Role of Isoprenylcysteine Carboxyl Methyltransferase in Tumor Necrosis Factor-α Stimulation of Expression of Vascular Cell Adhesion Molecule-1 in Endothelial Cells

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Abstract—We have previously shown that cytokine stimulation of the expression of vascular cell adhesion molecule-1 (VCAM-1), but not that of intercellular adhesion molecule-1 (ICAM-1), is redox sensitive in endothelial cells. Here, we investigated the role of isoprenylcysteine carboxyl methyltransferase (ICMTase), which methylates isoprenylated CAAX (where C indicates cysteine; A, aliphatic amino acids; and X, almost any other amino acid) proteins, including Rac1, a component of superoxide-generating NAD(P)H oxidase, in the expression of VCAM-1. Pretreatment of endothelial cells with N-acetyl-S-farnesyl-L-cysteine (AFC) or N-acetyl-S-geranylgeranyl-L-cysteine (AGGC), specific inhibitors of ICMTase, inhibited the tumor necrosis factor-α (TNF-α) stimulation of mRNA expression of VCAM-1 but not that of ICAM-1. Endothelial cells expressed constitutively active ICMTase, as suggested by the presence of methylated Rac1 and the methylation of AFC by the cells. TNF-α stimulation of the cells significantly increased the methylation of AFC and Rac1 in endothelial cells. That ICMTase was a component of the redox-sensitive signaling pathway was also suggested by the AFC inhibition of the generation of reactive oxygen species by TNF-α. Interestingly, the dominant-negative isoform of Rac1 was not selective but inhibited the TNF-α stimulation of the mRNA expression of VCAM-1 and ICAM-1. Thus, ICMTase is a critical component of the redox-sensitive VCAM-1–selective signaling pathway, at least in part, through the methylation of Rac1. (Arterioscler Thromb Vasc Biol. 2002;22:759-764.)

Key Words: cell signaling ■ gene regulation ■ growth factors ■ oxidant stress

Cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β, activate the cell surface expression of adhesion molecules (vascular cell adhesion molecule-1 [VCAM-1] and intercellular adhesion molecule-1 [ICAM-1]) and E-selectin in endothelial cells (ECs). The changes in the cell surface expression of adhesion molecules provide a mechanism by which the interaction of ECs with leukocytes is regulated, a step that is critical for the recruitment of leukocytes into the extravascular space and the initiation of inflammatory diseases, such as atherosclerosis.

Antioxidants selectively inhibited cytokine stimulation of the expression of VCAM-1 as opposed to ICAM-1 or E-selectin in ECs. These observations provided early evidence of a role for reactive oxygen species (ROS) in the selective stimulation of discrete inflammatory signaling pathways in ECs. Recent studies indicate that the small GTP-binding protein Ha-Ras is involved in regulating VCAM-1 expression. Many of the effects of Ras, including those associated with the generation of ROS, are mediated through the activation of Rac1, a multifunctional member of the Rho family of small GTP-binding proteins. ECs contain a neutrophil-type NAD(P)H oxidase, a multicomponent enzyme that is considered to be a major source of superoxide generation in these cells. Rac1 associates with this enzyme and is critical for its activation. Several studies have suggested that Rac1 is involved also in the generation of superoxide in nonphagocytic cells, including endothelium. ROS act as essential signaling intermediates for cytokines in many cell types. Thus, Rac1 may be important in the regulation of VCAM-1 expression.

Rac1 belongs to the families of proteins including small GTPases and the γ subunits of heterotrimeric G proteins that terminate with a CAAX (where C indicates cysteine; A, aliphatic amino acids; and X, almost any other amino acid) sequence. After isoprenylation on cysteine, the CAAX sequence is cleaved by an endoplasmic reticulum resident enzyme, isoprenylcysteine carboxyl methyltransferase (ICMTase). These “post-isoprenylation” steps are believed to provide additional targeting mechanisms to cell membranes for proteins, many of which participate in cell signaling.
evidence of the critical functional importance of ICMTase. The ICMTase-deficient mouse could not survive beyond mid gestation.16 Thus, methylation of CAAAX proteins, potentially including Rac1, appears to play a critical role in regulating various cell functions involving appropriate membrane targeting.

In the present study, we tested the hypothesis that ICMTase is involved in regulating the cytokine stimulation of VCAM-1 expression in ECs. Using specific inhibitors of ICMTase, N-acetyl-S-farnesyl-l-cysteine (AFC) and N-acetyl-S-geranylgeranyl-l-cysteine (AGGC), we show that methylated Rac1 may be a critical component of the TNF-α stimulation of mRNA expression of VCAM-1 but not ICAM-1. However, the dominant-negative mutant of Rac1 was not selective but inhibited the TNF-α stimulation of expression of VCAM-1 and ICAM-1. Thus, methylated Rac1 may be a critical component of the TNF-α-stimulated redox-sensitive signaling pathway up-regulating VCAM-1 expression in ECs.

**Methods**

**Cell Culture and Reagents**

Human aortic ECs (HAECs) were purchased from Clonetics and were grown according to their protocol. Human dermal microvascular ECs immortalized with simian virus 40 product large T antigen (HMEC-1) were grown as described earlier.19 AFC and AGGC were purchased from Calbiochem. Antibodies to Rac1, myc epitope, VCAM-1, and ICAM-1 were from Santa Cruz Biotechnology, and the antibody to ICMTase was also referred to as pcMTase.20 was kindly provided by Dr Douglas C. Eaton, Department of Physiology, Emory University, Atlanta, Ga. Ad.N17Rac1, the adenovirus encoding the myc epitope–tagged dominant-negative isoform of Rac1 was kindly provided by Dr Toren Finkel.21 National Institutes of Health, Bethesda, Md. Northern analyses were performed as described earlier.19

**Cell Extract Preparation and Western Analysis**

The cells (in 100-mm dishes) were lysed in 0.7 mL ice-cold lysis buffer (10 mmol/L Tris-HCl, pH 7.2, 1% Triton X-100, 150 mmol/L NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/mL each of leupeptin, aprotinin, and antipain, and 1 mmol/L phenylmethylsulfonyl fluoride), 50 mmol/L sodium fluoride, and 1 mmol/L sodium orthovanadate and, after vortexing, were put on a rotor for 1 hour at 4 °C. Equal amounts of total protein were size-fractionated by SDS-PAGE. Cell lysates were prepared as described above.19

**Cytotoxicity Assays**

The cytotoxicity assays were performed by using a lactate dehydrogenase–based toxicology assay kit (TOX-7) from Sigma Chemical Co.

**Hydrogen Peroxide Measurements**

The hydrogen peroxide measurements were performed by using an Amplex Red–based assay kit from Molecular Probes.

**ICMTase Assays**

HAECs were grown to confluence in 100-mm dishes and were labeled with [methyl-3H]methionine (50 μCi/mL) in 4 mL of methionine-free medium (Clonetics) at 37 °C overnight. The cells were then incubated with AFC (25 to 50 μmol/L) for 3 hours before stimulation with TNF-α for 1 hour. The cells were washed 3 times with isotonic buffer, and lysates were prepared as described above. To extract AFC from cell lysates, lysate and heptane in a ratio of 1:4 were mixed for 10 seconds by vortexing, followed by centrifugation at 16 000g for 10 minutes at 4 °C. The organic phase was transferred to another tube and dried. The methanol released from AFC was quantified by vapor phase equilibrium (VPE) assays.

**Methylation Measurements by VPE Assays**

The methyl esterification of proteins by ICMTase is base labile. To determine the level of methylation of proteins, whole-cell lysate or immunoprecipitate or a specific protein band cut out from SDS-PAGE was carefully placed in 1.5-mL Eppendorf centrifuge tubes (without caps) containing 200 to 500 μL of 1N NaOH. The tubes were then placed in 20-mL scintillation vials containing 5 mL scintillation fluid. The vials were then capped and left at 37 °C overnight to release the [3H]methanol resulting from hydrolysis of the methyl esters, and the radioactivity was determined by scintillation spectrometry.

**Rac1 Methylation Assays**

The cell lysates were prepared as described above. The cell extracts were incubated with agaro-conjugated protein A/G (Santa Cruz Biotechnology) for 2 hours at 4 °C. After centrifugation at 5000g for 5 minutes, the supernatant was collected and incubated overnight with polyonal antibodies raised against Rac1 at 4 °C. Immune complexes were captured by using agaro-conjugated protein A/G and separated by centrifugation. Pellets were washed 3 times with 1× lysis buffer. The base labile [3H]methanol released in the immunoprecipitates was quantified after adding 200 μL of 1 mol/L NaOH. Alternatively, Rac1 from the immunoprecipitates was separated on SDS-PAGE and immunodetected by Western analysis, and the level of methylation in the Rac1-containing bands was measured by VPE assays.

**Results**

**Inhibitors of ICMTase, AFC, and AGGC Inhibit the TNF-α Stimulation of the Expression of VCAM-1 but Not That of ICAM-1**

To determine the role of carboxyl methylation (a post-isoprenylation step) in the TNF-α stimulation of mRNA expression of VCAM-1, we used AFC and AGGC, selective inhibitors of ICMTase.22–26 Preincubation of the cells with AFC overnight dose-dependently inhibited the TNF-α stimulation of mRNA expression of VCAM-1, with almost complete inhibition at 15 μmol/L (Figure 1A). However, shorter preincubation times required higher concentrations of AFC for its inhibitory effect. The effect of 1-hour pretreatment of the cells with AFC and AGGC on the TNF-α stimulation of mRNA expression of VCAM-1 and ICAM-1 is shown in Figure 1B and 1C. AFC at 25 μmol/L inhibited (70±8%, P<0.05; n=6) the TNF-α stimulation of the mRNA expression of VCAM-1. Under the same conditions, the ICAM-1 mRNA expression was potentiated to 144±32% (n=6), although it did not reach statistical significance. AGGC at 5 μmol/L inhibited the TNF-α stimulation of VCAM-1 mRNA expression (49±5%, P<0.05; n=5). Under the same conditions, the mRNA expression of ICAM-1 was significantly potentiated to 152±39% (P<0.05, n=5). The fact that AFC and AGGC both inhibited the TNF-α stimulation of mRNA expression of VCAM-1 is consistent with previous results suggesting that a single enzyme activity can yield carboxymethylation of geranylgeranylated and farnesylated proteins.23 As has been observed with HAECs, AFC and AGGC also inhibit the ability of TNF-α to stimulate the...
mRNA expression of VCAM-1 without affecting that of ICAM-1 in HMEC-1 (data not shown). Furthermore, AFC affected the protein expression of VCAM-1 and ICAM-1 in a similar fashion (Figure 1D). Thus, TNF-α/H9251 stimulation of expression of VCAM-1 but not that of ICAM-1 requires ICMTase in ECs.

Assessment of Cytotoxic Effects of AFC and AGGC on ECs

To determine whether AFC and AGGC have any cytotoxic effects on ECs, HMEC-1 were incubated with AFC overnight and AGGC for 2 hours. The incubation with AFC and AGGC was continued for an additional 5 hours in the presence or absence of TNF-α, and cell viability was assessed by measuring lactate dehydrogenase in intact cells as well as in the medium. More than 90% of LDH was found to be present in intact cells, and no significant difference was observed between cells treated with or without AFC (25 to 100 mmol/L) or AGGC (5 mmol/L), as shown in Figure 2. Thus, AFC and AGGC do not exert any cytotoxic effects on ECs under our experimental conditions.

**Figure 1.** Effect of AFC and AGGC on the TNF-α stimulation of expression of VCAM-1 and ICAM-1. A, HMEC-1 were pretreated with different concentrations of AFC overnight before stimulation with TNF-α (100 U/mL) for 4 hours and Northern analysis were performed. B, HAECs were pretreated with AFC or AGGC for 1 hour before stimulation with TNF-α (100 U/mL) for 4 hours and Northern analysis were performed with the use of VCAM-1-specific, ICAM-1-specific, and GAPDH-specific cDNA probes. C, The data presented in panel B are expressed as fold stimulation or inhibition and are the mean±SE of 5 or 6 experiments. D, HAECs were pretreated with AFC for 1 hour before stimulation with TNF-α for 9 hours and Western analysis were performed with the use of VCAM-1– and ICAM-1–specific antibodies (representative of 2 experiments).

**Figure 2.** Effect of AFC and AGGC on total cytoplasmic lactate dehydrogenase (LDH). HMEC-1 were treated with AFC overnight or AGGC for 2 hours in serum-free medium before stimulation with TNF-α for 5 hours. The cell culture medium was then removed, and the total LDH was measured in the adherent cells.

**Figure 3.** TNF-α Stimulates the Methylation of AFC in HAECs

As shown in Figure 3A, HAECs constitutively express ICMTase, a ≈33-kDa protein, which is consistent with the results reported earlier.16 To measure the activity of the enzyme, we first developed a dose-response curve by measuring the level of methanol released after alkaline hydrolysis of the methyl esters in cell lysates (Figure 3B). To measure the constitutive activity of ICMTase, AFC, an artificial substrate, was incubated with HAECs, and the level of methylated AFC was determined in the cell extracts by measuring the level of methanol released. As shown in Figure 3C, AFC was efficiently methylated (>8- to 10-fold more than the endogenous proteins) by HAECs. Under the same conditions, TNF-α stimulation of the cells increased the methylation of AFC in vivo 2-fold (Figure 3D). These results were also confirmed by measuring the level of methylated AFC after its extraction in heptane from the cell lysates (Figure 3E). Thus, constitutively active ICMTase is expressed in HAECs, and TNF-α stimulation of the cells further increases the methylation of AFC.

**Figure 4.** Effect of AFC on the TNF-α Stimulation of Generation of H₂O₂ in HAECs

We hypothesized that the role of ICMTase in VCAM-1 expression by TNF-α might be through a role for the enzyme in the cytokine stimulation of ROS. A lower level of H₂O₂ was
The Expression of a Dominant-Negative Isoform of Rac1 (N17Rac1) in ECs Inhibits the Upregulation of VCAM-1 and ICAM-1 mRNA by TNF-α

To determine whether Rac1 plays a role in the TNF-α stimulation of generation of ROS in ECs, we used an adenovirus to express N17Rac1 in early passages of HAECs. As shown in Figure 6A and 6C, the expression of N17Rac1 in HAECs inhibited the TNF-α stimulation of mRNA expression of VCAM-1 (82±1% [P<0.05, n=3] and 94±1% [P<0.05, n=3] at a multiplicity of infection of 1 and 5, respectively) and ICAM-1 (71±5% [P<0.05, n=3] and 87±7% [P<0.05, n=3] at a multiplicity of infection of 1 and 5, respectively). This inhibition was paralleled by an increase in the expression of N17Rac1 protein (Figure 6B). Under the same conditions, the adenovirus expressing LacZ affected neither the mRNA expression of VCAM-1 nor that of ICAM-1 (Figure 6A). As observed with HAECs, the expression of N17Rac1 but not that of LacZ also markedly inhibited the TNF-α stimulation of mRNA expression of VCAM-1 and ICAM-1 in ECV304 cells (Figure 6D). This inhibition was paralleled by an increase in the expression of N17Rac1 protein (Figure 6E). Under the same conditions, the adenovirus expressing LacZ affected neither the mRNA expression of VCAM-1 nor that of ICAM-1 (Figure 6F). As observed with HAECs, the expression of N17Rac1 but not that of LacZ also markedly inhibited the TNF-α stimulation of mRNA expression of VCAM-1 and ICAM-1 in ECV304 cells (Figure 6G).
ICMTase was found to be a critical component of the VCAM-1 signaling pathway. This is based on the inhibition of VCAM-1 expression by N17Rac1 and ICMTase inhibitors AFC (25 μmol/L) and AGGC (5 μmol/L). AFC and AGGC have been used at 100 μmol/L and 10 μmol/L, respectively, to competitively inhibit ICMTase. Because AFC and AGGC can also inhibit the methylation of H-Ras, which has been shown to regulate the TNF-α stimulation of the expression of VCAM-1, we cannot rule out the possibility that methylated H-Ras also contributes to the mRNA expression of VCAM-1.

In recent studies, methylation has been demonstrated to be required for proper membrane targeting of K-Ras and several other Ras-related proteins in mammalian cells. Methylation has also been proposed to affect protein-protein interaction. The methylation of K-Ras regulates its binding to microtubules. Whether methylation affects Rac1 localization within the membrane or its translocation to the membrane is not understood. Further studies are under way to determine the mechanism by which methylation affects the activity of Rac1 in ECs.

Previous studies have shown that AFC inhibits the chemotactrant N-formyl-Met-Leu-Phe stimulation of the generation of superoxide in neutrophils and HL-60 granulocytes. We demonstrate that AFC inhibits the generation of ROS in HAECS by TNF-α. Thus, methylation also appears to be a critical step in the generation of ROS in HAECS by TNF-α.

TNF-α stimulated the methylation of exogenously added AFC and endogenous Rac1. However, the increase in methylation of Rac1 (1.3- to 1.5-fold) was less than that of AFC (>2-fold). These differences could have several explanations. First, the concentration of AFC (25 μmol/L) was used much higher than that of endogenous Rac1. Second, Rac1 is constitutively methylated in unstimulated HAECS. Therefore, the fold induction of Rac1 methylation after TNF-α stimulation of the cells is limited by the actual level of demethylated Rac1 present in unstimulated HAECS. Third, the level of methylation of Rac1 at any time may be determined by the balance between the activity of ICMTase and methyl esterase, which demethylates methyl-esterified proteins. Methyl esterase may act effectively on Rac1 but not on AFC. Regardless, these results suggest that TNF-α stimulates the methylation of AFC and Rac1. Methylation of CAAX proteins has also been shown to be stimulated by glucose and N-formyl-Met-Leu-Phe. Thus, methylation appears to be a common mechanism by which various activators stimulate the activity of signaling proteins.

Rac1 is a multifunctional GTPase. In the present study, N17Rac1 was not selective but inhibited the TNF-α stimulation of mRNA expression of VCAM-1 and ICAM-1. N17Rac1 prevents the activation of endogenous Rac1 by TNF-α. The data presented in panel A are expressed as percentage inhibition of stimulation by TNF-α and are the mean±SE of 3 experiments.

Discussion
Our data suggest that ICMTase is a critical component of the TNF-α-mediated signaling events that stimulate the mRNA expression of VCAM-1 but not that of ICAM-1 in ECs. ECs expressed constitutively active ICMTase, and TNF-α stimulation of the cells increased the methylation of AFC, an artificial substrate, and Rac1, which is believed to be involved in the production of ROS in ECs. Pretreatment of the cells with AFC inhibited the TNF-α stimulation of ROS in ECs. Interestingly, N17Rac1 was not selective but inhibited the TNF-α stimulation of mRNA expression of VCAM-1 and ICAM-1.

To identify the VCAM-1–selective signaling pathway, we used ICAM-1 as a reference point, which we have previously shown to be redox insensitive. On the basis of this criterion, ICMTase was found to be a critical component of the VCAM-1–selective TNF-α signal transduction pathway in ECs. In fact, ICMTase inhibition by AFC and AGGC potentiated the TNF-α stimulation of mRNA expression of ICAM-1. This could be due to an increase in the level of demethylated Rac1 or to an effect on another GTPase. ICMTase methylates farnesylated and geranylgeranylated CAAX proteins. Based on this finding, the present study suggests that none of the methylated proteins was involved in the TNF-α signal transduction regulating ICAM-1 mRNA expression.

Our data suggest that methylated Rac1 is a critical component of the redox-sensitive VCAM-1–selective signaling pathway. This is based on the inhibition of VCAM-1 expression by N17Rac1 and ICMTase inhibitors AFC (25 μmol/L) and AGGC (5 μmol/L). AFC and AGGC have been used at 100 μmol/L and 10 μmol/L, respectively, to competitively inhibit ICMTase. Because AFC and AGGC can also inhibit the methylation of H-Ras, which has been shown to regulate the TNF-α stimulation of the expression of VCAM-1, we cannot rule out the possibility that methylated H-Ras also contributes to the mRNA expression of VCAM-1.
and demethylated Ras1 activate 2 distinct TNF-α signal transduction pathways to stimulate VCAM-1 and ICAM-1 mRNA expression.

Our data implicate ICMTase into specific molecular pathways that may be involved in the redox-mediated selectivity of cytokine stimulation of VCAM-1 expression in ECs. ICMTase modulation of small GTPase membrane targeting and activation, essentially to stimulate the generation of ROS, may be important in the pathogenesis of atherosclerosis. Because methylation can occur only on isoprenylated proteins, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, which are also known to inhibit isoprenylation, may mediate, at least in part, their cholesterol-lowering–independent beneficial effects through preventing the methylation step. Thus, inhibitors of ICMTase may prove to be useful tools as anti-inflammatory agents in the vasculature.

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