Chapter 5

Endothelial barrier disruption: A role for AMPK

Authors
Jurjan Aman, Jan van Bezu, Etto C. Eringa, Victor W.M. van Hinsbergh, Geerten P. van Nieuw Amerongen.

Preliminary data
ABSTRACT

Introduction Adenosine monophosphate activated protein kinase (AMPK) is a key regulator of energy metabolism involved in many endothelial functions, amongst others. The contribution of AMPK to endothelial barrier regulation, however, remains incompletely understood. Studies after the role of AMPK in endothelial barrier regulation have yielded paradoxical results. Many tools are available to modify AMPK activity, which yields AMPK a suitable target for treatment of vascular leakage. As AMPK was previously described to inhibit RhoA, we hypothesized that AMPK protects against barrier dysfunction during endothelial stimulation with barrier-disruptive agents.

Methods and Results Electrical cell-substrate impedance sensing and macromolecule passage were used to evaluate the effect of AMPK inhibition (Compound C) and activation (AICAR, 2mM) on endothelial barrier function. Under non-stimulated conditions Compound C dose-dependently enhanced endothelial electrical resistance (36% increase at 10µM). AICAR slightly decreased endothelial resistance (8% decrease at 2mM). Compound C attenuated endothelial barrier disruption by thrombin (69% reduction of thrombin response), while AICAR enhanced the thrombin response (34% increase in thrombin response). Similar results were obtained during macromolecule passage. Endothelial stimulation with thrombin or vascular endothelial growth factor resulted in transient AMPK activation as measured by phosphorylation of AMPK and its downstream target Acetyl CoA Carboxylase. As Compound C did not affect RhoA or Rho kinase activity and had additive effects to inhibition of Rho kinase, AMPK mediates endothelial barrier disruption independently of the RhoA/Rho kinase pathway.

Conclusion In contrast to our hypothesis, this study strongly suggests involvement of AMPK in disruption of the endothelial barrier, independent of the RhoA/Rho kinase pathway. Although AMPK may form a suitable target for treatment of vascular leakage, additional experiments are required to elucidate how AMPK contributes to endothelial barrier disruption.
INTRODUCTION

In mammalian cells, the adenosine monophosphate activated protein kinase (AMPK) serves as a sensor of the cell’s energy state. It is activated in catabolic conditions like hypoxia and hypoglycemia as a direct result from an increased adenosine monophosphate / adenosine triphosphate (AMP/ATP) ratio. AMPK is widely expressed in the endothelium, although expression level and subunit isotype expression show large heterogeneity throughout the endothelium. Evaluation of the role of AMPK in endothelial barrier regulation has yielded paradoxical results. Since various tools are available to modulate (both activate and inhibit) AMPK activity, AMPK may form a suitable target for treatment of vascular leakage. However, the exact role of AMPK in endothelial barrier regulation are currently unknown.

The AMPK consists of 3 subunits, the catalytic α-subunit and the regulatory β-, and γ-subunit [1]. There are two α-subunit isoforms, the α1- and the α2. In the endothelium, expression of the α1-subunit is predominant [2]. Yet, endothelial cell phenotype may determine α-subunit expression, as one study demonstrated that in the lung vasculature the α1-subunit is predominantly expressed in the capillary-derived microvascular endothelial cells, and the α2-subunit is predominantly expressed in pulmonary conduit vessels [3]. Thus far, endothelial AMPK has been described to play a role in glucose uptake, cell metabolism, endothelium-dependent vasorelaxation, angiogenesis, inflammation and many others [1].

Studies have indicated a role for AMPK in acute lung injury (ALI) and sepsis. The exact role, however, remains unclear, because there is evidence for both a contributing and a protecting role of AMPK in acute lung injury. On the one hand, genetic depletion of the AMPKα1 subunit attenuated ALI development in mice [4], on the other hand AMPK activation by AICAR [3,5] or metformin [6] reduced severity of ALI [5] and mortality from sepsis [6], respectively. The targeting of a specific AMPK subunit (AMPKα1) versus AMPK in general, and the tissue-specific expression pattern of AMPK may have contributed to the paradoxical results. The association of AMPK with the inflammatory response was demonstrated in endothelial cells stimulated with vasoactive agents. Stimulation of endothelial cells with thrombin [7], bradykinin [8] or vascular endothelial growth factor (VEGF) [9] resulted in fast activation of AMPK.

Vasoactive agents stimulate AMPK independent of the energy status of the cell (AMP/ATP ratio) [1], via the AMPK kinases calmodulin-dependent protein kinase β and LKB1 [7]. It remains unclear whether AMPK activity (during stimulation with barrier-disruptive agents) mediates endothelial barrier disruption or forms a defensive mechanism that contributes to barrier restoration in a later phase. Among the many downstream targets of AMPK RhoA is of particular interest for vascular leakage. Recent smooth muscle cell studies demonstrated that AMPK inhibits RhoA,
either directly via inhibitory phosphorylation of RhoA [10], or indirectly via activation of the RhoA-GAP p190RhoGAP [11]. Because RhoA is a strong mediator of endothelial barrier disruption, we hypothesized that activation of AMPK attenuates endothelial barrier disruption by inhibition or deactivation of the RhoA/Rho kinase pathway. The current study demonstrates that, in contrast to our hypothesis, pharmacological inhibition of AMPK enhances endothelial barrier function under non-stimulated conditions, and strongly attenuates barrier disruption by the barrier-disruptive agent thrombin. AMPK stimulation with AICAR enhanced endothelial barrier disruption. Endothelial stimulation with thrombin and VEGF resulted in fast activation of AMPK. The contribution of AMPK to endothelial barrier disruption did not involve the RhoA/Rho kinase pathway.

**METHODS**

**Reagents and Materials**

Endothelial cells were stimulated with thrombin (Sigma Aldrich, Zwijndrecht, The Netherlands) or VEGF (Invitrogen, Camarillo, CA). The Rho kinase inhibitor Y27632 was purchased from Tocris Cookson Ltd (London, United Kingdom) and the AMPK inhibitor Compound C from Calbiochem/Merck (Darmstadt, Germany). For AMPK activation AICAR (Calbiochem/Merck, Darmstadt, Germany) or adiponectin (Enzo Life Sciences, Farmingdale, NY) was used. Pharmacological inhibitors were dissolved in dimethylsulfoxide (DMSO, Sigma Aldrich).

The following antibodies were used: anti-phosphoThr172 AMPK (Cell Signaling Technologies, Danvers, MA), anti-phosphoSer79 Acetyl CoA Carboxylase (Cell Signaling Technologies), anti-phosphoThr567ezrin / phosphoThr564radixin / phosphoThr558moesin (Cell Signaling Technologies) and anti-Ezrin/Radixin/Moezin (Cell Signaling Technologies).

**Endothelial Cell Culture**

For isolation of primary human umbilical vein endothelial cells, umbilical cords were obtained from the Department of Obstetry of the Amstelland Ziekenhuis (Amstelveen, The Netherlands). The Medical Ethical Committee of the VU University Medical Center approved the use of human tissue. Endothelial cells were isolated as described previously, and were extensively characterized for the presence of endothelial markers and the absence of epithelial and smooth muscle cell markers [12]. After isolation, cells were resuspended and cultured in M199 (Lonza, Verviers, Belgium), supplemented with penicilline 100U/mL and streptomycin 100μg/mL (Biowhittaker/Lonza), heat inactivated human serum 10% (Sanquin Blood Supply, Amsterdam, The Netherlands),
heat inactivated new-born calf serum 10% (Gibco, Grand Island, NY), crude endothelial cell growth factor 150µg/mL (prepared from bovine brains), L-glutamine 2mmol/L (Biowhittaker/Lonza), and heparin 5U/mL (Leo Pharmaceutical Products, Weesp, The Netherlands). Cells were cultured at 37°C and 5% CO₂, with a change of culture medium every other day.

**Endothelial Barrier Assays**

Endothelial barrier function was evaluated with electrical cell-substrate impedance sensing (ECIS) and with macromolecule passage. For ECIS measurements, confluent endothelial cells were seeded (0.63 x10⁵ cells/cm²) on gelatin-coated ECIS arrays, containing 8 wells/array and 10 electrodes/well (8W10E, Applied Biophysics, Troy, NY). Cells were grown to confluence in 48-72h. For experiments, cells were washed with M199 containing 1% human serum albumin (HSA, Sanquin Blood Supply) and pretreated with designated agents (diluted in 1%HSA/M199) for 60min. After 60min of pretreatment, thrombin was added to the wells for a final concentration of 1U/mL. The endothelial resistance was measured at 4000Hz, which best reflects endothelial barrier function [13]. For quantification of the thrombin response, the resistance was normalized to the initial resistance value just before addition of the thrombin. The area between the initial resistance value (y = 1.0 due to normalization) and the resistance curve was calculated, since it reflects the total loss of barrier function throughout the whole thrombin response.

For measurement of macromolecule passage, endothelial cells were seeded in 1:1 density on gelatin- and fibronectin-coated Costar polycarbonate filters, pore-size 3.0µm (Corning, Lowell, MA), and grown to confluence in 5 days with change of culture medium every other day. For experiments, cells were washed with 1%HSA/M199 and pretreated with designated agents (diluted in 1%HSA/M199). After 60min of pretreatment, medium was changed to 1%HSA/M199 containing designated agents, horse radish peroxidase (HRP) 5µg/mL (Sigma Aldrich, Zwijndrecht, The Netherlands) and thrombin 1U/mL (Sigma Aldrich). 1%HSA/M199 was added to the lower compartment. Samples were taken from the lower compartment for measurement of the HRP concentration. HRP was detected by chemoluminscence upon addition of TMB/E (Upstate/Millipore, Temecula, CA).

**Protein analysis**

Endothelial cells were grown to confluence in 5cm² culture wells. For experiments, cells were washed with 1%HSA/M199 and pretreated with 1%HSA/M199, 0.1% DMSO or Compound C. After 60min of pretreatment, cells were stimulated with thrombin 1U/mL or adiponectin (1µg/mL). At indicated intervals cells were washed with ice-cold phosphate-buffered saline (PBS), and lysed in lysisbuffer containing phosphatase (PhosStop, Roche Applied Sciences, Basel, Switzerland).
and protease inhibitors (Complete, Roche Applied Sciences). Cell lysates (20μg protein/condition) were loaded on SDS-PAGE gels, electrophoresed and transferred to nitrocellulose membranes. Membranes were blocked, and the protein content was analysed by incubation with antibodies against phosphoThr172 AMPK (1:1000), phosphoSer79 Acetyl CoA Carboxyl (1:1000), phosphoThr567ezrin/ phosphoThr564radixin / phosphoThr558moesin (1:1000) and ezrin/radixin/moesin (1:1000).

**RhoA activity assays**

Endothelial activity of RhoA was measured with a RhoA G-LISA Activity Assay kit (Cytoskeleton, Denver, CO). Cells were grown to confluence in 5cm² culture wells. For experiments, cells were washed with 1%HSA/M199 and pretreated with 0.1% DMSO or 10μM Compound C dissolved in 1%HSA/M199 for 60min. Subsequently thrombin was added to the cells for a final concentration of 1U/mL. At indicated intervals cells were washed with ice-cold PBS and lysed with lysis buffer provided by the manufacturer. Cell lysates were centrifuged, and the supernatant was snap frozen in liquid nitrogen. RhoA activity was measured with G-LISA, according to the manufacturers protocol.

**Statistics**

All experiments were performed with human umbilical vein endothelial cells. Data represent mean (range), N refers to the number of measurements, unless indicated otherwise.

**RESULTS**

**Inhibition of AMPK with Compound C enhances endothelial barrier function**

To study the role of AMPK in endothelial barrier regulation, human umbilical vein endothelial cells were treated with increasing concentrations of the AMPK-inhibitor Compound C. In monolayers of resting endothelial cells, Compound C dose-dependently enhanced the electrical resistance, as a measure of endothelial barrier function (Figure 1A). Optimal barrier enhancement was reached with 3 to 10μM Compound C (Figure 1A). Increasing the Compound C concentration to 30μM severely impaired endothelial resistance (data not shown), suggesting toxicity at Compound C levels above 10μM. Compound C strongly attenuates thrombin-induced endothelial barrier disruption (Figure 1B). These barrier-protective effects also were optimal at concentrations of 3-10μM (Figure 1B,C).
To further elucidate the contribution of AMPK to endothelial barrier disruption, cells were treated with the pharmacological AMPK activator, AICAR, with Compound C 10μM or the combination. AICAR did not affect the electrical resistance of resting endothelial monolayers (Figure 1D), but it enhanced the thrombin-induced barrier disruption (Figure 1E, Supplementary Figure 1A), in particular during the recovery phase (Supplementary Figure 1A). In contrast, the thrombin-induced barrier dysfunction was attenuated in Compound C pretreated cells. In cells pretreated with both AICAR and Compound C, the AMPK inhibiting effect of Compound C balanced the AMPK activating effect of AICAR (Figure 1E). However, continuous resistance measurements demonstrated that combined pretreatment with AICAR and Compound C attenuates the initial drop (0-1h) in resistance (comparable to Compound C pretreatment alone), but perturbs barrier recovery at a later phase (1-3h), comparable to AICAR pretreatment alone (Supplementary Figure 1A). In our hands, the physiological AMPK activator adiponectin (1μg/mL) barely affected thrombin-induced endothelial barrier disruption (Supplementary Figure 1B).

Compound C, AICAR or the combination had no effect on the macromolecule passage over resting endothelial monolayers (Figure 1F). However, during thrombin stimulation Compound C strongly attenuated the thrombin-induced macromolecule passage, while AICAR tended to enhance the thrombin-induced macromolecule passage. In line with the resistance measurements, the combination of AICAR and Compound C also attenuated thrombin-induced macromolecule passage at the initial phase (30min thrombin) (Figure 1F).

Altogether, these data indicate that in human umbilical vein endothelial cells, AMPK mediates disruption of the endothelial barrier by thrombin. Inhibition of AMPK at the initial phase of the thrombin response (0-1h) largely prevented the thrombin-induced drop in endothelial resistance. Activation of AMPK slightly enhanced the thrombin-induced drop in resistance, and severely impairs restoration.

**AMPK is activated during endothelial stimulation with barrier-disruptive agents**

Next, we investigated AMPK activity in endothelial cells stimulated with barrier-disruptive agents. AMPK activity was evaluated by westernblot analysis of phosphorylation of AMPK at Thr172 (an activation residue at AMPK) and phosphorylation of Acetyl CoA Carboxyl (ACC) at Ser79 (a downstream target of AMPK). Stimulation of endothelial cells with thrombin (1U/mL) resulted in a strong increase in phosphorylation of AMPK and its downstream target ACC (Figure 2). AMPK activity was maximal at 2-5 after thrombin stimulation. Similar results were obtained after endothelial stimulation with vascular endothelial growth factor (10ng/mL), which resulted in a transient activation of AMPK (Figure 2). Therefore AMPK is activated during endothelial stimulation with diverse barrier-disruptive agents.
AMPK contributes to barrier dysfunction independent of the RhoA/Rho kinase pathway

The RhoA/Rho kinase importantly contributes to the contractile response that follows endothelial stimulation with thrombin, and has been reported to be activated upon AMPK activation. To evaluate whether AMPK contributes to endothelial barrier disruption via this pathway, we...
measured RhoA activity in endothelial cells pretreated with vector or Compound C. Compound C did not affect RhoA activity, neither under unstimulated conditions, nor during thrombin stimulation (Figure 3A). Phosphorylation of the Rho kinase downstream targets ezrin, radixin and moezin (ERM), serving as surrogate markers for Rho kinase activity, did not differ between Compound C- or vector-pretreated cells (Figure 3B). In line with this, we demonstrated that pretreatment with the combination of the Rho kinase inhibitor Y27632 and Compound C had an additive protective effect compared to Compound C or Y27632 alone (Figure 3D). Together, this indicates that inhibition of AMPK does not affect the RhoA/Rho kinase pathway activity during thrombin stimulation, and that AMPK thus contributes to endothelial barrier function independent of this pathway.

**Figure 2 – Thrombin-induced changes in AMPK activity** Phosphorylation of AMPK (pAMPK) and its downstream target ACC (pACC) during stimulation of endothelial cells with thrombin (1U/mL) and the growth factor VEGF (10ng/mL). Ponceau panprotein staining served as loading control. ACC = acetyl coA carboxylase; AMPK = adenosine mono-phosphate activated protein kinase; VEGF = vascular endothelial growth factor.

**DISCUSSION**

This study demonstrates that the serine/threonine kinase AMPK is a mediator of endothelial barrier disruption. Using pharmacological inhibitors (Compound C) and activators (AICAR) of AMPK, we show that inhibition of AMPK improves endothelial barrier function under unstimulated conditions, and attenuates endothelial barrier dysfunction upon stimulation with barrier disruptive agents like thrombin. Activation of AMPK by AICAR enhances thrombin-induced endothelial barrier dysfunction. Endothelial stimulation with thrombin or VEGF resulted in fast activation of AMPK. The effects of AMPK on the endothelial barrier were independent of the RhoA/Rho kinase pathway.

**Comparison current results with other studies**

First of all, this study provides strong evidence for a beneficial effect of AMPK inhibition on endothelial barrier function under unstimulated conditions and during thrombin stimulation.
Figure 3 – Effects of AMPK on the RhoA/Rho kinase signaling pathway. A) Thrombin-induced RhoA activation in endothelial cells pretreated with DMSO 0.1% or Compound C (CompC) 10µM. Mean (range) of N = 2 measurements with cells from 1 donor. B) Phosphorylation of ERM, a downstream target of Rho kinase during stimulation of endothelial cells with 1U/mL thrombin (upper blot) or the AMPK-activator adiponectin (lower blot). C) Effect of pharmacological inhibition of AMPK (Compound C) or Rho kinase (Y27632) or the combination on barrier integrity of a resting endothelial monolayer. Endothelial barrier integrity was measured by electrical cell-substrate impedance sensing. Mean (range) of N = 2-4 measurements with cells from 1 donor. D) Quantification of the thrombin response in endothelial cells pretreated with DMSO, Compound C, Y27632 or the combination. A.U. = arbitrary units; AUC = area under the curve; DMSO = dimethyl sulfoxide; RhoA = Ras homolog A; ERM = ezrin, radixin and moezin.

Together with the finding that AMPK is activated at an early timepoint during endothelial stimulation with thrombin or VEGF (within minutes), it indicates that AMPK mediates endothelial barrier dysfunction. This is in clear contrast with previous studies after the role of AMPK in endothelial barrier regulation. Creighton et al. [3] demonstrated that neither AICAR, nor Compound C affected basal barrier function of a microvascular endothelial monolayer. AMPK inhibition by Compound C or knockdown of the α1 subunit perturbed the growth to confluence of subconfluent endothelial cells. AICAR could reverse the perturbed growth to confluence in α1 subunit depleted cells, and could reverse pulmonary edema development if given after induction of lung injury [3].
Several explanations might be given for the differences observed. First, there is a difference in phenotype between the two studies, as the barrier experiments in the study of Creighton et al. were performed with pulmonary microvascular endothelial cells. Endothelial phenotype is a key determinant of a subunit expression [3]. The differences in endothelial phenotype and in effects on endothelial barrier between the studies might indicate that the \( \alpha \) subunit importantly determines whether AMPK has barrier-disruptive or barrier-protective effects. Second, the Compound C concentration used by Creighton et al. was 50\( \mu \)M. We decided to use 10\( \mu \)M, because this concentration gave optimal protection effects and because we observed cell toxicity at 30\( \mu \)M (data not shown). This is in line with the study of Kim et al., which showed that endothelial cell toxicity increases linearly at Compound C concentrations above 5\( \mu \)M [14]. Although pulmonary microvascular endothelial cells may be more resistant to toxic effects than HUVECs used in the study of Kim et al. and in our study, the use of 50\( \mu \)M Compound C concentration is likely to carry off-target effects. Moreover, assuming that the AMPK \( \alpha \) subunits have a different IC\(_{50}\) for Compound C, the Compound C concentration may influence the balance of \( \alpha \) subunit activity.

Mechanisms by which AMPK may contribute to endothelial barrier disruption

The effects of AMPK on the endothelial barrier were independent of the RhoA/Rho kinase pathway, leaving unanswered the question how AMPK contributes to endothelial barrier dysfunction. The events downstream AMPK activation described thus far are beneficial for the endothelium, as AMPK has been shown to attenuate inflammation [15,16] and reactive oxygen formation [17], and to enhance endothelial repair [18] and production of nitric oxide [16]. Yet, one hypothetical mechanism may deserve some attention. Given the observations that: a) AMPK is a generator of intracellular ATP [1] that is quickly activated upon thrombin stimulation (Figure 2) [7], b) active cytoskeletal rearrangements and changes in cell shape due to actomyosin contraction require high amounts of localized energy, preferably as high energy phosphates [19], and c) intracellular ATP drops during endothelial stimulation by thrombin and histamin [20], it is tempting to speculate that AMPK fuels the energy-demanding processes that mediate endothelial barrier disruption (actin polymerization, actomyosin contraction). AMPK is activated in parallel to, but independent from, these processes, and our data simply suggest that closing the fuel supply stops cell movement. This mechanism would explain the paradoxical findings that AMPK not only mediates endothelial repair [18] and growth to confluence [3], but also angiogenesis [9] and endothelial barrier disruption [this chapter], because all these processes depend on active, energy-dependent cytoskeletal rearrangement and changes in cell shape. The proposed mechanism provides valuable information on the pathophysiology of vascular leakage, but at the same time limits therapeutic application of AMPK inhibition in diseases like acute...
lung injury and sepsis, in which recovery of vascular integrity depends on endothelial repair and reendothelialization.

**AMPK-independent effects of Compound C and AICAR**
Although the use of both AMPK agonists and antagonists points towards involvement of AMPK in endothelial barrier regulation, off-target effects of the compounds used in this study may confound the association of AMPK with endothelial barrier regulation. Numerous studies have shown effects of AICAR and Compound C independent of AMPK, both in endothelial cells [14] and other cell types [21,22]. These matters urge genetic approaches that evaluate the effects of AMPK on endothelial barrier. Preferably, such experiments evaluate the effect of depletion of either AMPK α subunit on endothelial barrier function in both macro- and microvascular cells. In collaboration with the group of dr. Heller [7,9], in vivo experiments are scheduled to evaluate vascular leakage in endothelial-specific AMPKα1⁻/⁻ and AMPKα2⁻/⁻ mice. These experiments may also confirm previous hypotheses that AMPK α subunit expression determines whether AMPK contributes to or protects against endothelial barrier dysfunction.

**Conclusion**
This study strongly suggests the involvement of AMPK in disruption of the endothelial barrier, which is independent of the RhoA/Rho kinase pathway. Although AMPK may form a suitable target for treatment of vascular leakage, additional experiments are required to elucidate how AMPK contributes to endothelial barrier disruption.
REFERENCES


Chapter 5
Supplementary Information
Supplementary Figure 1 – Endothelial barrier integrity during pharmacological inhibition or activation of AMPK. A) Endothelial barrier function during thrombin (1U/mL) stimulation of endothelial cells pretreated with vector (0.1% DMSO), the AMPK inhibitor Compound C or the AMPK activator AICAR. Endothelial barrier function was measured by electrical cell-substrate impedance sensing, and the resistance was normalized to the moment before addition of thrombin. The arrow indicates the moment of thrombin stimulation. Curves represent average values of N = 5-6 measurements with cells from 2 donors. B) Endothelial barrier function during thrombin (1U/mL) stimulation of endothelial cells pretreated with vector or adiponectin (1µg/mL). Curves represent average values of N = 2-4 measurements with cells from 1 donors. AICAR = 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide; AMPK = adenosine mono-phosphate activated protein kinase; DMSO = dimethylsulfoxide.