoding the human prenylcysteine carboxyl methyltransferase cDNA (pcCMT-GFP) (referred to as pICMT-GFP in this text) was a generous gift from Dr. Mark Philips, New York University School of

Medicine, New York, NY (Dai *et al.*, 1998). pGFP-C1 was purchased from Clontech (Palo Alto, CA). The GST-Raf 1-bound glutathione agarose beads were purchased from Upstate Biotechnology (Lake Placid, NY). The pUSEamp, pUSE-H-Ras(wt), pUSE-H-Ras(L61), and pUSE-H-Ras(dn) constructs were obtained from Upstate Biotechnology.

Antibodies directed against green fluorescent protein (GFP) were obtained from Molecular Probes (Eugene, OR). Antibodies specific for Akt, ERK-1/ERK-2 (p44/42), phosphorylated Akt (Akt^{serine473}), and phosphorylated ERK-1/ERK-2 (p44/42^{Thr202/Tyr204}) were purchased from Cell Signaling (Beverly, MA). Monoclonal antibodies directed against Ras were purchased from Upstate Biotechnology.

Transfection and Culture Conditions

Pulmonary artery endothelial cells (PAECs) were cultured and transfected with pGFP-C1, pICMT-GFP, pUSEamp, or the H-Ras cDNA constructs as previously described (Bellas *et al.*, 2002).

For the ICMT transient overexpression studies, PAECs grown on coverslips were transiently transfected with the indicated cDNA using the calcium phosphate method and incubated for 24 h. The cells were then incubated for 20 h with Ado/HC in HEPES or TNF- α in serum-free MEM. As controls, endothelial cells were treated with HEPES or serum-free MEM in parallel. In other experiments, the cells were exposed to UV light ($\lambda_{254\text{nm}}$) for 0, 1, 2, or 3 min and incubated in serum-free MEM for 20 h. All samples were analyzed for apoptosis by fluorescence microscopy, as described below.

Assessment of Apoptosis

PAECs were cultured and apoptosis was assessed as described previously (Bellas *et al.*, 2002).

Caspase-3 Activity Assay

PAECs were harvested, and the washed pellets were resuspended in caspase lysis buffer (10 mM HEPES, pH 7.5, 40 mM β -glycerophosphate, 50 mM NaCl, 2 mM MgCl₂, and 5 mM EGTA). The cells were lysed with freeze-thaw cycles, and insoluble cellular debris was removed by centrifugation. Caspase activity was quantified as the release of the fluorescent conjugate AMC from the peptide substrate DEVD, as described previously (Harrington *et al.*, 2001).

Immunoblot Analysis

For confirmation of overexpression of ICMT, GFP, H-Ras proteins, and Akt and ERK-1/ERK-2 activation/phosphorylation, PAECs were cultured and treated as described. Cells were harvested directly into Laemmli buffer, and equivalent volumes of proteins were resolved by SDS-PAGE. Immunoblot analysis was performed as previously described (Harrington *et al.*, 2001).

ICMT Enzymatic Activity Assay

ICMT enzymatic activity was assessed using alkaline hydrolysis of methyl esters in a vapor-phase assay (Pillinger *et al.*, 1994; Desrosiers *et al.*, 1999). PAECs were harvested by scraping. The cells were collected by centrifugation at

$5500 \times g$ for 5 min. The pellet was resuspended in lysis buffer containing 250 mM sucrose, 5 mM HEPES, pH 7.5, 5 mM Tris-Cl, pH 7.5, 20 $\mu\text{g}/\text{ml}$ aprotinin, 20 $\mu\text{g}/\text{ml}$ leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The pellet suspension was incubated on ice for 30 min and homogenized in a Dounce homogenizer. The suspension was centrifuged at $500 \times g$ for 10 min at 4°C to separate cell nuclei. The supernatant was collected, and protein concentrations were determined by Lowry assay.

In this *in vitro* assay, AGGC was used as an artificial substrate, and S-[methyl- ^3H]-adenosyl-L-methionine (^3H -SAM) was used as the methyl donor. Negative controls either lacked substrate or contained the inactive analogue AGC as artificial substrate. Cell lysates were incubated with 720 nM ^3H -SAM and 20 μM AGGC in 50 mM Tris-HCl, 1 mM EDTA, pH 8.0, at 37°C for 1 h. Reactions were initiated by the addition of cell lysates, stopped by addition of 50 μl of 20% trichloroacetic acid, and vortexed for 10 s. Heptane was added to each sample, followed by vortexing for 10 s. Samples were centrifuged at $15,000 \times g$ for 3 min. The recovered organic phase (300 μl) was placed in open Eppendorf tubes, and heptane was removed by vacuum centrifugation. Carboxyl methyl esters were hydrolyzed by addition of 100 μl 1N NaOH. The Eppendorf tubes were carefully lowered into sealed scintillation vials containing 8 ml of scintillation fluid (Ultima Gold; Packard, Meridian, CT) and incubated for 48 h at room temperature. The radioactive methanol released as a result of base-mediated hydrolysis of carboxyl esters was counted. Carboxyl ^3H -SAM counts were corrected for background of base-labile radioactivity present in ^3H -SAM alone. ICMT activity is expressed as picomoles methylated AGGC per milligram protein per minute.

Protein Carboxyl Methylation Assay

PAECs were pretreated with methionine-free MEM with or without 20 μM AGGC, 20 μM AGC, or 100 μM Ado/HC for 1 h. Cultures were metabolically labeled by incubation with 100 μCi [^3H]methyl-methionine in methionine-free MEM with 20 μM AGC or 20 μM AGGC for 4 h or 100 μM Ado/HC overnight at 37°C . Cells were lysed *in situ* with fresh lysis buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 $\mu\text{g}/\text{ml}$ aprotinin, 1 μM PMSF, 1 μM pepstatin) and dissociated by 10–15 passages through a 21-gauge needle. Cellular debris was removed by two centrifugations at $500 \times g$ for 15 min at 4°C and again at $11,000 \times g$ for 15 min at 4°C . Total cell lysate (100 μg) was resolved on 15% SDS-PAGE and dried. A SDS-PAGE was run in parallel and immunoblotted for H-Ras (21 kDa). This autoradiograph was used as a reference for the dried gel to excise bands at 21 kDa. The excised band was hydrolyzed with 1N NaOH for 24 h at 37°C in an open microfuge tube suspended in scintillation fluid (Wang *et al.*, 1997). Results are reported as cpm per microgram of 21 kDa protein.

In other experiments, Ras GTPase was immunoprecipitated and resolved on 15% SDS-PAGE. Gels were immunoblotted with Ras antibody, and illuminated bands were excised and hydrolyzed as described above.

Ras Subcellular Localization and Activation

PAECs were cultured and treated as described. For subcellular localization of Ras, cells were lysed in a buffer contain-

ing 20 mM Tris-Cl, pH 7.5, 3 mM MgCl_2 , 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin, and 10 $\mu\text{g}/\text{ml}$ aprotinin. The lysates were sonicated and centrifuged at $100,000 \times g$ for 1 h at 4°C . The supernatant (cytosolic fraction) was removed, and the insoluble pellet (membrane fraction) was solubilized by sonication in the lysis buffer supplemented with 2% Triton X-100. Equivalent amounts of cytosolic and membrane proteins were resolved on SDS-PAGE and immunoblotted for Ras.

For determination of Ras activity, the cells were harvested by scraping, rinsed once with PBS, and lysed in 25 mM HEPES, pH 7.5, 1% NP-40, 10% glycerol, 150 mM NaCl, 10 mM MgCl_2 , 1 mM EDTA, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin, 25 mM NaF, and 1 mM sodium orthovanadate (Ren and Schwartz, 2000). Cleared lysates were incubated with the glutathione-S-transferase (GST)-fused Ras-binding protein, GST-Raf 1, bound to glutathione agarose beads. The beads were then collected by centrifugation and washed three times with lysis buffer. Beads were resuspended in Laemmli buffer and boiled for 10 min. The protein complexes were resolved by SDS-PAGE. Parallel gels were run with corresponding crude cell lysates. The proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) according to the manufacturer's recommendations. Gels were then immunoblotted for Ras. The amount of activated Ras was normalized to the total amount of Ras in cell lysates. Data were expressed as the fold change in GTP-bound Ras relative to buffer control.

Statistical Analysis

Data are presented as mean $\pm$ SEM. Analysis of variance followed by the least significant difference test was used to analyze differences among groups. Differences among means were considered significant at a value of $p < 0.05$.

RESULTS

ICMT Inhibition Promotes Endothelial Apoptosis

We have previously demonstrated that adenosine and homocysteine caused endothelial cell apoptosis as assessed by DNA ladder formation, [^3H]thymidine release, and terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) (Dawicki *et al.*, 1997; Rounds *et al.*, 1998). In the present study, we assessed apoptosis by change in nuclear morphology using Hoechst 33342 (as described in Bellas *et al.*, 2002). Similar to previous studies (Dawicki *et al.*, 1997; Rounds *et al.*, 1998), the number of endothelial cells with apoptotic nuclear morphology was significantly increased in endothelial cells incubated overnight with buffer in the presence of 100 μM Ado/HC ($53.1 \pm 3.1\%$) compared with cells incubated with buffer alone ($7.6 \pm 3.1\%$) ($n = 2$; $p < 0.01$).

Next, we investigated the effects of ICMT inhibition on PAEC apoptosis. AFC and AGGC are prenylcysteine analogues that compete with endogenous proteins for methylation by ICMT (Volker *et al.*, 1991) and thus are competitive inhibitors of ICMT. Inhibition of ICMT by AFC or AGGC promoted PAEC apoptosis (Figure 2a) compared with PAECs incubated with the inactive analogue, AGC, or with buffer alone. In addition, competitive inhibition of ICMT with either FTA (Perez-Sala *et al.*, 1998) or FTS (Marom *et al.*, 1995) promoted endothelial cell apoptosis compared with

Figure 2. Endothelial cell apoptosis is induced by ICMT inhibition. PAECs were incubated with buffer in the absence (control) or presence of 20 μ M AGC, 20 μ M AGGC, or 20 μ M AFC (a) or in the absence (control) or presence of 50 μ M FTA or 10 μ M FTS (b) for 4 h. Cells were visualized by fluorescence microscopy and scored for change in nuclear morphology consistent with apoptosis. c, Asp-Glu-Val-Asp-ase (DEVDase) activity was determined in equivalent quantities of PAEC lysate treated with buffer (control), 100 μ M Ado/HC, 20 μ M AGC, or 20 μ M AGGC for varying times. All data are expressed as mean $\pm$ SEM. $n = 4$. * $p < 0.05$ compared with control.

buffer control (Figure 2b). It should be noted that none of these chemicals are specific inhibitors of ICMT but may also bind to other proteins that interact with isoprenyl groups (Stephenson and Clarke, 1990; Young *et al.*, 2001).

As another means of assessing apoptosis, we measured caspase activity in lysates from PAECs treated with Ado/HC or AGGC. The level of caspase activity was significantly higher in PAECs incubated with AGGC for 4 and 8 h than in PAECs incubated with the inactive analogue AGC (Figure 2c). Caspase activity also trended higher at 4 h in PAECs incubated with 100 μ M Ado/HC than in buffer control, reaching significance by 8 h. Hence, inhibition of ICMT causes apoptosis of endothelial cells, as assessed by nuclear morphological change and increased caspase activity.

Effect of ICMT Inhibitors on Protein Carboxyl Methylation

To demonstrate that AGGC was indeed inhibiting protein carboxyl methylation in endothelial cells, we assessed the effect of AGGC on carboxyl methylation of proteins of molecular weight 21 kDa, the size of small GTPases. The carboxyl methylation level of 21-kDa proteins was reduced by ~ 2.8 -fold in endothelial cells treated with 20 μ M AGGC (7.6 ± 1.6 cpm/ μ g protein, $n = 5$) compared with 20 μ M AGC (20.9 ± 2.3 cpm/ μ g protein, $n = 6$) or buffer alone (20.6 ± 2.8 cpm/ μ g protein, $n = 6$) ($p < 0.05$).

Overexpression of ICMT Blunts Endothelial Apoptosis

Because chemical inhibitors may have nonspecific effects, we also examined the ability of ICMT overexpression to protect against endothelial cell apoptosis. PAECs were transiently transfected with the eukaryotic expression vector encoding ICMT cDNA, pICMT-GFP, or with the empty expression vector, pGFP-C1, as control and assessed for their tolerance to exposure to various apoptotic agonists. The overexpression of GFP and ICMT-GFP was confirmed by fluorescence microscopy (Figure 3, a and b) and by immunoblot analysis (Figure 3c). The cellular localization of GFP was more diffuse than was the perinuclear localization of ICMT-GFP (Figure 3b), consistent with reports localizing ICMT protein expression to the endoplasmic reticulum (ER) (Dai *et al.*, 1998). Finally, functional overexpression of ICMT was confirmed by assaying the level of enzyme activity. The ICMT activity was threefold higher in PAECs transiently transfected with pICMT-GFP than in untransfected cells or cells transfected with pGFP-C1 vector alone (Figure 3d).

Next, we examined the ability of ICMT overexpression to protect against Ado/HC-induced apoptosis. There was a significant rise in the percentage of GFP-expressing endothelial cells with apoptotic nuclei after 20 h of incubation with 100 μ M Ado/HC in cells transfected with pGFP-C1 vector control (Figure 4a), an effect similar to that seen in untransfected cells. However, overexpression of ICMT blocked apoptotic effects of Ado/HC. These findings support the notion that Ado/HC induces endothelial cell apoptosis by inhibiting ICMT.

To determine whether the protective effects of ICMT overexpression were specific for Ado/HC, we determined the effect of ICMT overexpression on apoptosis caused by exposure to UV light or TNF- α . PAECs transfected with pGFP-C1 vector control and then exposed to UV light ($\lambda_{254\text{nm}}$) for 1, 2, or 3 min displayed an enhanced number of apoptotic nuclei (Figure 4b). Yet, overexpression of ICMT protected the endothelial cells against apoptosis caused by UV light. Incubation with TNF- α promoted apoptosis in endothelial cells transfected with pGFP-C1. Again, ICMT overexpression prevented apoptosis caused by TNF- α (Figure 4c). Thus, the protective effects of overexpression of ICMT in endothelial cells were not unique to Ado/HC-induced apoptosis.

Effect of Ado/HC on TNF- α -Induced Apoptosis

We then asked whether Ado/HC would enhance TNF- α -induced apoptosis. We speculated that if TNF- α caused apoptosis, in part because of inhibition of ICMT, further inhibition of ICMT with Ado/HC should augment TNF- α -induced apoptosis. PAECs were incubated with serum-free MEM control in the absence or presence of Ado/HC or TNF- α . TNF- α ($14.78 \pm 3.23\%$) significantly increased apoptosis compared with untreated cells ($4.7 \pm 1.08\%$) or cells incubated with lower doses (50 μ M) of Ado/HC ($3.95 \pm 0.46\%$) alone ($n = 4$; $p = 0.0052$). Coincubation with Ado/HC further enhanced the level of TNF- α -induced apoptosis ($19.89 \pm 2.39\%$) ($n = 4$; $p = 0.0003$ compared with untreated or Ado/HC-treated cells).

Figure 3. Overexpression of ICMT-GFP in endothelial cells. PAECs transiently overexpressing pGFP-C1 (a) or pICMT-GFP (b) cDNAs were visualized by fluorescence microscopy. In parallel, protein overexpression was confirmed by immunoblot analysis for GFP (c), and overexpression of a functional enzyme was confirmed by assaying for methyltransferase (ICMT) activity (d). Data in d are expressed as mean $\pm$ SEM. $n = 3$. * $p < 0.05$ compared with untransfected cultures or cultures transfected with pGFP-C1.

Ras GTPase Carboxyl Methylation, Activation, and Signaling Are Dependent on ICMT Activity

We speculated that ICMT exerted a protective effect against apoptosis by increasing carboxyl methylation of small GTPases, a modification that may enhance membrane localization and/or activity of Ras GTPases (Parish and Rando, 1996). Thus, we hypothesized that inhibition of ICMT would decrease the amount of activated GTP-bound Ras GTPase and diminish the levels of membrane-associated Ras. To test this idea, we assayed PAECs incubated with buffer control, Ado/HC, AGGC, or the inactive analogue, AGC, for changes in activated Ras GTPase by using an affinity precipitation assay for GTP-bound Ras and for alterations in subcellular localization of Ras. The level of activated Ras after incubation of PAECs with either Ado/HC or AGGC was significantly reduced compared with buffer control (Figure 5a). In addition, PAECs exposed to AFC, AGGC, and Ado/HC had decreased amounts of membrane-associated Ras and concomitant increased amounts of cytosol-associated Ras (Figure 5b). Consistent with this finding, experiments demonstrated that levels of Ras carboxyl methylation were significantly diminished in Ado/HC- or AGGC-exposed endothelial cells compared with buffer control (Figure 5c).

Parallel experiments were performed to assess the effects of UV light and TNF- α exposure on Ras activity and post-translational modifications in PAECs. The levels of activated Ras (Figure 6a) and carboxyl methylated Ras (Figure 6b) were also significantly blunted in PAECs exposed to UV light compared with nonexposed, buffer-treated cells. In contrast, TNF- α had no significant effect on either the level of activated Ras or carboxyl methylated Ras (K.L. Sheahan

Figure 4. ICMT overexpression protects against endothelial cell apoptosis. PAECs were transfected with pGFP-C1 or pICMT-GFP and incubated with: a, buffer (control) or 100 μ M Ado/HC; b, exposed to UV light for indicated time; or c, serum-free MEM (control) or 20 ng/ml TNF- α and incubated for 20 h at 37°C. The cells were then fixed and stained with Hoechst. Cells were visualized by fluorescence microscopy and scored for change in nuclear morphology consistent with apoptosis. Data are expressed as mean $\pm$ SEM. $n = 3$. * $p < 0.05$ compared with control or 0 min UV light treatment (in a, c, or b, respectively). ** $p < 0.05$ compared with pGFP-C1 transfected endothelial cells with respective treatment.

Figure 5. ICMT inhibition attenuates Ras GTPase activity. PAECs were incubated in buffer in the absence (control) or presence of 20 μ M AGC or 20 μ M AGGC for 4 h or 100 μ M Ado/HC overnight at 37°C. **a**, Cell lysates were harvested and active Ras GTPase was purified with GST-fused Raf-1 protein bound to glutathione agarose beads. Parallel gels were run with corresponding crude lysates. All gels were immunoblotted for Ras GTPase. Immunoblot signals were quantified by densitometry and are presented as the mean $\pm$ SEM of the ratio of GST-Raf-1-bound Ras GTPase to total Ras GTPase present in crude lysate. **b**, Membrane and cytosolic fractions were harvested, resolved by SDS-PAGE, and immunoblotted for Ras. Immunoblot signals were quantified and are presented as the mean $\pm$ SEM of the ratio of membrane/cytosol Ras normalized to buffer control. **c**, To determine the level of carboxyl methylation, Ras GTPase was immunoprecipitated, resolved on SDS-PAGE, and immunoblotted for respective GTPase. Illuminated bands were excised and hydrolyzed, and the level of [³H]methyl incorporation was determined. $n = 3$. * $p < 0.05$.

Figure 6. Ras GTPase activity and carboxyl methylation are blunted by UV light. PAECs were exposed to UV light for indicated times and incubated in serum-free MEM overnight at 37°C. The levels of active Ras GTPase (**a**) and Ras GTPase carboxyl methylation (**b**) were measured and quantified as stated in Figure 5 legend. $n = 3-4$. * $p < 0.05$.

and J. Newton, data not shown). These results suggest that UV light-induced endothelial cell apoptosis is associated with ICMT inhibition and disruption of its downstream signaling pathway through Ras GTPase. Conversely, endothelial cell apoptosis caused by exposure to TNF- α may not be caused by perturbation of ICMT-mediated signaling through Ras GTPase.

Next, we examined downstream mediators known to be important in the Ras GTPase signaling pathway in protecting against cellular apoptosis (Khwaja *et al.*, 1997; Gire *et al.*, 2000). ICMT inhibition with AGGC significantly diminished the level of activated Akt in endothelial cells by 2 h of treatment compared with time 0 h (Figure 7a). The level of phosphorylated Akt was significantly reduced in AGGC-treated endothelial cells for 4 h compared with buffer control- and AGC-treated endothelial cells. Similarly, inhibition of ICMT with AGGC significantly reduced the levels of active ERK-1 and ERK-2 in PAECs compared with buffer control- and AGC-treated endothelial cells (Figure 7b). Finally, we determined the effects of overexpression of dominant active, wild-type, and dominant negative H-Ras on endothelial cell apoptosis caused by Ado/HC. Overexpres-

Figure 7. Akt and ERK-1/ERK-2 activities are blunted by ICMT inhibition. PAECs were incubated in buffer in the absence (control) or presence of 20 μM AGC or 20 μM AGGC at 37°C for indicated times. Equivalent volumes of protein were resolved on SDS-PAGE and immunoblotted for phosphorylated Akt (a) or ERK-1/2 (b). Respective immunoblots were subsequently stripped and reprobed for total Akt or ERK-1/2 protein. Immunoblot signals were quantified by densitometry and are presented as the mean $\pm$ SEM of the ratio of phosphorylated protein to total protein. Representative immunoblots are shown. $n = 5$ for control and AGC; $n = 3$ for AGGC. * $p < 0.05$ compared with time 0 of respective treatment (a) or compared with control or AGC (b).

sion of wild-type and dominant active H-Ras(L61) attenuated Ado/HC-induced apoptosis compared with cultures transiently transfected with vector alone (Figure 8). Furthermore, overexpression of dominant negative H-Ras(dn) promoted PAEC apoptosis.

Together, our data suggest that ICMT overexpression may protect against endothelial cell apoptosis by enhancing the carboxyl methylation posttranslational modification, activity, and subsequent intracellular signaling pathway of Ras GTPase.

DISCUSSION

These results show that inhibition of ICMT with Ado/HC or with the competitive inhibitors AGGC or AFC caused endothelial cell apoptosis, as characterized by nuclear morphological change and increased caspase activity. In addition, overexpression of ICMT prevented apoptosis caused by Ado/HC. These results support our idea that Ado/HC

Figure 8. H-Ras GTPase overexpression protects against Ado/HC-induced endothelial cell apoptosis. PAECs transiently cotransfected with pGFP-C1 and vector (pUSEamp), wild-type [pUSE H-Ras(wt)], dominant active [pUSE H-Ras(L61)], or dominant negative [pUSE H-Ras(dn)] H-Ras GTPase cDNAs were incubated in buffer in the absence or presence of 100 μM Ado/HC. The cells were then fixed and stained with Hoechst. Endothelial cells were visualized with a fluorescence microscope, and GFP expressing cells were scored for change in nuclear morphology consistent with apoptosis. Data are expressed as mean $\pm$ SEM. $n = 3$. * $p < 0.05$ compared with cotransfected endothelial cells with respective buffer treatment. ** $p < 0.05$ compared with vector and dominant negative H-Ras GTPase-overexpressing endothelial cells.

causes endothelial cell apoptosis by inhibition of protein carboxyl methyltransferase activity. Furthermore, ICMT-mediated protection against endothelial cell apoptosis induced by Ado/HC, TNF- α , or UV light suggests that protein carboxyl methylation may be critical for endothelial cell integrity. In addition, we found that inhibition of ICMT decreased Ras GTPase methylation and activity, resulting in diminished activation of the downstream signaling molecules Akt, ERK-1, and ERK-2. Finally, overexpression of wild-type or dominant active H-Ras constructs protected against Ado/HC-induced apoptosis. These results further suggest that ICMT may modulate endothelial cell apoptosis by regulating protein carboxyl methylation and activation of Ras GTPase.

Carboxyl methylation of proteins is a posttranslational modification that may be important in signal transduction. In the present study, we showed that enhanced expression of ICMT prevented programmed cell death of endothelial cells. ICMT is a 32-kDa protein that localizes to the ER (Dai *et al.*, 1998; Romano *et al.*, 1998). The C-terminal CAAX motif on proteins confers subcellular targeting to the ER (Choy *et al.*, 1999), where the CAAX motif proteins are methylated by ICMT, generating a hydrophobic C-terminal domain (Parish and Rando, 1996). ICMT catalyzes carboxyl methylation of several protein substrates, some of which are involved in cell signaling (e.g., Ras and Rho GTPases, phosphorylase kinase, or oncogenic protein tyrosine phosphatases) and others are not (e.g., nuclear lamins A and B) (Cheng and Blumenthal, 2000). ICMT has been shown to be critical to normal embryonic development (Bergo *et al.*, 2001). ICMT-deficient mice died by midgestation, and cell lysates from these embryos lacked the ability to methylate K-Ras or AGGC (Bergo *et al.*,

2001). The data indicate ICMT to be important in normal development and suggest that there are no apparent redundant pathways for CAAX protein carboxyl methylation.

Ras GTPases are signaling proteins important in many cellular functions, including the organization of cytoskeletal proteins necessary for cell motility, adhesion, and proliferation (Mackay and Hall, 1998). It is possible that ICMT modulates apoptosis through effects on carboxyl methylation of small GTPases. Indeed, the absence of ICMT caused mislocalization of K-Ras from the plasma membrane to cytoplasm in cells derived from ICMT knock-out embryos (Bergo *et al.*, 2000), suggesting that carboxyl methylation of C-terminal isoprenyl cysteine is important in subcellular localization and possibly in normal enzymatic function of K-Ras GTPase. Wang *et al.* (1997) demonstrated that coinubation of endothelial cells with homocysteine and the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)-adenosine, maneuvers that inhibit carboxyl methyltransferase activity, decreased plasma membrane localization and the level of carboxyl methylation of v-H-Ras. These data again suggest the importance for the role for posttranslational carboxyl methylation of Ras GTPase in protein subcellular localization. Like Wang *et al.*, we found that Ado/HC and AGGC decreased membrane localization and activation of Ras. We also showed that ICMT inhibition blunted Akt, ERK-1, and ERK-2 activation. Wang *et al.* showed decreased endothelial cell proliferation on treatment with homocysteine and an adenosine deaminase inhibitor. These results are consistent with our findings of increased endothelial cell apoptosis.

TNF- α has been shown to be cytotoxic to many cell types, including endothelial cells (Polunovsky *et al.*, 1994; Petrache *et al.*, 2001). The mechanism of TNF- α -induced cell death includes caspase-dependent and -independent pathways (Jones *et al.*, 2000), mitochondrial membrane permeability changes (Higuchi *et al.*, 1998; Lemasters *et al.*, 1998), and activation of the G protein-linked phospholipase A₂ (Mutch *et al.*, 1992; Hayakawa *et al.*, 1993). Ratter *et al.* (1999) showed that Ado/HC exacerbated TNF- α -mediated death in L929 cells. These results are consistent with our findings that Ado/HC enhanced TNF- α -induced endothelial cell apoptosis. Interestingly, ICMT overexpression protected against TNF- α -induced endothelial cell apoptosis. However, we were unable to demonstrate changes in Ras GTPase carboxyl methylation or activity on TNF- α exposure. It is possible that overexpression of ICMT provided an alternative mode of protection to the endothelial cells from the proapoptotic effects of TNF- α by maximally carboxyl methylating other small GTPases. Indeed, Petrache *et al.* (2001) suggest involvement of the RhoA GTPase/Rho kinase signaling pathway and actomyosin contraction in mediating TNF- α proapoptotic signals. Thus, at present, our data suggest that Ras GTPase is not directly involved in TNF- α -mediated endothelial cell apoptosis. Nevertheless, we cannot exclude the possibility that other GTPases are affected by ICMT inhibition by TNF- α .

UV light is of the class of apoptotic stimuli causing programmed cell death through direct effects on cells. Thus, we were surprised to find that overexpression of ICMT protected against UV light-induced apoptosis of endothelial cells. UV light diminished Ras GTPase carboxyl methylation and activity, suggesting that UV light promoted apoptosis

by inhibiting ICMT-mediated posttranslational modification, and hence activation, of Ras GTPase.

Inhibition of ICMT by AGGC or Ado/HC caused apoptosis of endothelial cells and decreased carboxyl methylation and levels of activated GTP-bound Ras GTPase. Because inhibition of ICMT decreased activity of Ras GTPase and increased apoptosis of endothelial cells, it is possible that apoptosis was a result of altered Ras GTPase function. In support of this idea, overexpression of wild-type and dominant active H-Ras cDNAs protected against Ado/HC-induced apoptosis. Further work is needed to determine the details of this pathway. Although Ado/HC and AGGC may exert effects on other methyltransferases, these data support the idea that ICMT modulates endothelial cell apoptosis.

Elevated homocysteine levels *in vivo* have been associated with atherosclerotic vascular injury in humans and endothelial cell loss in baboons (Harker *et al.*, 1976, 1983; Welch and Loscalzo, 1998). Thus, we have speculated that endothelial cell apoptosis may contribute to homocysteine-induced vascular injury *in vivo*. We have previously demonstrated that increased concentrations of Ado/HC altered the ratio of intracellular concentrations of SAM/SAH, suggesting inhibition of methyltransferases (Dawicki *et al.*, 1997). Also, we have shown that Ado/HC exposure promotes the disruption of focal adhesion complexes and caspase-induced proteolysis of selected focal adhesion complex components in endothelial cells (Harrington *et al.*, 2001). We have further shown the apoptotic effects induced in endothelial cells by Ado/HC exposure to be blunted by overexpression of focal adhesion kinase and not paxillin or p130^{CAS} (Bellas *et al.*, 2002). We now demonstrate that Ado/HC most likely acts via ICMT inhibition. Our present results indicate that inhibition of ICMT decreases carboxyl methylation of Ras GTPase, resulting in impaired activation of the GTPase. This, in turn, leads to diminished Akt phosphorylation and caspase activation. Because ICMT may mediate the carboxyl methylation of both Ras and Rho GTPases, we speculate that ICMT inhibition causes apoptosis by altering both the Ras GTPase antiapoptotic pathway via PI-3 kinase, Akt, and ERK-1/ERK-2 inhibition and the Rho GTPase anoikis pathway via focal adhesion contact disruption and degradation. Inhibition of both Ras GTPase and Rho GTPase may result in enhanced caspase activation and ultimately in DNA fragmentation and programmed cell death.

In summary, these experiments show that ICMT may be important in modulating apoptosis caused by a variety of harmful agents. We speculate that this effect may be a result of alterations in Ras and Rho GTPase function. Our results suggest that posttranslational protein carboxyl methylation may be an important mechanism of cell signaling. Further elucidation of the protective effects of ICMT may be important in establishing preventive or therapeutic strategies against vascular injury seen with critical illness.

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REFERENCES

- Bellas, R.E., Harrington, E.O., Sheahan, K.L., Newton, J., Marcus, C., and Rounds, S. (2002). FAK blunts adenosine-homocysteine-induced endothelial cell apoptosis: requirement for PI 3-kinase. *Am. J. Physiol.* 282, L1135–L1142.
- Bergo, M.O., Leung, G.K., Ambroziak, P., Otto, J.C., Casey, P.J., Gomes, A.Q., Seabra, M.C., and Young, S.G. (2001). Isoprenylcysteine carboxyl methyltransferase deficiency in mice. *J. Biol. Chem.* 276, 5841–5845.
- Bergo, M.O., Leung, G.K., Ambroziak, P., Otto, J.C., Casey, P.J., and Young, S.G. (2000). Targeted inactivation of the isoprenylcysteine carboxyl methyltransferase gene causes mislocalization of K-Ras in mammalian cells. *J. Biol. Chem.* 275, 17605–17610.
- Boivin, D., Bilodeau, D., and Beliveau, R. (1996). Regulation of cytoskeletal functions by Rho small GTP-binding proteins in normal and cancer cells. *Can. J. Pharmacol.* 74, 801–810.
- Chatterjee, M., and Wu, S. (2001). Cell line dependent involvement of ceramide in ultraviolet light-induced apoptosis. *Mol. Cell. Biochem.* 219, 21–27.
- Cheng, X., and Blumenthal, R.M. (2000). *S-Adenosylmethionine-Dependent Methyltransferases: Structures and Functions*, Singapore: World Scientific Publishing.
- Choy, E., Chiu, V.K., Silletti, J., Feoktistov, M., Morimoto, T., Michaelson, D., Ivanov, I.E., and Philips, M.R. (1999). Endomembrane trafficking of Ras: the CAAX motif targets proteins to the ER and Golgi. *Cell* 98, 69–80.
- Clarke, S., Vogel, J.P., Deschenes, R.J., and Stock, J. (1988). Post-translational modification of the Ha-Ras oncogene protein: evidence for a third class of protein carboxyl methyltransferases. *Proc. Natl. Acad. Sci. USA* 85, 4643–4647.
- Dai, Q., Choy, E., Chiu, V., Romano, J., Slivka, D.R., Steitz, S.A., Michaelis, S., and Philips, M.R. (1998). Mammalian prenylcysteine carboxyl methyltransferase is in the ER. *J. Biol. Chem.* 273, 15030–15034.
- Dawicki, D., Chatterjee, D., Wyche, J., and Rounds, S. (1997). Extracellular ATP and adenosine cause apoptosis of pulmonary artery endothelial cells. *Am. J. Physiol.* 279, L733–L742.
- Desrosiers, R.R., Nguyen, Q., and Beliveau, R. (1999). The carboxyl methyltransferase modifying G proteins is a metalloenzyme. *Biochem. Biophys. Res. Commun.* 261, 790–797.
- Frisch, S.M., and Francis, H. (1994). Disruption of epithelial cell-matrix interactions induces apoptosis. *J. Cell Biol.* 124, 619–626.
- Gire, V., Marshall, C., and Wynford-Thomas, D. (2000). PI-3-kinase is an essential anti-apoptotic effector in the proliferative response of primary human epithelial cells to mutant RAS. *Oncogene* 19, 2269–2276.
- Han, Z., and Wyche, J.H. (1994). Guanosine induces necrosis of cultured aortic endothelial cells. *Am. J. Pathol.* 145, 427–433.
- Harker, L.A., Harlan, J.M., and R. Ross. (1983). Effect of sulfinpyrazone on homocysteine-induced endothelial injury and arteriosclerosis in baboons. *Circ. Res.* 53, 731–739.
- Harker, L.A., Slichter, S.J., and C.R. Scott. (1976). Homocysteine-induced arteriosclerosis. The role of endothelial cell injury and platelet response in its genesis. *Journal of Clinical Investigation* 58, 731–741.
- Harrington, E.O., Smeglin, A., Newton, J., Ballard, G., and Rounds, S. (2001). Protein tyrosine phosphatase-dependent proteolysis of focal adhesion complexes in endothelial cell apoptosis. *Am. J. Physiol.* 280, L342–L353.
- Hayakawa, M., Ishida, N., Takeuchi, K., Shibamoto, S., Hori, T., Oku, N., Ito, F., and Tsujimoto, M. (1993). Arachidonic acid-selective cytosolic phospholipase A2 is crucial in the cytotoxic action of tumor necrosis factor. *J. Biol. Chem.* 268, 11290–11295.
- Higuchi, M., Proske, R.J., and Yeh, E.T. (1998). Inhibition of mitochondrial respiratory chain complex I by TNF results in cytochrome c release, membrane permeability transition, and apoptosis. *Oncogene* 17, 2515–2524.
- Hoyt, D.G., Mannix, R.J., Rusnak, J.M., Pitt, B.R., and Lazo, J.S. (1995). Collagen is a survival factor against LPS-induced apoptosis in cultured sheep pulmonary artery endothelial cells. *Am. J. Physiol.* 269, L171–L177.
- Jones, B.E., Lo, C.R., Liu, H., Srinivasan, A., Streetz, K., Valentino, K.L., and Czaja, M.J. (2000). Hepatocytes sensitized to tumor necrosis factor- α cytotoxicity undergo apoptosis through caspase-dependent and caspase-independent pathways. *J. Biol. Chem.* 275, 705–712.
- Khwaja, A., Rodriguez-Viciana, P., Wennstrom, S., Warne, P.H., and Downward, J. (1997). Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *EMBO J.* 16, 2783–2793.
- Lemasters, J.J., Qian T., Elmore S.P., Trost L.C., Nishimura Y., Herman B., Bradham C.A., Brenner D.A., and Nieminen A.L. (1998). Confocal microscopy of the mitochondrial permeability transition in necrotic cell killing, apoptosis and autophagy. *Biofactors* 8, 283–285.
- Mackay, D.J.G., and Hall, A. (1998). Rho GTPases. *J. Biol. Chem.* 273, 20685–20688.
- Marom, M., Haklai, R., Ben-Baruch, G., Marciano, D., Egozi, Y., and Kloog, Y. (1995). Selective inhibition of Ras-dependent cell growth by farnesylthiosalicylic acid. *J. Biol. Chem.* 270, 22263–22270.
- Mebmer, U.K., Briner, V.A., and Pfeilschifter, J. (1999). Tumor necrosis factor α and lipopolysaccharide induce apoptotic cell death in bovine glomerular endothelial cells. *Kidney Int.* 55, 2322–2337.
- Mutch, D.G., Powell, C.B., Kao, M.S., and Collins, J.L. (1992). Resistance to cytolysis by tumor necrosis factor alpha in malignant gynecological cell lines is associated with the expression of protein(s) that prevent the activation of phospholipase A2 by tumor necrosis factor alpha. *Cancer Res.* 52, 866–872.
- Parish, C.A., and Rando, R.R. (1996). Isoprenylation/methylation of proteins enhances membrane association by a hydrophobic mechanism. *Biochemistry* 35, 8473–8477.
- Perez-Sala, D., Gilbert, B.A., Rando, R.R., and Canada, F.J. (1998). Analogs of farnesylcysteine induce apoptosis in HL-60 cells. *FEBS Lett.* 426, 319–324.
- Perna, A.F., Ingrosso, D., De Santo, N.G., Galletti, P., Brunone, M., and Zappia, V. (1997). Metabolic consequences of folate-induced reduction of hyperhomocysteinemia in uremia. *J. Am. Soc. Nephrol.* 8, 1899–1905.
- Petrache, I., Verin, A.D., Crow, M.T., Birukova, A., Liu, F., and Garcia, J.G. (2001). Differential effect of MLC kinase in TNF- α -induced endothelial cell apoptosis and barrier dysfunction. *Am. J. Physiol.* 280, L1168–L1178.
- Philips, M.R., and Pillinger, M.H. (1995). Prenylcysteine-directed carboxyl methyltransferase activity in human neutrophil membranes. *Methods Enzymol.* 256, 49–63.
- Philips, M.R., Pillinger, M.H., Staud, R., Volker, C., Rosenfeld, M.G., Weissmann, G., and Stock, J.F. (1993). Carboxyl methylation of

- Ras-related proteins during signal transduction in neutrophils. *Science* 259, 977–980.
- Pillinger, M.H., Volker, C., Stock, J.B., Weissmann, G., and Philips, M.R. (1994). Characterization of a plasma membrane-associated prenylcysteine-directed α carboxyl methyltransferase in human neutrophils. *J. Biol. Chem.* 269, 186–192.
- Polunovsky, V.A., Chen, B., Henke, C., Snover, D., Wed, T.C., Ingbar, D.H., and Bitterman, P.B. (1993). Role of mesenchymal cell death in lung remodeling after injury. *J. Clin. Invest.* 92, 388–397.
- Polunovsky, V.A., Wendt, C.H., Ingbar, D.H., Peterson, M.S., and Bitterman, P.B. (1994). Induction of endothelial cell apoptosis by TNF- α : modulation by inhibitors of protein synthesis. *Exp. Cell Res.* 214, 584–594.
- Ratter, F., Gabner, C., Shatrov, V., and Lehmann, V. (1999). Modulation of tumor necrosis factor α -mediated cytotoxicity by changes of the cellular methylation state: mechanism and in vivo relevance. *Int. Immunol.* 11, 519–527.
- Ren, X.D., and Schwartz, M.A. (2000). Determination of GTP loading on Rho. *Methods Enzymol.* 325, 264–272.
- Romano, J.D., Schmidt, W.K., and Michaelis, S. (1998). The *Saccharomyces cerevisiae* prenylcysteine carboxyl methyltransferase Ste14p is in the endoplasmic reticulum membrane. *Mol. Biol. Cell* 9, 2231–2247.
- Rounds, S., Yee, W.L., Dawicki, D.D., Harrington, E.O., Parks, N., and Cutaia, M.V. (1998). Mechanism of extracellular ATP and adenosine induced apoptosis of cultured pulmonary artery endothelial cells. *Am. J. Physiol.* 275, L379–L388.
- Stephenson, R.C., and Clarke, S. (1990). Identification of a C-terminal carboxyl methyltransferase in rat liver membranes utilizing a synthetic farnesyl cysteine-containing peptide substrate. *J. Biol. Chem.* 265, 16248–16254.
- Volker, C., Miller, R.A., McCleary, W.R., Rao, A., Poenie, M., Backer, J.M., and Stock, J.B. (1991). Effects of farnesylcysteine analogs on protein carboxyl methylation and signal transduction. *J. Biol. Chem.* 268, 21515–21522.
- Wang, H., Yoshizumi, M., Lai, K., Tsai, J.C., Perrella, M.A., Haber, E., and Lee, M.E. (1997). Inhibition of growth and p21Ras methylation in vascular endothelial cells by homocysteine but not cysteine. *J. Biol. Chem.* 272, 25380–25385.
- Welch, G.N., and Loscalzo, J. (1998). Homocysteine and atherothrombosis. *N. Engl. J. Med.* 338, 1042–1050.
- Young, S.G., Ambroziak, P., Kim, E., and Clarke, S. (2001). Postisoprenylation protein processing: CXXX (CaaX) endoproteases and isoprenylcysteine carboxyl methyltransferase. In: *The Enzymes*, vol 2, ed. F. Tamanoi and D.S. Sigman, New York: Academic Press, 155–213.