Oxidative-Nitrosative Stress and Post-Translational Protein Modifications: Implications to Lung Structure–Function Relations

Inhibition of ICMT Induces Endothelial Cell Apoptosis through GRP94

Qing Lu, Elizabeth O. Harrington, Julie Newton, Matthew Jankowich, and Sharon Rounds

Vascular Research Laboratory, Providence Veterans Affairs Medical Center; and Department of Medicine, Brown Medical School, Providence, Rhode Island

Isoprenylcysteine-O-carboxyl methyltransferase (ICMT) catalyzes methylation of proteins containing a C-terminal CAAX motif. We have previously shown that chemical inhibition of ICMT caused endothelial cell apoptosis, an effect correlated with decreased Ras and RhoA carboxyl methylation and GTPase activities. In the current study, proteomic analysis of pulmonary artery endothelial cells (PAEC) exposed to the ICMT inhibitor, N-acetyl-geranylgeranyl-cysteine (AGGC), demonstrated a shift in the isoelectric points (pI) of the glucose-regulated protein (GRP) 94. Two-dimensional PAGE and immunoblot analysis further documented that ICMT inhibition caused multiple changes in the pI of GRP94. GRP94 is an endoplasmic reticulum molecular chaperone, a component of the unfolded protein response (UPR), and is involved in apoptosis. Immunofluorescence analyses revealed redistribution and aggregation of GRP94 after 3 h exposure to AGGC. A similar finding was noted with calnexin. In addition, GRP94 protein levels were significantly diminished upon 18 h AGGC exposure or ICMT suppression. The effects of ICMT inhibition on changes in GRP94 subcellular localization and protein content were blunted by overexpression of constitutively active RhoA or a caspase inhibitor. Furthermore, GRP94 depletion augmented endothelial cell apoptosis induced by ICMT inhibition. These results indicate that ICMT inhibition leads to GRP94 relocation, aggregation, and degradation; effects were dependent upon the activities of RhoA and caspases. We speculate that changes in the pI, subcellular localization, and protein level of GRP94 cause endothelial cell apoptosis, possibly through UPR dysfunction. These studies suggest a novel link between RhoA GTPases and the UPR.

Keywords: ICMT; GRP94; RhoA GTPase; unfolded protein response; apoptosis

Endothelial cells are exposed to multiple biochemical and biomechanical stresses, which can cause endothelial cell apoptosis. Apoptosis is initiated by a death-inducing signal and mediated by receptor-dependent extrinsic pathway and/or mitochondrial-

CLINICAL RELEVANCE

Endothelial cell apoptosis plays important roles in homeostasis and diseases. This study has potential importance in the treatment of lung diseases in which abnormal endothelial apoptosis occurs, such as emphysema, primary pulmonary hypertension, and cancer.

dependent intrinsic pathway (1). Apoptosis also occurs upon endoplasmic reticulum (ER) stress (2). Increased levels of adenosine triphosphate (ATP) or adenosine may occur in blood vessels upon platelet exocytosis or cytolytic release from necrotic cells (3). We have previously demonstrated that ATP and adenosine cause endothelial cell apoptosis (4, 5). We have also shown that homocysteine exacerbates adenosine-induced endothelial cell apoptosis (5). Furthermore, we have demonstrated that adenosine and homocysteine cause endothelial cell apoptosis by inhibiting isoprenylcysteine-O-carboxyl methyltransferase (ICMT) activity (6). ICMT is a membrane protein localized to the ER (7, 8) catalyzing post-translational carboxyl methylation of proteins encoding a C-terminal CAAX motif (C, cysteine; A, an aliphatic amino acid; X, any amino acid). ICMT-deficient mice die by mid-gestation (9), suggesting that protein carboxyl methylation is critical to embryonic development and no redundant protein exists for methylation of these proteins.

Small GTPases, such as Ras and RhoA, possess a preserved C-terminal CAAX motif, which is carboxyl methylated by ICMT (10). Post-translational modification of small GTPases is essential for localization to cell membrane and biological function (11–13). We have previously shown that ICMT inhibition blunts carboxyl methylation and subsequent activation of Ras and RhoA (6, 14). Furthermore, inhibition of ICMT induces endothelial cell apoptosis by a mechanism involving decreased Ras carboxyl methylation and activation (6). The roles of RhoA GTPase in the initiation and/or progression of apoptosis are unknown.

Protein synthesis, post-translational modification, and folding take place within the ER. Disruption of protein folding results in accumulation of unfolded or misfolded proteins in the ER, thereby eliciting ER stress (15, 16). In unstressed cells, glucose-regulated protein (GRP) 78, an ER molecular chaperone, is bound to and represses the unfolded protein response (UPR) sensors, inositol requiring kinase 1 (IRE1), double-stranded RNA-activated protein kinase-like ER kinase (PERK), and

(Received in original form August 14, 2006 and in final form February 12, 2007)

This work was supported by grants HL 64936 and VA Merit Review (S.R.), HL 67795 and VA Merit Review (E.O.H.), a Rhode Island Foundation Medical Research Grant, American Lung Association Research Grant RG-1140-N, and a Parker B. Francis Fellowship (Q.L.).

Correspondence and requests for reprints should be addressed to Sharon Rounds, M.D., Providence VA Medical Center, Pulmonary/Critical Care Medicine Section, 830 Chalkstone Avenue, Providence, RI 02908. E-mail: Sharon_Rounds@brown.edu

Am J Respir Cell Mol Biol Vol 37, pp 20–30, 2007

Originally Published in Press as DOI: 10.1165/rcmb.2006-03015M on March 8, 2007

Internet address: www.atsjournals.org

Figure 1. Effects of ICMT inhibition on GRP94 post-translational modifications in pulmonary artery endothelial cells. Human PAEC were incubated with vehicle or 20 μ M AGGC in serum-free medium for 18 h. Equivalent amounts of lysates were resolved by two-dimensional PAGE. (a) Proteins were stained with Coomassie blue and analyzed by Phoretix 2D image analysis software. Proteins that differed significantly between vehicle and AGGC treatment were selected, excised, digested, and analysed by mass spectrometry. (b) Two-dimensional PAGE-resolved lysates were immunoblotted for GRP94. Arrows indicate GRP94 with distinct pI.

activating transcription factor 6 (ATF6). During ER stress, unfolded or misfolded proteins accumulate in the ER lumen. This in turn causes titration of GRP78 away from IRE1, PERK, and ATF6, resulting in their activation. Sensor activation promotes ER-associated protein degradation, reduces protein translation of the malformed proteins, and increases transcription of ER molecular chaperones (16). Through UPR, cells correct the accumulation of unfolded or misfolded proteins, thus eliminating ER stress (16). However, prolonged or unresolved ER stress may lead to apoptosis (2). A growing number of diseases are now recognized to result from abnormal function of the UPR, including cystic fibrosis (17) and hepatic cirrhosis in ZZ form of α_1 -antitrypsin deficiency (18).

Glucose-regulated protein (GRP) 94, also known as gp96 and tumor rejection antigen (19), is another ER molecular chaperone important in the folding and export of proteins from the ER (19). GRP94 is phosphorylated on the C-terminal domain (20–23) and

binds ATP in the N-terminal domain (24). Overexpression of GRP94 suppresses ER stress-induced apoptosis of neuronal cells (25). Also, suppression of GRP94 expression accelerates ER stress-induced apoptosis (25, 26). These findings suggest that GRP94 protects against ER stress-induced apoptosis.

Proteomics analysis demonstrated a shift in the pI of GRP94 upon ICMT inhibition. In addition, GRP94 is involved in UPR and apoptosis. Thus, we hypothesized that ICMT inhibition caused endothelial cell apoptosis through dysfunction of UPR. We demonstrated that inhibition of ICMT caused subcellular relocation, aggregation, and decrease in protein content of GRP94 in endothelial cells. These effects were blunted by overexpression of constitutively active RhoA and a caspase inhibitor. Furthermore, knockdown of GRP94 exacerbated endothelial cell apoptosis induced by ICMT inhibition. These results suggest that subcellular redistribution, aggregation, and decrease in protein level of GRP94 contribute to endothelial cell apoptosis upon ICMT inhibition, possibly through induction of UPR dysfunction.

TABLE 1. PEPTIDE SEQUENCES DETECTED IN SPOTS #2 AND #3

Spot #2	Spot #3
-EESDDEAAVEEEEEK.-	-EESDDEAAVEEEEEK.-
-VFITDDFHDMMPK.-	-VFITDDFHDMMPK.-
-GLFDEYGSK.-	-GLFDEYGSK.-
-LSLNIDPDAK.-	-LSLNIDPDAK.-
-GVVDSDDLPLNVSR.-	

MATERIALS AND METHODS

Cell Lines and Reagents

Bovine pulmonary artery endothelial cells (BPAEC) were isolated and characterized, as previously described (4). Human pulmonary artery endothelial cells (HPAEC) and human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (Walkersville, MD). BPAEC

Figure 2. Effect of ICMT inhibition on subcellular localization of GRP94 and calnexin. Bovine PAEC were incubated with vehicle or 10 μ M AGGC in serum-free medium for 3 h. Cells were immunofluorescently stained for GRP94 (*a*, red) or double labeling of GRP94 and calnexin (*c*: GRP94, red; calnexin, green). The nuclei were counterstained with DAPI (*blue*). (*a* and *c*) Images are representative of multiple independent experiments. Arrows indicate perinuclear localization; block arrows indicate diffuse subcellular relocalization; arrowheads indicate aggregates. (*b*) The GRP94 fluorescence was quantified ($n = 12-16$, $*P < 0.05$ versus vehicle or AGC).

were used in most experiments; however, some experiments were performed in HPAEC or HUVEC due to availability of compatible reagents.

Antibodies directed against GRP94, calnexin, Ras, RhoA, and β -catenin were purchased from Assay Designs (Ann Arbor, MI), Upstate, Inc. (Charlottesville, VA), and Santa Cruz Biotechnologies (Santa Cruz, CA), respectively. pUSEampH-Ras(Q61L) and pUSEamp constructs

were obtained from Upstate, Inc. pEGFP-C1 construct was purchased from Clontech (Palo Alto, CA). pcDNA3-EGFP-RhoA(Q63L) construct was a generous gift from Dr. Klaus M. Hahn (Scripps Research Institute, La Jolla, CA).

ICMT siRNA was purchased from Qiagen (Valencia, CA) with the sense strand sequence of 5'-CCA UAG CUU AUA UUC UCA AdTdT-3' and the antisense strand sequence of 5'-UUG AGA AUA

Figure 3. Effect of ICMT inhibition on GRP94 protein level. Bovine PAEC were incubated with vehicle, 10 μ M AGC, or 10 μ M AGGC in serum-free medium for (a) 3 h or (b) 18 h. (c) HUVEC were transfected with 80 nM ICMT siRNA or equivalent amount of control siRNA for 72 h. Suppression of ICMT protein was confirmed by real-time RT-PCR and by measuring ICMT methyltransferase activity. Equivalent amounts of lysates were resolved by SDS-PAGE and immunoblotted for GRP94, using actin as a loading control. Representative immunoblots are shown. Densitometric values are presented as mean $\pm$ SE relative to vehicle or control siRNA ($n = 3-4$, * $P < 0.05$ versus vehicle, AGC, or control siRNA). Panel a: $P = NS$.

UAA GCU AUG GdTdA-3'. GRP94 siRNA was purchased from Santa Cruz Biotechnologies. Control (non-silencing) siRNA was purchased from Qiagen with the sense strand sequence of 5'-UUC UCC GAA CGU GUC ACG UdTdT-3' and the antisense strand sequence of 5'-UUC UCC GAA CGU GUC ACG UdTdT-3'.

N-acetyl-S-geranylgeranyl-L-cysteine (AGGC) and N-acetyl-S-geranyl-L-cysteine (AGC) were obtained from Biomol (Plymouth Meeting, PA). S-[3 H-methyl]-adenosyl-L-methionine and [3 H]-methyl-methionine were purchased from Perkin Elmer (Wellesley, MA). zVAD-fmk was obtained from Axxora (San Diego, CA). The fluorogenic peptide, N-acetyl-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC), was purchased from BD Biosciences Pharmingen (San Diego, CA). Ampholyte (pH 3.5–10) was purchased from Amersham Biosciences (Piscataway, NJ). Lipofectamine 2000 reagent and TRIZOL reagent were purchased from Invitrogen Life Technologies (Carlsbad, CA). iScript cDNA synthesis kit was obtained from BioRad (Philadelphia, PA). TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assay Mix were purchased from Applied Biosystems (Foster City, CA).

Two-Dimensional PAGE and Mass Spectrometry

HPAEC were treated as described and lysed in SDS homogenization buffer (1% SDS, 10% glycerol, 20 mM DTT), as previously described (14, 27). Lysates were first resolved by isoelectric point using preblended pH 3.5–10 carrier ampholytes and then resolved by molecular weight using 12% SDS-PAGE. After electrophoresis, the gels were stained with Coomassie blue and photographed. Matching analysis of images was performed by using Phoretix 2D image analysis software. The spots that differed significantly between vehicle and AGGC treatments were excised, trypsin digested, and processed for mass spectrometry, followed by database search and peptide mapping at the Harvard NIEHS Center for Environmental Health Proteomics Facility, according to standard procedures. Fully tryptic peptides were matched with SEQUEST at a delta correlation (Cn) of > 0.08 and correlation (Xcorr) > 1.9 , 2.2, and 3.5 for charged states of +1, +2, and +3, respectively, as described (28). The search was performed against the whole NCBI nonindexed human GRP94 database.

Gel Electrophoresis and Immunoblot Analysis

Proteins were resolved by two-dimensional PAGE or SDS-PAGE. The resolved proteins were then transferred to Immobilon PVDF membranes and immunoblotted with indicated antibodies, as previously described (14).

GRP94 Carboxyl Methylation Assay

Endothelial cells pre-incubated in methionine-free MEM for 1 h were metabolically labeled with 300 μ Ci [3 H]-CH₃-methionine for 4 h, as previously described (6). GRP94 was immunoprecipitated from lysates, and the immunoprecipitates were resolved by two 7.5% SDS-PAGE. Proteins in the first SDS-PAGE gel were transferred to Immobilon PVDF membranes, which were immunoblotted for GRP94 and used as a reference for excision of GRP94 band from the second gel. The excised GRP94 band was hydrolyzed with 1 N NaOH and processed to determine the amount of carboxyl methylated GRP94 protein using the vapor phase assay, as described (6).

Immunofluorescence Microscopy

Endothelial cells grown on coverslips were treated as described, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100, as described (14). Cells were stained with primary antibody followed by Texas red- or FITC-conjugated species-specific secondary antibody. Images were visualized using a Nikon Eclipse E400 fluorescence microscope (Nikon Corp., Kawasaki, Japan). at $\times 1,000$ magnification and recorded.

Transfections

Endothelial cells were transfected with cDNA, or 120 nM GRP94 siRNA, 80 nM ICMT siRNA, or equivalent amount of control vector or control siRNA using Lipofectamine 2000 reagent according to the manufacturer's protocol, as described (29). Protein overexpression or suppression was confirmed by real-time RT-PCR, immunoblot analysis, or fluorescence microscopy.

Real Time RT-PCR

Total RNA was purified from lysates using TRIZOL reagent and reversely transcribed into cDNA using iScript cDNA synthesis kit. ICMT

Figure 4. Effect of GRP94 knockdown on endothelial cell apoptosis induced by ICMT inhibition. Bovine PAEC were transfected with 120 nM GRP94 siRNA or equivalent amount of control siRNA for 48 h. Cells were then treated with vehicle or 10 μ M AGGC for 18 h. Knockdown of GRP94 protein expression was confirmed by immunoblot analysis, using vinculin as a loading control (a). Representative blot of multiple independent experiments is presented. Endothelial cell apoptosis was assessed by measuring caspase-3 activity (b). The data are presented as mean $\pm$ SE relative to control siRNA in the absence of AGGC ($n = 3$, $^{\#}P < 0.05$ versus control siRNA and treated with vehicle; $^*P < 0.05$ versus control siRNA and treated with AGGC). (c and d) Bovine PAEC were treated with vehicle, 10 μ M AGC, or 10 μ M AGGC in the absence or presence of 100 nM MG132 for 18 h, and the levels of GRP94 (c) and active (cleaved) caspase-3 (d) were detected by immunoblot analysis using antibodies directed against GRP94 and cleaved caspase-3, respectively ($n = 1$).

cDNA or 18S rRNA was then amplified using TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assay Mix for ICMT or 18S rRNA containing labeled TaqMan probe and forward and reverse ICMT or 18S rRNA specific primers. The levels of ICMT mRNA were detected and quantified by 7300 Real Time PCR System (Applied Biosystems).

ICMT Enzymatic Activity Assay

ICMT activity was assessed using alkaline hydrolysis of methyl esters in a vapor phase assay, as previously described (14). Briefly, AGGC was used as an artificial substrate and S-[3 H-methyl]-adenosyl-L-methionine (3 H-SAM) was used as the methyl donor. Negative controls either lacked substrate or contained the inactive analog, AGC, as an artificial substrate. Equivalent amounts of lysates were incubated with 720 nM 3 H-SAM and 20 μ M AGGC or AGC in reaction buffer at 37°C for 1 h. ICMT enzymatic activity is presented as pmol methylated AGGC $\text{mg}^{-1} \text{min}^{-1}$.

Caspase-3 Activity Assay

Cells were lysed in caspase lysis buffer (10 mM HEPES, pH 7.5, 40 mM β -glycerophosphate, 50 mM NaCl, 2 mM MgCl₂, and 5 mM EGTA) by freeze-thaw cycles, and caspase-3 activity was assessed and presented as nmol $\text{mg}^{-1} \text{min}^{-1}$, as previously described (30).

Data Analysis

All experiments were performed at least in triplicate. Data are presented as mean $\pm$ SE. ANOVA tests and unpaired t test analyzed differences among groups. Differences among means were considered significant when $P < 0.05$.

RESULTS

Identification of Proteomic Changes upon ICMT Inhibition

We have previously shown that ICMT inhibition causes endothelial cell apoptosis (6). To identify proteins potentially involved

in the apoptotic response to ICMT inhibition in endothelial cells, we assessed the effects of chemical inhibition of ICMT on expression and post-translational modifications of proteins from human PAEC. Equivalent amounts of lysates from PAEC treated with vehicle or AGGC for 18 h were resolved by two-dimensional PAGE and the gels were stained with Coomassie blue. Image analysis of the stained two-dimensional PAGE gels revealed 82 spots that differed significantly between vehicle- and AGGC-treated lysates in regards to intensity level. Selected spots were excised, digested, and subjected to mass spectrometry. Peptide sequences detected in spots # 2 and # 3 (Figure 1a, insets) are shown in Table 1. By database search and peptide mapping, we noted that the sequences detected in spots # 2 and # 3 match sequences of GRP94 protein, indicating both spots contain GRP94 with distinct isoelectric points (pI). In addition, image mapping analysis revealed that spot # 1 (Figure 1a, insets) matched spot # 2 (Figure 1a, insets). These results indicate that AGGC caused a shift in the pI of GRP94. Similar to a previous report (31), immunoblot analysis of two-dimensional PAGE demonstrated that GRP94 existed in multiple forms, each with distinct pI in endothelial cells (Figure 1b, upper panel). Although 3 h exposure to AGGC did not alter the pI of GRP94 (data not shown), 18 h exposure to AGGC caused appearance of a new form of GRP94 and disappearance of several other forms of GRP94 (Figure 1b, lower panel). These results suggest that ICMT inhibition may differentially affect post-translational modifications of GRP94.

Because of the known involvement of GRP94 in the UPR and apoptosis, we decided to further explore these changes. Although GRP94 peptide encodes an internal CAAX (CALV) motif at amino acid 645–648, it lacks C-terminal CAAX motif (32), suggesting that it is unlikely to be a substrate of ICMT carboxyl methylation. To determine if ICMT inhibition affected GRP94 carboxyl methylation, bovine PAEC exposed to vehicle,

Figure 5. Effects of caspase activation on ICMT inhibition-induced GRP94 subcellular localization and protein degradation. Bovine PAEC were preincubated with vehicle or 100 μ M zVAD-fmk for 1 h and then incubated with vehicle, 10 μ M AGC, or 10 μ M AGGC in the absence or presence of zVAD-fmk for (a) 3 h or (b) 18 h. (a) cells were immunofluorescently stained for GRP94 (red) and β -catenin (green). The nuclei were counterstained with DAPI (blue). Images are representative of three independent experiments. Arrows indicate GRP94 perinuclear localization; block arrows indicate diffuse subcellular relocation of GRP94; arrowheads indicate GRP94 aggregates. (b) Lysates were resolved by SDS-PAGE and immunoblotted for GRP94. The immunoblot signals were quantified by densitometry. The data are presented as mean $\pm$ SE relative to vehicle ($n = 3$, * $P < 0.05$ versus vehicle or AGC in the absence of zVAD-fmk; # $P < 0.05$ versus AGGC in the absence of zVAD-fmk).

AGC, or AGGC for 18 h were metabolically labeled with [3 H]CH $_3$ -methionine for 4 h, and GRP94 was immunoprecipitated from equivalent amount of lysates. The levels of GRP94 carboxyl methylation in endothelial cells treated with vehicle, AGC, and AGGC were low, as expected (0.0144, 0.0218, 0.027 CPM/ μ g total protein, respectively; $n = 1$). Thus, changes in carboxyl methylation of GRP94 do not likely contribute to post-translational modifications upon ICMT inhibition.

GRP94 Subcellular Relocalization and Aggregation upon ICMT Inhibition

GRP94 localizes to the ER and participates in the UPR (19). Immunofluorescence analysis demonstrated GRP94 within the perinuclear region of bovine PAEC treated with vehicle (Figure

2a) or AGC, an inactive analog of AGGC (data not shown), consistent with ER localization. GRP94 relocated in endothelial cells exposed to AGGC for 3 h, with diffuse cytoplasmic staining and aggregation (Figure 2a). Quantitation of GRP94 immunofluorescence demonstrated a significant increase in the area of fluorescence in endothelial cells treated with AGGC (Figure 2b), suggesting relocalization from the ER. We noted variability in the size of aggregates at a single time point in a single cell. We also observed that aggregate formation begins at 2 h exposure to AGGC and that the numbers of aggregates increase by 3 h exposure (data not shown). Thus, aggregates may grow in time and ultimately form aggresomes. Similar to GRP94, we also observed relocalization and aggregation of calnexin, another ER molecular chaperone, upon ICMT inhibition by AGGC (Figure 2c).

We next investigated the correlation between changes in GRP94 post-translational modifications and subcellular redistribution. Immunoblot analysis of two-dimensional PAGE from endothelial cells exposed to AGGC for 3 h demonstrated no significant changes in the pI of GRP94, as compared to vehicle or AGC exposed endothelial cells (data not shown), suggesting that redistribution of GRP94 is independent of changes in the pI.

Inhibition of ICMT Decreases GRP94 Protein Level

It has been shown previously that GRP94 suppression enhanced ER stress-induced apoptosis (25, 26). Thus, we hypothesized

Figure 6. Effects of constitutively active Ras and RhoA on ICMT inhibition-induced decrease in GRP94 protein level. (a) Bovine PAEC were preincubated with vehicle or 100 μ M zVAD-fmk for 1 h and then incubated with vehicle, 10 μ M AGC, or 10 μ M AGGC in the absence or presence of zVAD-fmk for 18 h. Lysates were resolved by SDS-PAGE and immunoblotted for RhoA. The immunoblot signals were quantified by densitometry. The data are presented as mean $\pm$ SE relative to vehicle in the absence of zVAD-fmk ($n = 4$, * $P < 0.05$ versus vehicle or AGC in the absence of zVAD-fmk). (b and c) Bovine PAEC were transfected with pUSEampH-Ras(Q61L) using pUSEamp as a vector control (b), or transfected with pcDNA3-EGFP-RhoA(Q63L) using pEGFP-C1 as a vector control (c) for 24 h. Cells were then treated with 10 μ M AGC or 10 μ M AGGC for 18 h. Overexpression of Ras and GFP-RhoA was confirmed by immunoblot analysis using antibodies directed against Ras (b, top blot) and RhoA (c, top blot), respectively. GRP94 protein level was assessed by immunoblot analysis. The data are presented as mean $\pm$ SE relative to the cells transfected with vector control and treated with AGC ($n = 3-5$, * $P < 0.05$ versus respective AGC treatment; # $P < 0.05$ versus transfected with GFP and treated with AGGC).

that ICMT inhibition may promote endothelial cell apoptosis through suppression of GRP94. To determine if the protein levels of GRP94 were suppressed upon ICMT inhibition, bovine PAEC were treated with vehicle, AGC, or AGGC for 3 or 18 h. Although 3 h exposure to AGGC promoted GRP94 subcellular relocation (Figures 2a and 2b), GRP94 protein level was not significantly altered (Figure 3a). However, PAEC exposed to AGGC for 18 h had significantly lower level of GRP94 protein, as compared with vehicle or AGC treated endothelial cells (Figure 3b). Next, we assayed the effect of molecular suppression of ICMT on GRP94 protein levels. HUVEC transfected with ICMT siRNA demonstrated a 10-fold reduction in ICMT mRNA level (data are presented as percentage of untransfected cells. Control siRNA: 143 ± 6.4 ; ICMT siRNA: 13.3 ± 7.8 ; $n = 3-5$, $P < 0.05$, ICMT siRNA versus control siRNA) and 6-fold decrease in ICMT enzyme activity (Control siRNA: 5.52 ± 1.5 pmol/mg/min; ICMT siRNA: 0.88 ± 0.12 pmol/mg/min; $n = 7$, $P < 0.05$, ICMT siRNA versus control siRNA). Consistent with the effect of ICMT chemical inhibition, knockdown of ICMT protein expression also significantly attenuated GRP94 protein level (Figure 3c).

Knockdown of GRP94 Exacerbates AGGC-Induced Endothelial Cell Apoptosis

We next assayed the effect of GRP94 protein depletion on AGGC-induced endothelial cell apoptosis. Bovine PAEC transfected with control siRNA or GRP94 siRNA for 48 h were exposed to vehicle or AGGC for 18 h and endothelial cell apoptosis was assayed by measuring caspase-3 activity. Depletion of GRP94 protein was confirmed by immunoblot analysis (Figure 4a). Suppression of GRP94 expression did not alter caspase-3 activity significantly (Figure 4b), suggesting that depletion of GRP94 alone is not sufficient to cause endothelial cell apoptosis. ICMT inhibition by AGGC significantly increased caspase-3 activity in endothelial cells transfected with control siRNA (Figure 4b). This effect was significantly exacerbated by suppression of GRP94 (Figure 4b). These findings suggest that GRP94 may preserve or limit endothelial cell apoptosis in the setting of ICMT inhibition. These results also suggest that decreased GRP94 protein level induced by ICMT inhibition contributes to endothelial cell apoptosis.

We next explored if proteasome-mediated protein degradation played any role in decreasing GRP94 protein level and endothelial

Figure 7. Effect of constitutively active RhoA on ICMT inhibition-induced relocalization of GRP94. Bovine PAEC were transfected with pcDNA3-EGFP-RhoA(Q63L) using pEGFP-C1 as a vector control for 24 h. Cells were then treated with 10 μ M AGC or 10 μ M AGGC for 3 h. Overexpression of GFP and GFP-RhoA was confirmed by fluorescence microscopy (a, c, e, and g, green). GRP94 subcellular localizations were assessed by immunofluorescence microscopy using antibody directed against GRP94 (b, d, f, and h, red). The nuclei were counterstained with DAPI (blue) ($n = 8$, representative figures are shown). Arrows indicate GRP94 perinuclear localization; block arrows indicate diffuse subcellular relocalization of GRP94; arrowheads indicate GRP94 aggregates.

cell apoptosis upon ICMT inhibition. Co-incubation of bovine PAEC with AGGC and proteasome inhibitor MG132 did not prevent decrease in GRP94 protein level or caspase-3 activation induced by ICMT inhibition (Figures 4c and 4d). These results suggest that decrease in GRP94 protein level is through mechanism(s) other than proteasome-mediated protein degradation.

GRP94 Subcellular Relocalization and Decrease in Protein Content upon ICMT Inhibition Require Caspase Activation

To determine if ICMT inhibition caused GRP94 relocalization and decrease in protein level through a caspase-dependent mechanism, endothelial cells were preincubated with zVAD-fmk, a broad caspase inhibitor, for 1 h. The endothelial cells were

then exposed to vehicle, AGC, or AGGC in the absence or presence of zVAD-fmk for 3 or 18 h, and GRP94 subcellular localization and protein content were determined, respectively. Relocalization and aggregation of GRP94 upon AGGC treatment was prevented by zVAD-fmk (Figure 5a). In addition, zVAD-fmk also blunted the decrease in GRP94 protein level induced by ICMT inhibition (Figure 5b). These results suggest that zVAD-fmk-sensitive caspases are important for GRP94 relocalization, aggregation, and degradation.

ICMT Inhibition Reduces RhoA Protein Content in a Caspase-Dependent Manner

We have previously shown that 30 min exposure to AGGC decreased RhoA carboxyl methylation and GTPase activity (14). In this study, we noted that 18 h exposure of endothelial cells to AGGC dramatically reduced RhoA protein level, as compared to vehicle or AGC treatment (Figure 6a). This effect was prevented by inhibition of caspase activity using zVAD-fmk (Figure 6a).

RhoA Protects against GRP94 Degradation and Relocalization upon ICMT Inhibition

We have previously demonstrated a link between ICMT activity and the activities of Ras (6) and RhoA (14) in endothelial cells. Thus, we next examined the effect of transient overexpression of Ras and RhoA on GRP94 protein level and subcellular relocalization upon ICMT inhibition. GRP94 protein levels were assayed in bovine PAEC transiently transfected with cDNAs for constitutively active Ras or RhoA and then treated with AGC or AGGC for 18 h. Overexpression of Ras and GFP-RhoA was confirmed by immunoblot analyses using antibodies directed against Ras and RhoA, respectively (Figures 6b and 6c, *top blots*). Overexpression of GFP-RhoA was also confirmed by immunoblot analyses using antibody directed against GFP (data not shown). While endogenous Ras and RhoA were diminished upon 18 h exposure to AGGC, the levels of the overexpressed dominant active Ras (H-RasQ61L) and dominant active RhoA (GFP-RhoAQ63L) were preserved (Figures 6b and 6c, *top blots*). This allowed us to assess the effects of constitutively active Ras and RhoA on ICMT inhibition-induced alterations of GRP94. AGGC-induced decreases in GRP94 protein level were not rescued by constitutively active Ras (Figure 6b), but were blunted by overexpression of constitutively active RhoA (Figure 6c). These results suggest that decrease in active RhoA upon ICMT inhibition contributes to a reduction in GRP94 protein level. In addition, two-dimensional PAGE and immunoblot analysis of lysates from bovine PAEC transiently transfected with cDNAs for constitutively active RhoA and then treated with AGC or AGGC for 18 h demonstrated that overexpression of dominant active RhoA did not prevent changes in the pI of GRP94 induced by ICMT inhibition (data not shown). This result suggests that alterations in the pI of GRP94 upon ICMT inhibition may result from other undefined mechanisms.

Next, to determine if overexpression of constitutively active RhoA can prevent GRP94 relocalization and aggregation induced by ICMT inhibition, bovine PAEC transiently transfected with constitutively active RhoA were treated with AGC or AGGC for 3 h and immunofluorescently stained for GRP94. Overexpression of GFP or GFP-RhoA(Q63L) was confirmed by fluorescence microscopy (Figures 7a, 7c, 7e, and 7g). Similar to that in untransfected cells, exposure of GFP-transfected PAEC to AGGC caused GRP94 relocalization and aggregation (Figure 7d), compared with GFP-transfected, AGC-treated endothelial cells (Figure 7b). While transient overexpression of constitutively active RhoA did not affect GRP94 subcellular localization

in PAEC exposed to AGC (Figure 7f), it prevented AGGC-induced GRP94 relocalization and aggregation (Figure 7h). These results suggest that RhoA inactivation is necessary for GRP94 subcellular relocalization and aggregation upon ICMT inhibition.

DISCUSSION

Apoptosis is important in development, remodeling of tissues, and progression of diseases. We have previously demonstrated that inhibition of ICMT causes endothelial cell apoptosis by a mechanism involving decreased Ras carboxyl methylation and activity (6). In the current study, we determined that inhibition of ICMT caused subcellular relocalization, aggregation, and decrease in protein level of GRP94. We also demonstrated that ICMT inhibition altered the pI of GRP94. In addition, inhibition of caspases or overexpression of constitutively active RhoA blunted relocalization, aggregation, and reduction in protein content of GRP94 induced by ICMT inhibition. Furthermore, depletion of GRP94 exacerbated endothelial cell apoptosis upon ICMT inhibition. We speculate that relocalization and decrease in protein level of GRP94 upon ICMT inhibition may contribute to endothelial cell apoptosis, possibly through UPR dysfunction.

ICMT catalyzes carboxyl methylation of proteins possessing a C-terminal CAAX motif, including Ras (33) and RhoA (34). Decreased carboxyl methylation of RhoA reduces protein stability and half life (34). We have previously shown that exposure of endothelial cells to AGGC, an ICMT inhibitor, for 30 min significantly reduces RhoA carboxyl methylation (14). In this study, we observed that endogenous RhoA protein level began to decrease after 3 h exposure to AGGC (data not shown), and almost disappeared after 18 h exposure. Thus, the decrease in RhoA protein level upon ICMT inhibition is likely due to inhibition of carboxyl methylation of RhoA and subsequent decrease in RhoA stability. RhoA is a key regulator of the cytoskeleton. In the current study, constitutively active RhoA blunted redistribution, aggregation, and reduction in protein level of GRP94 induced by ICMT inhibition. These results suggest that RhoA inactivation is critical for GRP94 relocalization and aggregation.

Although cell surface localization of GRP94 has been observed in transformed cells (35, 36), as a molecular chaperone, GRP94 primarily resides in the ER due to an encoded C-terminal ER retention signal, Lys-Asp-Glu-Leu (KDEL) (37). Cleavage of KDEL motif leads to cytoplasmic localization (37). GRP94 has been shown to be cleaved to an 80-kD fragment, which contains KDEL motif, by calpain during etoposide-induced apoptosis of various cell lineages (26). Thus, calpain-dependent cleavage of GRP94 unlikely contributes to its redistribution. Misfolded or unfolded proteins can be translocated from ER to cytosol for degradation through an ER-associated degradation system. We demonstrated that proteasome inhibition did not preserve GRP94 protein level upon ICMT inhibition, suggesting that proteasome-mediated ER-associated degradation system does not play a role in GRP94 relocalization. GRP94 can be released in parallel with other ER luminal proteins upon permeabilization and/or disruption of ER membrane (38). We have demonstrated co-relocalization and aggregation of calnexin, another ER molecular chaperone, with GRP94 upon ICMT inhibition. These results suggest that GRP94 redistribution results from dysfunction of ER membrane. As stated previously, RhoA plays a critical role in maintaining functional cytoskeleton. Thus, we speculate that cytoskeletal dysfunction induced by RhoA inactivation upon ICMT inhibition may cause disruption of ER membrane, leading to redistribution of GRP94 and calnexin. We have also shown that caspase inhibitor prevented relocalization and aggregation of GRP94, which correlated with inhibition

Figure 8. Schematic representation of the intracellular signaling involved in ICMT inhibition-induced endothelial cell apoptosis. *Solid lines* indicate defined pathways, and *dashed lines* indicate speculative pathways.

of endothelial cell apoptosis. Thus, caspases may also participate in disruption of ER membrane, ultimately leading to GRP94 relocalization and aggregation. These results also suggest that relocalization and aggregation of GRP94 may be a prerequisite for endothelial cell apoptosis.

While relocalization of GRP94 occurs upon 3 h exposure to AGGC, significant changes in the pI and protein level of GRP94 occur after 18 h exposure, suggesting that redistribution of GRP94 is independent of post-translational modifications and protein degradation. However, we cannot rule out the possibility that redistribution of GRP94 may be a prerequisite for post-translational modifications or degradation. Proteins can be degraded through ER-associated degradation. In this study, proteasome inhibition did not prevent ICMT inhibition-induced decrease in GRP94 protein level, suggesting that decrease in GRP94 protein level is through mechanism(s) other than proteasome-mediated degradation. Indeed, we have shown that caspase inhibitor and constitutively active RhoA blunted the effect of ICMT inhibition on GRP94 relocalization, aggregation, and decrease in protein level. Thus, it is possible that relocalization and aggregation of GRP94 mediated by RhoA and caspases upon ICMT inhibition may lead to GRP94 degradation. We certainly cannot rule out the possibility that RhoA and caspases have direct effect on GRP94 protein level.

GRP94 is not likely to be a substrate of ICMT, due to absence of C-terminal CAAX motif (32). Indeed, our data indicate that carboxyl methylation of GRP94 in endothelial cells either unstimulated or exposed to ICMT inhibitor was undetectable. GRP94 has been reported to be phosphorylated at multiple sites by casein kinase II (CK2) (21, 22) and by Golgi apparatus casein kinase (G-CK) (20). GRP94 is also autophosphorylated on serine and threonine residues; loss of which correlated with apoptosis (23). In this study, inhibition of ICMT causes changes in the pI of GRP94. Whether such post-translational modifications of GRP94 relate to phosphorylation remains to be investigated.

Overexpression of GRP94 protects against ER stress-induced cell death *in vitro* and neuronal cell death in ischemia/reperfusion injury *in vivo* (25, 39). Reduction of GRP94 correlated with activation of caspase-3 and decrease in cell viability (26). Recent studies have shown that GRP94 protein level was decreased during ER stress-induced apoptosis (40, 41). Consistent with these findings, we found that GRP94 protein level was significantly reduced concomitantly with occurrence of apoptosis upon ICMT inhibition. Furthermore, depletion of GRP94 exacerbated ICMT inhibition-induced caspase-3 activation. These results suggest that reduction of GRP94 contributes to apoptosis upon ICMT inhibition. GRP94 participates in the correct protein folding in the ER. Relocalization, aggregation, changes in the pI,

and reduction in protein content of GRP94 may reduce protein folding capacity, leading to accumulation of unfolded or misfolded proteins in the ER, resulting in ER stress. This in turn activates the UPR, a mechanism by which cells are able to dispose of unfolded or misfolded proteins and thus alleviate ER stress. However, prolonged or unresolved ER stress may cause UPR dysfunction, ultimately resulting in apoptosis. This notion is supported by our as yet unpublished data demonstrating that ICMT inhibition by AGGC decreased levels of phosphorylated eIF2 α , a UPR component that in its phosphorylated state mediates both translational attenuation of malformed proteins and selective transcriptional initiation of ER chaperones. Decrease in eIF2 α phosphorylation upon ICMT inhibition indicates UPR dysfunction.

In summary, inhibition of ICMT causes endothelial cell apoptosis via Ras-dependent pathway and RhoA-mediated, GRP94-dependent UPR dysfunction pathway, as described in Figure 8. Our results indicate that ICMT inhibition causes GRP94 relocalization, aggregation, changes in the pI, and degradation. Our data suggest that ICMT inhibition-induced caspase activation and RhoA inactivation play an important role in GRP94 relocalization, aggregation, and degradation. We speculate that relocalization, aggregation, changes in the pI, and decrease in protein content of GRP94 promote endothelial cell apoptosis, possibly through UPR dysfunction. These studies suggest a novel link between RhoA GTPase and the unfolded protein response.

Conflict of Interest Statement: S.R. has received \$2,500 from Novartis and \$2,500 from Astra-Zeneca as unrestricted educational grants. None of the other authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: This material is the result of work supported with resources and the use of facilities at the Providence VA Medical Center. The authors thank Dr. Klaus M. Hahn (Scripps Research Institute, La Jolla, CA) for pcDNA3-EGFP-RhoA(Q63L) construct. Some of these results were presented at the American Thoracic Society 101st International Conference, May 20–25, 2005, San Diego, California and published in abstract form in *Proceedings of the American Thoracic Society* 2:A608, 2005. Q.L. is a Parker B. Francis Fellow.

References

- Lu Q, Harrington EO, Rounds S. Apoptosis and lung injury. *Keio J Med* 2005;54:184–189.
- Groenendyk J, Michalak M. Endoplasmic reticulum quality control and apoptosis. *Acta Biochim Pol* 2005;52:381–395.
- Dubyak G, el-Moatassim C. Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. *Am J Physiol* 1993;265:C577–C606.
- Dawicki DD, Chatterjee D, Wyche J, Rounds S. Extracellular ATP and adenosine cause apoptosis of pulmonary artery endothelial cells. *Am J Physiol* 1997;273:L485–L494.
- Rounds S, Yee W, Dawicki DD, Harrington EO, Parks N, Cutaia MV. Mechanism of extracellular ATP- and adenosine-induced apoptosis of cultured pulmonary artery endothelial cells. *Am J Physiol* 1998;275:L379–L388.
- Kramer K, Harrington EO, Lu Q, Bellas R, Newton J, Sheahan KL, Rounds S. Isoprenylcysteine carboxyl methyltransferase activity modulates endothelial cell apoptosis. *Mol Biol Cell* 2003;14:848–857.
- Dai Q, Choy E, Chiu V, Romano J, Slivka S, Steitz SA, Michaelis S, Philips MR. Mammalian prenylcysteine carboxyl methyltransferase is in the endoplasmic reticulum. *J Biol Chem* 1998;273:15030–15034.
- Romano JD, Schmidt WK, Michaelis S. The *Saccharomyces cerevisiae* prenylcysteine carboxyl methyltransferase Ste14p is in the endoplasmic reticulum membrane. *Mol Biol Cell* 1998;9:2231–2247.
- Bergo MO, Leung GK, Ambroziak P, Otto JC, Casey PJ, Gomes AQ, Seabra MC, Young SG. Isoprenylcysteine carboxyl methyltransferase deficiency in mice. *J Biol Chem* 2001;276:5841–5845.
- Clarke S. Protein isoprenylation and methylation at carboxyl terminal cysteine residues. *Annu Rev Biochem* 1992;61:355–386.
- Bergo MO, Leung GK, Ambroziak P, Otto JC, Casey PJ, Young SG. Targeted inactivation of the isoprenylcysteine carboxyl methyltransferase gene causes mislocalization of K-Ras in mammalian cells. *J Biol Chem* 2000;275:17605–17610.

12. Choy E, Chiu VK, Silletti J, Feoktistov M, Morimoto T, Michaelson D, Ivanov IE, Philips MR. Endomembrane trafficking of Ras: the CAAX motif targets proteins to the ER and Golgi. *Cell* 1999;98:69–80.
13. Li X, Liu L, Tupper JC, Bannerman DD, Winn RK, Sehti SM, Hamilton AD, Harlan JM. Inhibition of protein geranylgeranylation and RhoA/RhoA kinase pathway induces apoptosis in human endothelial cells. *J Biol Chem* 2002;277:15309–15316.
14. Lu Q, Harrington EO, Hai C-M, Newton J, Garber M, Hirase T, Rounds S. Isoprenylcysteine carboxyl methyltransferase (ICMT) modulates endothelial monolayer permeability: involvement of RhoA carboxyl methylation. *Circ Res* 2004;94:306–315.
15. Lee AS. The glucose-regulated proteins: stress induction and clinical applications. *Trends Biochem Sci* 2001;26:504–510.
16. Schroder M, Kaufman RJ. The mammalian unfolded protein response. *Annu Rev Biochem* 2005;74:739–789.
17. Chevet E, Jakob CA, Thomas DY, Bergeron JJ. Calnexin family members as modulators of genetic diseases. *Semin Cell Dev Biol* 1999;10:473–480.
18. Lawless MW, Greene CM, Mulgrew A, Taggart CC, O'Neill SJ, McElvaney NG. Activation of endoplasmic reticulum-specific stress responses associated with the conformational disease Z alpha 1-antitrypsin deficiency. *J Immunol* 2004;172:5722–5726.
19. Nicchitta CV, Reed RC. The immunological properties of endoplasmic reticulum chaperones: a conflict of interest? *Essays Biochem* 2000;36:15–25.
20. Brunati AM, Contri A, Muenchbach M, James P, Marin O, Pinna LA. GRP94 (endoplasmin) co-purifies with and is phosphorylated by Golgi apparatus casein kinase. *FEBS Lett* 2000;471:151–155.
21. Cala SE. GRP94 hyperglycosylation and phosphorylation in Sf21 cells. *Biochim Biophys Acta* 2000;1496:296–310.
22. Cala SE, Jones LR. GRP94 resides within cardiac sarcoplasmic reticulum vesicles and is phosphorylated by casein kinase II. *J Biol Chem* 1994;269:5926–5931.
23. Csermely P, Miyata Y, Schnaider T, Yahara I. Autophosphorylation of grp94 (endoplasmin). *J Biol Chem* 1995;270:6381–6388.
24. Rosser MF, Trotta BM, Marshall MR, Berwin B, Nicchitta CV. Adenosine nucleotides and the regulation of GRP94-client protein interactions. *Biochemistry* 2004;43:8835–8845.
25. Bando Y, Katayama T, Aleshin AN, Manabe T, Tohyama M. GRP94 reduces cell death in SH-SY5Y cells perturbed calcium homeostasis. *Apoptosis* 2004;9:501–508.
26. Reddy RK, Lu J, Lee AS. The endoplasmic reticulum chaperone glycoprotein GRP94 with Ca²⁺-binding and antiapoptotic properties is a novel proteolytic target of calpain during etoposide-induced apoptosis. *J Biol Chem* 1999;274:28476–28483.
27. Hai CM, Sadowska G, Francois L, Stonestreet BS. Maternal dexamethasone treatment alters myosin isoform expression and contractile dynamics in fetal arteries. *Am J Physiol* 2003;283:H1743–H1749.
28. Zhou C, Arslan F, Wee S, Krishnan S, Oliva A, Leatherwood J, Wolf DA. PCI proteins eIF3e and eIF3m define distinct translation initiation factor 3 complexes. *BMC Biol* 2005;3:14.
29. Lu Q, Harrington EO, Jackson H, Morin N, Shannon C, Rounds S. Transforming growth factor-β1-induced endothelial barrier dysfunction involves SMAD2-dependent p38 activation and subsequent RhoA activation. *J Appl Physiol* 2006;101:375–384.
30. Harrington EO, Smeglin A, Newton J, Ballard G, Rounds S. Protein tyrosine phosphatase-dependent proteolysis of focal adhesion complexes in endothelial cell apoptosis. *Am J Physiol* 2001;280:L342–L353.
31. Paris S, Denis H, Delaive E, Dieu M, Dumont V, Ninane N, Raes M, Michiels C. Up-regulation of 94-kDa glucose-regulated protein by hypoxia-inducible factor-1 in human endothelial cells in response to hypoxia. *FEBS Lett* 2005;579:105–114.
32. Maki RG, Old LJ, Srivastava PK. Human homologue of murine tumor rejection antigen gp96: 5'-regulatory and coding regions and relationship to stress-induced proteins. *Proc Natl Acad Sci USA* 1990;87:5658–5662.
33. Philips MR, Pillinger MH, Staud R, Volker C, Rosenfeld MG, Gerald W, Stock JB. Carboxyl methylation of Ras-related proteins during signal transduction in neutrophils. *Science* 1993;259:977–980.
34. Backlund PS Jr. Post-translational processing of RhoA. *J Biol Chem* 1997;272:33175–33180.
35. Altmeyer A, Maki RG, Feldweg AM, Heike M, Protopopov VP, Masur SK, Srivastava PK. Tumor-specific cell surface expression of the KDEL containing, endoplasmic reticular heat shock protein gp96. *Int J Cancer* 1996;69:340–349.
36. Robert J, Menoret A, Cohen N. Cell surface expression of the endoplasmic reticular heat shock protein gp96 is phylogenetically conserved. *J Immunol* 1999;163:4133–4139.
37. Munro S, Pelham HR. A C-terminal signal prevents secretion of luminal ER proteins. *Cell* 1987;48:899–907.
38. Nicchitta CV. Biochemical, cell biological and immunological issues surrounding the endoplasmic reticulum chaperone GRP94/gp96. *Curr Opin Immunol* 1998;10:103–109.
39. Bando Y, Katayama T, Kasai K, Taniguchi M, Tamatani M, Tohyama M. GRP94 (94 kDa glucose-regulated protein) suppresses ischemic neuronal cell death against ischemia/reperfusion injury. *Eur J Neurosci* 2003;18:829–840.
40. Bruneel A, Labas V, Mailloux A, Sharma S, Royer N, Vinh J, Pernet P, Vaubourdolle M, Baudin B. Proteomics of human umbilical vein endothelial cells applied to etoposide-induced apoptosis. *Proteomics* 2005;5:3876–3884.
41. Dey A, Kessova IG, Cederbaum AI. Decreased protein and mRNA expression of ER stress proteins GRP78 and GRP94 in HepG2 cells over-expressing CYP2E1. *Arch Biochem Biophys* 2006;447:155–166.