T-Cadherin Modulates Endothelial Barrier Function

ALEXANDRA V. ANDREEVA,1* JINGYAN HAN,1 MIKHAIL A. KUTUZOV,1
JASMINA PROFIROVIC,1 ISEVOLOD A. TKACHUK,2
AND TATYANA A. VOYNO-YASENETSKAIA1*

1Department of Pharmacology, University of Illinois at Chicago, Chicago, Illinois
2Cardiology Research Center, Moscow, Russia

T-cadherin is an atypical member of the cadherin family, which lacks the transmembrane and intracellular domains and is attached to the plasma membrane via a glycosylphosphatidylinositol anchor. Unlike canonical cadherins, it is believed to function primarily as a signaling molecule. T-cadherin is highly expressed in endothelium. Using transendothelial electrical resistance measurements and siRNA-mediated depletion of T-cadherin in human umbilical vein endothelial cells, we examined its involvement in regulation of endothelial barrier. We found that in resting confluent monolayers adjusted either to 1% or 10% serum, T-cadherin depletion modestly, but consistently reduced transendothelial resistance. This was accompanied by increased phosphorylation of Akt and LIM kinase, reduced phosphorylation of p38 MAP kinase, but no difference in tubulin acetylation and in phosphorylation of an actin filament severing protein cofilin and myosin light chain kinase. Serum stimulation elicited a biphasic increase in resistance with peaks at 0.5 and 4–5 h, which was suppressed by a PI3 kinase/ Akt inhibitor wortmannin and a p38 inhibitor SB 239063. T-cadherin depletion increased transendothelial resistance between the two peaks and reduced the amplitude of the second peak. T-cadherin depletion abrogated serum-induced Akt phosphorylation at Thr308 and reduced phosphorylation at Ser473, reduced phosphorylation of cofilin, and accelerated tubulin deacetylation. Adiponectin slightly peaks and reduced the amplitude of the second peak. T-cadherin depletion abrogated serum-induced Akt phosphorylation at Thr308 and reduced phosphorylation at Ser473, reduced phosphorylation of cofilin, and accelerated tubulin deacetylation. Adiponectin slightly improved transendothelial resistance irrespectively of T-cadherin depletion. T-cadherin depletion also resulted in a reduced sensitivity and delayed responses to thrombin. These data implicate T-cadherin in regulation of endothelial barrier function, and suggest a complex signaling network that links T-cadherin and regulation of barrier function.


Additional Supporting Information may be found in the online version of this article.

Jingyan Han’s present address is Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA.

*Correspondence to: Alexandra V. Andreeva and Tatyana A. Voyno-Yasenetskaya, Department of Pharmacology (MC 868), University of Illinois at Chicago, 909 S. Wolcott Ave. CoMRB, Chicago, IL 60612. E-mail: aandreev@uic.edu; tvy@uic.edu

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Malik, 2006). Overexpression of T-cadherin in resting cells does not affect actin cytoskeleton, adhesion or migration, unless recombinant T-cadherin or T-cadherin-specific antibody are added to the substrate (but not to the medium) (Ivanov et al., 2004), which activates the Rho-ROCK pathway (necessary for actin stress fiber formation) and Rac1 (which promotes actin-rich lamellipodia) (Phillipova et al., 2005). T-cadherin has been suggested to play a role in angiogenesis (Phillipova et al., 2006; Rubina et al., 2007; Hebbard et al., 2008).

The considerations outlined above prompted us to investigate the role of endogenous T-cadherin in regulation of transendothelial resistance in EC monolayers adjusted to low (1%) or high (10%) serum, as well as dynamic changes in resistance upon stimulation with serum. In parallel, we examined the responses of individual signal transduction molecules, such as kinases (Akt, p38, LIMK), coflin, myosin light chain (MLC)-2 and acetylated tubulin that might underlie the effects on transendothelial resistance.

Materials and Methods
Antibodies and reagents

The following antibodies were used: Akt, Akt-phospho-Ser473, α-tubulin and VE-cadherin (Santa Cruz Biotechnology (SCBT), Santa Cruz, CA); Akt-phospho-Thr308 (Signalway Antibody, Pearland, TX); acetylated tubulin (M2, Sigma, St. Louis, MO); coflin, phospho-cofilin, LIMK1, phospho-LIMK, MLIC2, phospho-MLC2, p38, phospho-p38, and PTEN (Cell Signaling Technology, Beverly, MA). The affinity-purified polyclonal T-cadherin antibody was described previously (Stambolsky et al., 1999). Anti-mouse and anti-rabbit antibodies conjugated with horseradish peroxidase were from Amersham Pharmacia Biotech (Little Chalfont, England). Recombinant HEK-derived human adiponectin (Acrp30 HEK Human) and thrombin were purchased from ProSpec (South Bend, IN), respectively. SB 239063 and wortmannin were from Sigma and were dissolved in DMSO.

Cell culture and small interfering RNA (siRNA) transfection

Human umbilical vein endothelial cells (HUVECs), were purchased from Lonza (Walkersville, MD) and cultured for up to five passages in EGM-2 BulletKit. Inhibition of T-cadherin expression was performed using siRNA designed by Dharmaco (Lafayette, CO). Cells were transfected using siRNA transfection reagent (SCBT). Control siRNA was purchased from SCBT. The final siRNA concentration was 20 nM.

Transendothelial electrical resistance (TER) measurements

The electrical resistance across the EC monolayer was measured using an electric cell substrate impedance sensor (ECIS, Applied BioPhysics, Inc., Troy, NY) as described previously (Tiruppathi et al., 1992). HUVECs were grown on the small gold electrode until they reached confluence. The electrodes were connected to a phase-sensitive lock-in amplifier. A constant current of 1 μA was applied between the small electrodes and the large counter electrode using a 1–V, 4,000-Hz ac signal supplied through a 1-MΩ resistor. The voltage changes between small electrodes and large electrode were recorded every 5 min over the period indicated. The data are presented as actual resistance values or as the change in the in-phase voltage normalized to its initial value (Tiruppathi et al., 1992).

Electrophoresis and immunoblotting

Cells were washed twice with ice-cold phosphate-buffered saline, resuspended in 20 mM sodium phosphate (pH 7.5), 25 mM NaF, 1 mM Na-orthovanadate, 5 mM EDTA, lysed by addition of a standard Laemmli SDS buffer, and boiled for 5 min. Cell lysates were separated on 5–20% gradient SDS–polyacrylamide gels and proteins transferred onto Protran nitrocellulose membrane (Millipore, Bedford, MA). Membranes were probed with appropriate antibodies and developed using SuperSignal ECL reagents (Pierce, Rockford, IL). Densitometry of protein bands was performed on scanned images using ImageJ program (National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/). To determine the levels of protein phosphorylation, membranes were probed with phospho-specific antibodies and then reprobed with antibodies recognizing respective proteins independently of their phosphorylation state. Phosphorylation data were normalized to total content of respective proteins.

Results
T-cadherin differentially regulates endothelial barrier function in resting and disturbed HUVECs

T-cadherin exists as two distinct forms with apparent molecular masses of 105 and 130 kDa, which are thought to correspond to mature T-cadherin and to unprocessed precursor, respectively (Stambolsky et al., 1999). Both T-cadherin forms could be efficiently depleted in primary cultures of human ECs using siRNA, with 130 kDa band disappearing first in the course of downregulation (Andreeva et al., 2009). In some experiments (Supplementary Fig. 1A), a faint 130 kDa band was present, while the 105 kDa form was almost undetectable, which may indicate reappearance of newly synthesized T-cadherin. Expression of VE-cadherin was not affected at 80–90% T-cadherin depletion (Supplementary Fig. 1A), although variations in VE-cadherin levels could be detected at 98–99% T-cadherin depletion (data not shown).

T-cadherin depletion in HUVECs resulted in a moderate yet reproducible decrease in transendothelial resistance in unstimulated monolayers adjusted to either 1% or 10% serum (Fig. 1A; cells were considered adjusted after >15 h incubation in either low or high serum). Serum contains a variety of factors (growth factors and hormones) that may affect endothelial resistance. Since it is largely unknown which of those factors may signal through or be modulated by T-cadherin, we examined a possible dynamic role of endogenous T-cadherin in responses of HUVEC monolayers to stimulation with whole serum, considering these studies as the first step towards a more detailed analysis of individual components present in serum.

Addition of 10% serum to the cells adjusted to 1% serum induced a biphasic increase in endothelial resistance both in the control and in T-cadherin-depleted cells (Fig. 1B). The first sharp increase in resistance, which peaked ~0.5 h after stimulation and lasted for 1 h, was followed by a second phase, which peaked at 4–5 h and lasted 6–7 h or longer (Fig. 1B). T-cadherin depletion affected the time course of the response and the ratio between the two peaks: the descending shoulder of the first peak was considerably steeper (Fig. 1B) and the ratio between the amplitudes of the second and the first peaks was higher (Fig. 1C) in the control cells as compared to T-cadherin-depleted cells. The above observations indicate that T-cadherin differentially regulates endothelial resistance in resting and serum-stimulated HUVECs, decreasing or enhancing the barrier, respectively.

T-cadherin differentially modulates signaling events in resting and serum-stimulated HUVECs

As the first step towards dissecting the signaling that may link T-cadherin with regulation of endothelial barrier, we explored the effects of T-cadherin depletion on Akt and p38 activation, as well as other signaling molecules and events known to affect transendothelial resistance and/or reported to occur downstream of T-cadherin, including LIMK1, components
affecting acto-myosin system (cofilin and MLC), and the level of tubulin acetylation.

**Akt.** phosphorylation at both Thr308 and Ser473 was increased in T-cadherin-depleted resting HUVECs (Fig. 2A,B). Serum stimulation induced a sharp decrease in Akt phosphorylation (Fig. 2C–E), the timing of which was similar to the timing of the first peak in transendothelial resistance (compare Figs. 2D,E and 1B). This phosphorylation peak was attenuated in T-cadherin-depleted cells. The effect of T-cadherin depletion was especially striking for Thr308: its phosphorylation (Fig. 2C–D, E) and transient phosphorylation of Ser473 in response to serum was reduced by ~50% in T-cadherin-depleted cells.

Since interference with function of other cadherins may affect the levels of PTEN (Kotelevets et al., 2001; Fournier et al., 2009), and PTEN is a well-known negative regulator of Akt (Cantley and Neel, 1999), we examined whether PTEN levels would be affected by T-cadherin depletion. However, we found no changes in PTEN levels in T-cadherin-depleted as compared with control cells (Supplementary Fig. 1B).

Addition of wortmannin (a PI3 kinase/Akt inhibitor) abrogated response of HUVECs to serum stimulation (Supplementary Fig. 2), indicating that these kinases are involved in serum-induced increase in transendothelial resistance.

**p38.** In resting T-cadherin-depleted cells adjusted to low serum, p38 phosphorylation was slightly decreased (Fig. 3A,B), in line with an increased Akt activity. Similar tendency was observed in cells adjusted to high serum, although this case p38 phosphorylation in T-cadherin-depleted cells was more variable, and the difference was not statistically significant (Fig. 3B).

Serum stimulation induced a biphasic increase in p38 phosphorylation (Fig. 3C,D). The first peak (which correlated in time with the first peak of endothelial resistance, see Fig. 1B) was not significantly affected by the absence of T-cadherin, while the smaller second peak (corresponding in time to the descending shoulder of the first peak of endothelial resistance, see Fig. 1B) was typically reduced in T-cadherin-depleted cells (in three out of four experiments). These data are consistent with p38 being under control of T-cadherin-regulated Akt activity at least in resting cells.

Addition of a p38 inhibitor SB 239063 inhibited response of HUVECs to serum stimulation (Supplementary Fig. 2A, B), suggesting that p38 may be involved in serum-induced increase in transendothelial resistance.

**LIM kinase.** LIMK is present as the major 70 kDa form and a minor poorly characterized 50 kDa form (Foletta et al., 2004), both of which are phosphorylated (Fig. 4A). In T-cadherin-depleted resting cells adjusted to high or low serum, 70 kDa LIMK form phosphorylation was increased, while 50 kDa form was not significantly affected (Fig. 4A,B).

Serum stimulation resulted in a slight (almost within experimental error) transient increase in LIMK (70 kDa) phosphorylation in control cells, while in T-cadherin-depleted cells a decrease in LIMK phosphorylation occurred with a similar time course (Fig. 4C,D). A qualitatively similar pattern was observed for the 50 kDa form (Fig. 4C,E). In both cases, the amplitudes of serum-induced changes in LIMK phosphorylation were small.

**Cofilin.** LIMK1 phosphorylates and thus inactivates cofilin and promotes actin polymerization (Arber et al., 1998; Bernard, 2007). In resting cells adjusted to either high or low serum, there was no detectable difference in cofilin phosphorylation, irrespectively of the presence of T-cadherin (Fig. 5A; data not shown). Serum stimulation resulted in a rapid (though moderate: up to 1.5-fold) cofilin phosphorylation, which was blunted in T-cadherin-depleted cells.

**Myosin light chain (MLC).** No significant difference could be detected in the level of phospho-MLC-2 between control cells and T-cadherin-depleted resting cells cultured in high or low serum (Fig. 5A,C and data not shown). Upon serum stimulation, phospho-MLC-2 content increased and peaked at ~1 h. The time course of MLC-2 phosphorylation and dephosphorylation was similar in T-cadherin-depleted cells and in the control cells (Fig. 5A,C).

**Microtubule stability.** Both LIMK1 and p38 affect the level of tubulin polymerization. We assessed the extent of tubulin polymerization as a ratio of acetylated (i.e., stable)
tubulin to total α-tubulin. No significant difference could be detected between resting control and T-cadherin-depleted cells cultured in high or low serum (Fig. 6 and data not shown).

Upon serum stimulation, the levels of acetylated tubulin similarly and rapidly increased (∼3.5- to 5-fold) both in the control and T-cadherin-depleted cells. This initial increase was followed by a rapid partial deacetylation, notably accelerated in T-cadherin-depleted cells (Fig. 6), which was followed by a slower deacetylation between 1 and 4 h. These data suggest that T-cadherin may contribute to stabilization of polymerized tubulin upon serum stimulation.

Effects of adiponectin

The experiments described above assessed possible signaling events that might link T-cadherin with regulation of transendothelial resistance in response to serum. Adiponectin might be one of serum components potentially active in this respect, since it is (1) known to enhance endothelial barrier (measured by fluorescein-conjugated dextran transit (Elbatarny et al., 2007) or transendothelial electric resistance in the presence of angiotensin II (Xu et al., 2008)), and (2) T-cadherin was reported to be a novel receptor for adiponectin (Hug et al., 2004).

We examined whether the effect of adiponectin would be reduced or abolished in T-cadherin-depleted cells. In resting HUVECs adjusted to either low (data not shown) or high serum (Fig. 7A), addition of adiponectin resulted in a slight (up to 10%) increase in resistance both in control cells and in T-cadherin-depleted cells. This difference was statistically significant in T-cadherin-depleted cells, suggesting that this effect of adiponectin is T-cadherin independent.

We also assessed the effect of adiponectin under conditions of barrier function challenged by thrombin. Thrombin elicited a temporary drop in endothelial resistance (Fig. 7B,C). The amplitude of these responses increased with increasing concentrations (0.1–10 nM) of thrombin (Fig. 7B). In high serum, T-cadherin depletion decreased sensitivity to thrombin at concentrations below 10 nM (Fig. 7B,C) and delayed the peak of thrombin response (Fig. 7C,D). These effects were similar in the absence and in the presence of adiponectin (Fig. 7C,D). No effects of T-cadherin depletion on thrombin responses could be detected in low serum (data not shown).

Discussion

In this work, we assessed the effect of T-cadherin on transendothelial resistance as measured using electric cell substrate impedance approach, and followed dynamic changes in signaling events that might be affected by T-cadherin. Since T-cadherin expression level in ECs relative to total protein is already at least one order of magnitude higher than in several other cell lines tested (PC12, NIH 3T3, COS-7, and HEK 293A; our unpublished observations), we employed siRNA-mediated
Fig. 3. Effect of T-cadherin depletion on p38 phosphorylation in resting and serum-stimulated HUVECs. Cell lysates from the experiment shown in Figure 2 were analyzed for phosphorylated and total p38. p38 phosphorylation levels were normalized to total p38 content in respective samples. A,B: Effect of T-cadherin depletion on p38 phosphorylation in HUVECs adjusted to 1% or 10% serum. Data are normalized to control siRNA in 1% serum, and are means of four experiments (error bars, SD; *P < 0.05 in a Student’s test). C,D: Effect of T-cadherin depletion on the time course of serum-induced p38 phosphorylation and dephosphorylation in the samples shown in Figure 2C. The data shown are means from four experiments ±SD.

Fig. 4. Effect of T-cadherin depletion on phosphorylation of LIM kinase (LIMK) in resting and serum-stimulated HUVECs. Cell lysates from the experiment shown in Figure 2 were analyzed for total and phosphorylated LIM kinase. Phosphorylation levels of the 50 and 70 kDa LIMK forms were normalized to their total content in respective samples. A,B: Effect of T-cadherin depletion on LIMK phosphorylation in HUVECs adjusted to 1% or 10% serum. Data are normalized to control siRNA in 1% serum, and are means of three experiments (error bars, SD; **P < 0.01 in a Student’s test). C–E: Effect of T-cadherin depletion on the time course of serum-induced LIMK phosphorylation and dephosphorylation. The data shown in (D) and (E) (70 and 50 kDa LIMK forms, respectively) are normalized to phosphorylation levels in the control cells before serum stimulation, and are means from three experiments ±SD.
knockdown of endogenous T-cadherin. siRNA-mediated depletion of T-cadherin in ECs proved to be efficient and specific, since expression of VE-cadherin was unaffected by 80–90% depletion of T-cadherin. A decreased expression of VE-cadherin associated with 98–99% T-cadherin depletion is likely due to a well-known ability of cadherins to influence expression of each other (see the Discussion Section and references in Joshi et al., 2009). In comparison with some other stimuli that affect endothelial permeability (e.g., thrombin), serum is far less explored, yet in the case of T-cadherin is most relevant, since T-cadherin function seems to be sensitive to some serum components (Niermann et al., 2000; Hug et al., 2004).

Resting T-cadherin-depleted cells adjusted to low or high serum showed a modest but reproducible decrease in transendothelial resistance compared to cells transfected with the control siRNA. Similar levels of tubulin acetylation in T-cadherin-depleted and in control cells indicate that regulation of microtubule stability is not involved in this effect. Although the increased LIMK activity would be consistent with the reduced transendothelial resistance, the absence of any changes in cofilin phosphorylation indicates that the LIMK–cofilin–actin axis is also unlikely to be a mechanism underlying reduced transendothelial resistance in the resting HUVECs. This is in line with a report that alterations in T-cadherin expression per se have no effect on actin cytoskeleton (Ivanov et al., 2004).

Serum stimulation elicited responses with a clearly distinct shape in control and in T-cadherin-depleted cells. We followed serum-induced signaling events that correlate in time with the

**Fig. 5.** Effect of T-cadherin depletion on serum-induced phosphorylation of cofilin and myosin light chain (MLC) in HUVECs. Cell lysates from the experiment shown in Figure 2 were analyzed for phosphorylated and total cofilin (A,B) and MLC (A,C). Phosphorylation levels of cofilin and MLC were normalized to their total content in respective samples. The data shown in (B) and (C) (cofilin and MLC, respectively) are normalized to phosphorylation levels of respective proteins in the control cells before serum stimulation, and are means from three experiments ±SD.

**Fig. 6.** Effect of T-cadherin depletion on serum-induced tubulin acetylation in HUVECs. Cell lysates from the experiment shown in Figure 2 were analyzed for acetylated and total α-tubulin (A). Tubulin acetylation levels were normalized to total α-tubulin content in respective samples. The data shown in (B) are normalized to tubulin acetylation levels in the control cells before serum stimulation, and are means from three experiments ±SD.
first sharp peak in resistance (which is similar irrespectively of T-cadherin expression) and with its descending shoulder (where endothelial resistance in T-cadherin-depleted cells was elevated). All parameters examined (except MLC-2 phosphorylation) showed a time course reminiscent of that of transendothelial resistance, that is, a sharp peak within 1 h after stimulation, followed by a shallower phase.

Suppression of serum-induced transendothelial resistance response by PI3K/Akt and p38 inhibitors suggests the involvement of these kinases. One of the strongest effects of T-cadherin depletion observed in response to serum was suppression of Akt phosphorylation at Thr308 (i.e., in the activation loop), followed by lagging phosphorylation in the following hours, while Akt Thr308 was rapidly dephosphorylated in the control. Persisting Akt activity is consistent with the observed increased resistance, since Akt has been reported to positively regulate endothelial barrier (Dossumbekova et al., 2008; Singleton et al., 2009). We observed p38 activation in response to serum, similar to reported earlier p38 activation in response to HGF (Liu et al., 2002). p38 may have dual effects on transendothelial resistance (see the first section). Reduced p38 phosphorylation in T-cadherin-depleted cells in the second peak is in line with both increased Akt activity and increased endothelial resistance within a similar time interval. This would be consistent with p38 exerting a negative effect on endothelial barrier and being itself negatively regulated by Akt.

We also examined serum-induced changes in phosphorylation state of three proteins expected to affect the acto-myosin complex: MLC, LIMK, and coflin. Although
T-cadherin depletion appeared to affect serum-induced LIMK activation and phosphorylation of cofillin (but not MLC), the amplitude of these responses was small, suggesting that LIMK–cofilin pathway may not play a major role in regulation of cytoskeleton upon stimulation with serum.

Microtubule polymerization is thought to enhance barrier function in ECs (Verin et al., 2001; Birukova et al., 2004; Bogstcheva et al., 2007; Sehrawat et al., 2008). Indeed, serum stimulation resulted in a more than fourfold increase in tubulin acetylation (i.e., stabilization), in line with the data published for fibroblasts (Warren et al., 2006). However, the following decrease in tubulin acetylation was accelerated in T-cadherin-depleted cells, while endothelial resistance remained higher than in control, which suggests that a decrease in microtubule stability does not result in a reduction in monolayer resistance under these conditions. Taken together, our data are consistent with Akt and perhaps p38 acting downstream of T-cadherin in regulation of transendothelial resistance upon serum stimulation.

An interesting observation in its own right is that a similar initial rate of tubulin acetylation upon serum stimulation is followed by a sharp decrease in acetylation in T-cadherin-depleted cells, which is suggestive of higher activity of tubulin desacetylase in the absence of T-cadherin. Microtubules may serve as a substrate for HDAC6, a member of the histone deactylase family, which can function as a tubulin deacteylate (Hubbert et al., 2002; Palazzo et al., 2004). It would be interesting to see whether HDAC6 activity may be regulated in a T-cadherin-dependent manner.

Adiponection is one of the most obvious serum components that might be relevant to the observed effects, since it (1) may be responsible for increased monolayer resistance and (2) may act as a T-cadherin ligand. Yet, our data point out that in our experimental system the effect of adiponectin on endothelial barrier is likely unrelated to its ability to bind T-cadherin. It should be noted that two other adiponectin receptors may exert distinct and even opposite effects as compared to T-cadherin (Lee et al., 2008). Since T-cadherin knockout mice have high level of adiponectin in the serum (Hebbard et al., 2008), it seems possible that the main function of T-cadherin as adiponectin receptor is to regulate adiponectin synthesis or turnover.

We also found that upon challenge with thrombin (10 nM) in the presence of serum T-cadherin depletion resulted in a decreased sensitivity and delayed the peak of thrombin response. The requirement of high serum for this effect suggests that T-cadherin may act to enhance an action of some serum component that sensitizes signaling downstream of thrombin.

Our current findings indicate that endogenous T-cadherin enhances barrier in resting confluent HUVEC monolayers, but reduces transendothelial resistance in response to at least two different stimuli (serum and thrombin), and thus suggest a T-cadherin role in regulation of endothelial barrier function.


