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CAT-1 as a novel CAM stabilizes endothelial integrity and mediates the protective actions of L-Arg *via* a NO-independent mechanism

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ABSTRACT

Interendothelial junctions play an important role in the maintenance of endothelial integrity and the regulation of vascular functions. We report here that cationic amino acid transporter-1 (CAT-1) is a novel interendothelial cell adhesion molecule (CAM). We identified that CAT-1 protein localized at cell–cell adhesive junctions, similar to the classic CAM of VE-cadherin, and knockdown of CAT-1 with siRNA led to an increase in endothelial permeability. In addition, CAT-1 formed a *cis*-homo-dimer and showed Ca^{2+}-dependent *trans*-homo-interaction to cause homophilic cell–cell adhesion. Co-immunoprecipitation assays showed that CAT-1 can associate with β-catenin. Furthermore, we found that the sub-cellular localization and function of CAT-1 are associated with cell confluency, in sub-confluent ECs CAT-1 proteins distribute on the entire surface and function as L-Arg transporters, but most of the CAT-1 in the confluent ECs are localized at interendothelial junctions and serve as CAMs. Further functional characterization has disclosed that extracellular L-Arg exposure stabilizes endothelial integrity *via* abating the cell junction disassembly of CAT-1 and blocking the cellular membrane CAT-1 internalization, which provides the new mechanisms for L-Arg paradox and *trans*-stimulation of cationic amino acid transport system (CAAT). These results suggest that CAT-1 is a novel CAM that directly regulates endothelial integrity and mediates the protective actions of L-Arg to endothelium *via* a NO-independent mechanism.
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1. Introduction

Cell junctions are essential for tissue morphogenesis and the maintenance of tissue architecture. In blood vessels, interendothelial junctions play important roles in the maintenance of endothelial integrity and in the regulation of vascular functions. A variety of diseases or pathophysiologic conditions, such as ischemia/reperfusion injury, primary or secondary pulmonary hypertension, sepsis and acute respiratory distress, are characterized by endothelial cell (EC) dysfunction and endothelial integrity injury [1–4]. So far, three types of intercellular junctions have been reported in ECs, *i.e.*, tight junctions, adherens junctions and gap junctions [1,4–6]. Among them, adherens junctions are the major structure of the interendothelial junctions; and cadherins and nectins are the known transmembrane adhesion molecules of adherens junctions.

In addition to the aforementioned CAMs as physical determinants of endothelial integrity, NO is an important signaling molecule in regulating various vasomediators responsible for vascular permeability [7–10]. The role of NO in vascular permeability has been extensively investigated by employing endothelial-NO synthase (eNOS) inhibitors or NO donors. The exact mechanism, however, remains ambiguous. One of the possible mechanisms is *via* the regulation of EC shape and intercellular junction formation in which excessive NO may augment vascular permeability [11–13].

The first member of CAT-1 (mCAT-1, for mouse CAT-1) was originally identified as the receptor for moloney murine leukemia virus (MuLVR) [14]. Amino acid similarities observed between the MuLVR and L-histidine and L-Arg permeases from *Saccaromyces cerevisiae* led to the discovery of its physiological function as the Na^{+}-independent transporter of cationic amino acids. The importance of this protein in vascular biology was emphasized by further findings as follows. L-Arg is the exclusive precursor of NO production in ECs [15–21]; nitric oxide synthase (NOS)-mediated NO formation is dependent upon an

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adequate and continuing supply of L-Arg. However, the intracellular L-Arg content is derived primarily from plasma membrane-dependent transport of extracellular L-Arg. Although L-Arg transport can be mediated by several independent transport activities in mammalian cells, CAT-1 is the predominant mediator of L-Arg transport in ECs [22,23] and overexpression of CAT-1 dramatically increases NO generation in ECs [22,24–26].

Since CAT-1 drives endothelial NO generation and excessive NO increases vascular permeability, we originally hypothesized that CAT-1 upregulation will augment endothelial permeability due to the increased NO generation. It is somewhat unexpected however that CAT-1 overexpression reduced rather than increased the endothelial permeability despite higher NO production as presented in our study. This intriguing finding led us to investigate whether CAT-1 itself-*via* a NO-independent mechanism, such as strengthening intercellular junctions, regulates endothelial integrity. We used a CAT-1-GFP fusion construct to examine the role of CAT-1 in regulating intercellular junctions in ECs. Using confocal microscopy analysis, our study has revealed that CAT-1 protein localized at cell–cell adhesive junctions in the confluent ECs, similar to the classic CAM of VE-cadherin. Cell aggregation and cross-linking assays indicated that CAT-1 protein exist as *trans*-interaction form and *cis*-homodimers. Cell density assays have demonstrated that CAT-1 mainly serves as intercellular junction molecules in confluent cells, whereas it serves as cationic acid transporter mediating L-Arg transport in sub-confluent cells. Further functional characterization has disclosed that extracellular L-Arg exposure stabilizes endothelial integrity *via* abating the cell junction disassembly of CAT-1 and blocking the cellular membrane CAT-1 internalization, which provides the new mechanisms for L-Arg paradox and *trans*-stimulation of CAAT. These results suggest a multifunctional and conformation-dependent role of the CAT-1 as a membrane protein, which is a novel mechanism that has never been reported previously. In ECs, CAT-1 acts as a novel CAM that directly regulates endothelial integrity and mediates the protective actions of L-Arg in a NO independent way.

2. Methods

2.1. Cell isolation and culture

PAECs were obtained from the main pulmonary artery of lungs derived from 6–7 month old pigs as previously reported [27]. Third-to-eighth passage cells were used for all experiments. Monolayers were maintained in RPMI 1640 medium containing 4% fetal bovine serum and antibiotics (penicillin, gentamicin and fungizone). Human umbilical vein endothelial cells (HUVECs) were purchased and cultured with ECM medium (Sciencell, America). Phase contrast and electron microscopies were used for the cell identification and confirmation of purity based on the presence of factor VIII antigen and uptake of acetylated LDL. Cell viability was assessed using trypan blue staining and cell counting. CHO-S cells were purchased and cultured with BW12009 medium (Biowit, China).

2.2. Expression of GFP, CAT-1-GFP and CAT-4-GFP in ECs and CHO-S cells

Plasmid encoding the human CAT-1-GFP fusion protein was constructed as described below [27]. Briefly, a 1890-bp human CAT-1 cDNA containing the full-length CDS of human CAT-1 was obtained using one-step RT-PCR amplification and the following PCR primers (Forward, 5′-GGATCCCACCATGGGGTGCAAAGTCCT-3′; Reverse, 5′-ACCGGTTTGCACTGGTCCAAGTTG-3′). The reaction conditions of RT-PCR were 42 °C for 45 min, 94 °C for 10 min, followed by 35 cycles of amplification (94 °C for 30 s, 60 °C for 45 s, 68 °C for 2 min). After BglII/EcoRI digestion and gel purification, the PCR product was inserted in the BglII/EcoRI sites of pEGFP-N1 (BD Biosciences Clontech). The resulting plasmid was termed pCAT-1-GFP. After subcloning into pENTR-1A, LR recombination between pENTR-1A and adenovector as well as lentivector was performed; the resulting adenovector and lentivector expressing CAT-1-GFP were finally generated according to the standard protocols (Invitrogen) and used to infect PAEC, HUVEC and CHO-S cells after tiltation. The same procedure was applied to GFP and other isoform of CAT-GFP generation.

2.3. Measurement of L-Arg uptake

L-Arg uptake was measured using L-[3H]Arg (a mixture of 50 μM unlabeled L-Arg plus L-[3H]Arg, 10 μCi/ml) according to the method reported by us [25,27,28]. Briefly, ECs grown in 24-well plates were washed with 0.5 ml of the buffer of the following composition (in mM): 140 LiCl, 5 KCl, 2 Na_2HPO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 11 glucose, and 10 HEPES-Tris (pH 7.4, 37 °C, LiCl-Dulbecco solution) and then incubated with L-[3H]Arg in LiCl-Dulbecco solution. The incubation was stopped by washing the cells four times with 2 ml of ice-cold LiCl-Dulbecco solution. After solubilization of the cells, radioactivity was quantitated by liquid scintillation spectrometry. Aliquots of solubilized cells were also taken for measurement of protein content. All measurements of L-Arg uptake were normalized by subtracting the nonspecific component of uptake. The results are expressed as the ratio of CAT-1-GFP vs GFP, CAT-1 siRNA vs scramble siRNA. Bars represent means ± SEM. All experiments were repeated at least three times in triplicate wells. Statistical analysis was performed using paired Student *t* test.

2.4. Measurement of NO production

NO production was measured by monitoring the conversion of L-[3H]Arg into L-[3H] citrulline as previously described [29]. ECs were washed once in 1 ml of warm LiCl-DMEM and then incubated in 0.5 ml of LiCl-DMEM containing L-[3H]Arg (5 μCi/ml) for 15 min. After the 15-min incubation, PAECs were washed three times with 2 ml of ice-cold LiCl-DMEM containing 5 mM EDTA and were then lysed in 1 ml of 10 mM HCl containing 0.1% SDS. Two aliquots of lysates (100 μl each) were removed for measurements of protein content by the method of Lowry et al. [30]. To the remaining samples (0.8 ml), 0.1 ml of 0.2 M sodium acetate buffer, pH 13.0, containing 10 mM L-citrulline was added. The samples were then applied to a column of Dowex AG50WX 8 (H^+ form). The effluents, which contained L-[3H]citrulline, were collected in scintillation vials and subjected to scintillation spectrometry. The results are expressed as the ratio of CAT-1-GFP vs GFP. Bars represent means ± SEM. All experiments were repeated at least three times in triplicate wells. Statistical analysis was performed using paired Student *t* test. **, $p < 0.01$.

2.5. Confocal microscopy of paraformaldehyde-fixed cells

In order to analyze the subcellular localization of CAT-1, ECs grown on coverslips were observed using laser scanning confocal microscopy (Zeiss LSM 510) as reported previously [27,31] at 48 h after infection with the CAT-1-GFP construct (ad-CAT-1-GFP) or control ad-GFP. Cells were washed, fixed with 4% paraformaldehyde. The fluorescence at the midsection of cells was demonstrated. Original magnification 40×, section 1.2 μm.

To further confirm the cell–cell junctions localization of CAT-1, after ad-CAT-1-GFP-infected cells were grown on glass coverslips precoated with gelatin (1%), cells were washed with PBS and fixed in 4% paraformaldehyde for 15 min, blocked with 1% bovine serum albumin in PBS for 30 min, and incubated with VE-cadherin antibody (1:50; abcam) overnight at 4 °C. Images were captured with PerkinElmer Ultraview RS-3 spinning disk confocal microscope (Nikon ECLIPSE TE2000). Original magnification 40×, section 0.4 μm.

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