Adrenomedullin and endothelial barrier function

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Summary

Although loss of endothelial barrier function is a hallmark of every acute inflammation and contributes to fatal loss of organ function during severe infections, there is no sufficient therapy for stabilization of endothelial barrier function. Endogenous peptide adrenomedullin (AM) serum levels were shown to be increased during severe infection including sepsis and septic shock. In different in-vitro and in-vivo models AM acted as a potent therapeutic endothelial barrier function-stabilizing agent. Activation of specific receptors by AM results in elevation of second messenger cAMP. AM inhibits actin-myosin based endothelial cell contraction and junctional disassembly, thereby preventing paracellular permeability and oedema formation. The peptide furthermore possesses several protective cardiovascular qualities, including protection of the microcirculation during inflammation, and was proven as an efficient counter-regulatory molecule in various models of sepsis and septic shock. Overall, AM may be an attractive molecule to combat against cardiovascular malfunction during severe infection.

Keywords

Adrenomedullin, endothelium, permeability, inflammation, sepsis

Endothelial barrier dysfunction in inflammation

The vascular endothelium constitutes a cellular barrier that plays a crucial role in the maintenance of vessel integrity and controls exchange of small solutes and macromolecules between the intravascular and interstitial space. Increased endothelial permeability is a hallmark of virtually every acute inflammatory reaction (review articles [1–5]). It results in extravasation of fluid and plasma molecules as well as inflammatory mediators through the activated endothelium. Besides hyperpermeability, the pro-inflammatory endothelial phenotype is furthermore characterized by e.g. increased mediator release of the endothelium (chemo-, cytokines, nitric oxide, prostacyclin), procoagulant activities as well as increased adhesion molecule expression (review articles [2, 6–8]). In severe local (e.g. pneumonia, acute respiratory distress syndrome) or systemic (e.g. sepsis, systemic inflammatory response syndrome) inflammation endothelial hyperpermeability and subsequent oedema formation could result in fatal organ dysfunction (2, 7, 9−11). During septic shock extensive volume supply is needed to maintain sufficient circulatory function thereby aggravating deleterious tissue oedema formation (12). This is especially critical with respect to the lungs, when fluid flux into the pulmonary interstitium and airspace jeopardizes gas exchange function with the final result of severe hypoxemia. Loss of blood-brain barrier function with subsequent brain oedema formation is a further example of endothelial hyperpermeability resulting in severe vital organ function (13, 14). Although this pathophysiologically important endothelial malfunction is well known since many years, there is no causative therapy for the stabilization of endothelial barrier function.

Mechanisms of endothelial permeability regulation in inflammation

The endothelium regulates fluid flux and transport of small molecules by the trans- and paracellular pathway. Although transendothelial transport plays an important role for nutrients, excessive paracellular leakage of large fluid volume and macromolecules across the endothelial monolayer is the single most critical factor in the development of oedema in acute inflammation in most blood vessels (but not e.g. in the blood brain barrier). However, only a brief description of the complex mechanisms involved in endothelial barrier regulation can be given in this article, and the interested
reader may be referred to articles more specifically dealing with the interesting underlying phenomena (2, 3, 5, 7, 8, 11, 13–15).

Endothelial cell-cell contacts are formed by a highly sophisticated organization of tight junctions, and adherence junctions: Tight junctions consisting of transmembrane occludin, claudin family members and junctional adhesion molecules (JAMs) are found. Integral membrane proteins like occludin are linked to intracellular proteins like ZO-1 and cingulin thereby connecting the junction proteins to the actin cytoskeleton (for a review see [1, 3, 16]). For example, the role of occludin for endothelial barrier regulation is highlighted by the observation that its expression levels may determine tight junction permeability of the endothelium in different tissues (e.g. higher expression in the brain than in peripheral tissues) (17). Adherence junctions composed by cadherins and catenins are primary adhesions between adjacent endothelial cells. Molecules like JAM-C may interact with VE-cadherin-related adhesion and endothelial contractility (see below) thereby modulating vascular permeability and demonstrating the highly cooperative nature of different pathways in barrier function regulation (18).

Finally, platelet/endothelial cell adhesion molecule (PECAM), and integrin-based junctions are formed in the more basal zone of the endothelial layer (reviewed in [1, 3, 5, 14–16]). Perturbation of these adhesion structures results in loosening of endothelial cell-cell contacts, opening of intercellular gaps, and subsequent paracellular fluid flux.

In addition, a second important mechanism contributes to the opening of intercellular gaps between endothelial cells: endothelial cells contain an elaborated cytoskeleton allowing active cell contraction. Actin-myosin based contraction leads to force generation along F-actin stress fibers and the peripheral F-actin dense band in activated endothelium resulting in cell retraction and subsequent intercellular gap formation (reviewed in [3, 5, 15, 19–24]). Stimulation of e.g. the Rho-Rho kinase pathway or activation of myosin light chain kinase is shown to be important regulators of actin-myosin based endothelial contraction (5, 19–24). Moreover, recent studies evidenced that the stability of the microtubule network directly and by connections to the contractile actin-myosin system may also be important for barrier regulation (25–30). Even though all three pathways are known to be important for endothelial barrier regulation, it is still not entirely clear whether these pathways act in a parallel or sequential way and how they interact.

The great variety of inflammatory reactions accompanied by loss of endothelial barrier function already indicates that very different agents act on the above-mentioned mechanisms: Receptor-independent agents like proteases from pathogens (31, 32) or the host (e.g. elastase from leucocytes [33, 34]) directly could degrade critical junctional structures. Bacterial exotoxins, including Staphylococcus aureus α-toxin (35–38) or Escherichia coli hemolysin (37–41), attack endothelial cell membranes by toxin insertion resulting in cell activation and complete loss of barrier function. Hydroxyl radicals released by activated leucocytes are well known as stimulators of endothelial cell contraction and junctional reorganization (3, 5, 39, 40, 42, 43). Receptor-operated stimuli like bacterial lipopolysaccharide or host derived molecules (e.g. thrombin, tumour necrosis factor [TNF]-α) feed into complex signalling pathways stimulating both, Junctional disturbance and actin-myosin based cell contraction (3, 5, 20, 21, 23, 40, 43, 44). Thus, a crude mixture of agents increasing permeability may act together in inflammation finally overcoming the endothelial barrier.

Based on this comprehension, a potent endothelial barrier-stabilizing agent must be able to counteract the terminating signalling cascades (stabilization of junctional proteins, prevention of cell contraction) rather than the different diverse initial signalling events.

**Adrenomedullin, a multifunctional endogenous peptide**

Adrenomedullin (AM) was first isolated by Kitamura et al. from a human pheochromocytoma in 1993 (45). It is a 52-amino-acid peptide, belonging to the calcitonin gene-related peptide family (reviewed in [46–50]). The *adrenomedullin* gene encodes a 185-amino acid preprohormone, which after cleavage of the 21-residue N-terminal signal peptide, generates a 164-amino acid pro-AM peptide. Starting from here, in particular two biologically active peptides are processed: AM, and proAM N-terminal 20 peptide (PAMP) (46, 50–54). In addition, further fragments derived from the AM precursor or AM, like pro-AM145–92 (55), AM1–25 (56), and AM26–52 (57) have been identified. Enzymatic amydiation from a glycine-extended precursor by peptidylglycine alpha-amidating monooxygenase (PAM) forms the mature form of AM and is essential for its biological function (58–61). AM is a widely expressed peptide, and secretion has been demonstrated from e.g. endothelial cells, vascular smooth muscle cells, cardiac myocytes as well as human leucocytes (46, 47, 62–66).

AM seems to mediate its activities through binding to a complex receptor composed of the calcitonin receptor like-receptor (CRLR) associated with receptor activity modifying proteins (RAMP)-2 and RAMP-3 (48, 67–78). However, the existence of further, AM-specific, receptors is discussed. In most cells, including endothelial cells, activation of receptors by AM results in accumulation of the second messenger cAMP (39, 46, 77, 79–82). Activation of several kinases, like protein kinase (PK) A (83–85), Src (86), PKC (83), phosphatidylinositol 3-kinase/Akt (87–90) as well as e.g. p38 mitogen-activated protein kinase and ERK (84, 91), is implicated in AM-mediated signalling pathways.

As a consequence of widely spread expression of the peptide and its receptors, the peptide participates in the control of central body functions, such as vascular tone regulation, fluid and electrolyte homeostasis or regulation of the reproductive system (for a review see [46, 48–50, 92–100]). However, increasing evidence suggests an important role of AM in inflammatory reactions (101, 102), and in particular, in the regulation of endothelial barrier function.

**Adrenomedullin and inflammation**

Different pro-inflammatory cytokines, like interleukin (IL)-1α and IL-1β, TNF-α, and TNF-β, bacterial products such as lipopolysaccharide (LPS) or hypoxia are shown to increase AM expression in various cells (46, 62, 63). Most importantly, high ex-
pression of this peptide is also demonstrated in vivo in humans (48, 103–106) as well as in animals (65, 66, 107–110) suffering from severe infection. In particular, increased expression is observed in sepsis and septic shock as well as in LPS-exposed animals. In a model of cecal ligation and puncture in rats, the small intestine was identified as an important source of AM release during polymicrobial sepsis (66) and high expression was observed in the lung in endotoxaemia (109) as well as in acute lung injury induced by hypoxia and LPS (111). In addition, AM was shown to act as a potent antimicrobial peptide against Gram-positive and Gram-negative bacteria (101). Altogether, these observations raise the question whether AM acts as a detrimental or counteracting molecule in severe inflammation.

### Table 1: Mechanisms of adrenomedullin in endothelial barrier regulation (dysfunction).

<table>
<thead>
<tr>
<th>Site of endothelial permeability</th>
<th>Endothelial cell types involved</th>
<th>Permeability stimuli</th>
<th>Effect</th>
<th>Endothelial pathway implicated</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circulation</td>
<td><em>In vivo</em> mouse endothelial cells</td>
<td>Functional gene knock-out for AM</td>
<td>Lethal hydrops fetalis</td>
<td>Disruption of AM signaling</td>
<td>(70, 112, 113)</td>
</tr>
<tr>
<td>Brain</td>
<td>prCEC</td>
<td>none</td>
<td>AM increased TEER</td>
<td>Unknown</td>
<td>(116, 117, 120)</td>
</tr>
<tr>
<td>Lung</td>
<td>Isolated perfused rabbit lungs PAEC guinea pig airways</td>
<td><em>H₂O₂</em>, ovalbumin</td>
<td>Prevention of pulmonary vascular leakage by AM</td>
<td>cAMP induction</td>
<td>(39, 114, 115)</td>
</tr>
<tr>
<td>Gut</td>
<td>Capillary endothelium of Tunica submucosa and muscularis</td>
<td><em>S.aureus</em> α-toxin</td>
<td>Protection of endothelial barrier function by AM</td>
<td>Perpetuation of intercellular VE-cadherin connections</td>
<td>(114, 115)</td>
</tr>
<tr>
<td>Skin</td>
<td><em>In vivo</em> mouse skin endothelial cells</td>
<td>AM</td>
<td>AM induced edema formation</td>
<td>Indirect, unknown</td>
<td>(73, 119)</td>
</tr>
<tr>
<td>Other</td>
<td>HUVEC, baEC, other</td>
<td><em>H₂O₂</em>, Thrombin <em>S.a.-tox</em>, <em>E.c.-hemolysin</em></td>
<td>Protection of endothelial barrier function by AM</td>
<td>cAMP induction, Ca²⁺ influx</td>
<td>(39, 79, 82, 114, 115, 125)</td>
</tr>
</tbody>
</table>

AM: adrenomedullin; baEC: bovine aortic endothelial cells; CRLR: calcitonin receptor like-receptor; E.c.: Escherichia coli; H UVEC: human umbilical vein endothelial cells; *H₂O₂*: hydrogen peroxide; PAAM: peptide-dyglycine alpha-amidating monoxygenase; PAEC: porcine pulmonary artery endothelial cells; pMLC: phosphorylated myosin light chain; prCEC: primary rat cerebral endothelial cells; VE: vascular endothelial; S.a.: Staphylococcus aureus; TEER: transendothelial electrical resistance.

### Adrenomedullin as a regulator of endothelial permeability

Loss of endothelial barrier function is a central feature of acute inflammation and contributes essentially to organ dysfunction in severe infection. Thus, the ability to stabilize endothelial barrier function might characterize a powerful counteracting molecule in inflammation at the level of the vascular wall.

The first evidence that AM may play an important role in endothelial barrier function came from the observation of extreme hydrops fetalis in mice lacking the functional *adrenomedullin* gene (112). Moreover, deletion of the possible AM receptor gene, the *calcitonin receptor-like receptor*, thus potentially blocking AM signalling, results in the same phenotype (70). A similar phenotype was also found in mice lacking the *peptidylglycine alpha-amidating monoxygenase* gene thereby blocking AM function (113). Overall, three separate knockout mouse models affecting the AM system result in extreme generalized oedema formation suggesting a strong barrier stabilizing function of the peptide.

Several studies tested the potency of AM as a therapeutic molecule for the direct stabilization of endothelial barrier function during inflammation and infection. Direct effects of AM on endothelial barrier function was demonstrated in an in-vitro system in which endothelial monolayer permeability is analyzed under the application of physiological hydrostatic pressure. In this model, AM dose-dependently reduced hyperpermeability induced by stimuli such diverse as hydrogen peroxide, thrombin, *E. coli* hemolysin, or *S. aureus* α-toxin (39, 114, 115). Furthermore, AM tightened blood-brain barrier endothelial function *in vitro* (116, 117). These results indicate that AM acts on central downstream mechanisms in permeability regulation independent from the stimulus initiating the barrier dysfunction. Since endothelial cells from different species (human, rat, porcine) and vascular beds (lung pulmonary artery, umbilical vein,
Adrenomedullin – mechanisms of endothelial barrier stabilization

How might AM cause its barrier-stabilizing effect in endothelial cells? Active actin-myosin-based cell contraction and perturbation of endothelial cell junctions are key events promoting opening of intercellular gaps allowing paracellular fluid flux and oedema formation under inflammatory conditions (1–5). AM is shown to block phosphorylation of endothelial myosin light chains after stimulation of cells with thrombin, S. aureus α-toxin, and hydrogen peroxide (39, 114). This is accompanied by reduction of the typical formation of F-actin stress fibers known to be vital for force generation during actin-myosin based cell contraction in the endothelium (39, 114, 115). As this effect could be observed in resting as well as in hydrogen peroxide, thrombin or bacterial exotoxin (E. coli hemolysin, S. aureus α-toxin) exposed cells (39, 114, 115), AM may act as a general microfilament-relaxing peptide in endothelial cells. Although the microtubule system seems also to contribute to barrier regulation (26–29), the role AM on its regulation is unknown.

Recent reports indicate a strong stabilizing effect of AM on endothelial cell junctions. In-vitro experiments with cultured human brain) were tested. Barrier stabilisation seems to be a general effect of AM in vitro (Table 1).

The tightening effect of AM on endothelial barrier is also shown in ex-vivo and in-vivo models: Investigations using hydrogen peroxide exposed rabbit lungs or S. aureus α-toxin infused isolated rat ileum preparations demonstrated the barrier stabilising action of AM ex vivo (39, 114). The study by Ohbayashi et al. evidenced a protective effect of AM on ovalbumin-induced airway microvascular leakage in ovalbumin-sensitized guinea pigs (118). Importantly, in a model of septic shock induced by intravascular administration of S. aureus α-toxin, therapeutically infusion of AM started one hour after onset of α-toxin the peptide blocked hyperpermeability in lung, liver, ileum and kidney (Fig. 1). These studies using more integrated models therefore verified the results obtained in vitro. However, it should be noted that Grant et al. (73) and Tam et al. (119) found increased vascular permeability in the skin of mice after AM administration. In the skin, this observation may be due to increased vessel recruitment rather than direct hyperpermeability. Nevertheless, organ- or vascular bed-specific responses with respect to AM-related action on the endothelium have to be considered. However, overall AM is shown to stabilize endothelial barrier function in vitro, ex vivo and in vivo under several circumstances simulating inflammatory reactions in various vascular beds.

Figure 1: Stabilization of endothelial barrier function by therapeutic application of AM in vivo. Septic shock was induced by central venous infusion of pore-forming S. aureus α-toxin in rats. Infusion of α-toxin induced massive filtration of Evans-blue dye (EBD)-labelled albumin into lung, liver, ileum and kidney as indicated by the dark staining of α-toxin-exposed organs compared to controls. Therapeutic infusion of AM started one hour after beginning of α-toxin application completely prevented extravasation of the albumin-bound dye demonstrating the effective stabilization of endothelial barrier function in vivo (from Temmesfeld-Wollbrück et al. [131]. Reprinted with kind permission of Springer Science and Business Media/Verlag Berlin Heidelberg).

Figure 2: Representative for many stimuli, thrombin-treated HUVECs are demonstrated for the stabilization of VE-cadherin and occludin as well as the prevention of intercellular gap formation by AM. A) HUVECs treated with solvent showed an intact monolayer as visualized by double staining of VE-cadherin (green) and occludin (red) resulting in yellow (arrows). B) Exposure of endothelial cells to 1 µM ADM for 15 min resulted in linearization of the VE-cadherin adherence- and occludin tight junction protein bands (arrows). C) Gap formation and loss of both junctional proteins occurred after exposure of cells to 0.75 U/ml thrombin for 15 min (arrows). D) Pre-incubation with AM impeded loss of the junctional proteins and prevented the gap formation caused by thrombin (arrows).
Figure 3: ADM reduced VE-cadherin disassembly in α-toxin-treated isolated rat ileum. Immunoreaction of VE-cadherin was visualized by green Alexa 488 staining, nuclei were counter-stained with DAPI (blue) and tissue structure was visualized by differential interference contrast (grey). A) In solvent or 0.1 μM ADM (not shown) treated guts, capillaries of the tunica muscularis showed continuous VE-cadherin (green) distribution (arrows). B) Thirty minutes after the application of a bolus of 1µg S. aureus α-toxin into superior mesenteric artery, VE-cadherin was visualized. Discontinuous VE-cadherin staining indicated a loss of endothelial cell–cell contacts (arrows). C) Starting ADM infusion 5 min before the application of α-toxin sufficiently inhibited α-toxin-induced disassembly of interendothelial VE-cadherin (arrows). (from Hocke et al. [115]. Reprinted with kind permission of Springer Science and Business Media/Verlag Berlin Heidelberg).

Adrenomedullin – action on the endothelium and vascular system besides barrier function

Beyond its action on endothelial permeability, AM comprises important effects on the vascular system. Cumulating evidence demonstrates increased nitric oxide release, vasodilation in different vascular beds, and subsequent hypotension due to AM (95, 99, 104, 114, 126–130). With respect to hypotension in severe infection and sepsis it is noteworthy that septic mice over-expressing AM in their vasculature are resistant to endotoxin shock despite hypotension (127). Moreover, we recently demonstrated in a model of S. aureus α-toxin-induced shock, that normalized vascular permeability and increased cardiac output overcome the hypotonic effects in rats resulting in a significant reduction of mortality (131). AM-related reduction of mortality is also observed in models of polymicrobial sepsis (99, 100, 132–134), endotoxin-related shock (132), and hemorrhage (135). Beside its action on the macrocirculation, intravital microscopy studies demonstrated that AM diminishes microcirculatory disturbances under inflammatory conditions (35). In addition, AM attenuates endothelial cell apoptosis in polymicrobial sepsis possibly by increasing anti-apoptotic Bel-2 expression (136). In concert with the reduction of pro-inflammatory cytokine release (134) these vascular effects of AM may improve circulation during severe infection.

However, it should be noted that desensitizing mechanisms acting during severe infection may be active. For example, vascular responsiveness to AM might be decreased due to alteration of receptor expression and function (74, 77, 137–140). Combined application of AM with its binding-protein AMBP-1 might overcome desensitizing mechanisms and help to prevent the transition from hyper- to hypodynamic phase in sepsis (133, 134, 136, 141–145).

Besides protective effects on the vascular wall during acute inflammation, accumulating evidence suggests that AM may be
important in diseases affecting the cardiovascular system like myocardial infarction and heart failure (52, 94, 96, 146), systemic and pulmonary hypertension (94, 98, 146), and angiogenesis (96).

Concluding remarks

AM seems to be a very relevant peptide with respect to the protection of the vascular wall during various disorders of the cardiovascular system. More importantly, its characteristics turn it into an interesting novel target molecule to directly stabilize endothelial barrier function and to suppress further pro-inflammatory impairment of the vessel wall during severe infection. Massive volume substitution to stabilize circulation during sepsis and septic shock increases systemic oedema formation thereby impairing oxygenation in the lung and systemic oxygen delivery. Thus, a therapeutic strategy to stabilize endothelial barrier function and microcirculation by AM may be of benefit in severe infection.

In addition, the molecular action of AM has to be determined on a molecular level by straightforward characterization of its receptors, signalling pathways and functional consequences.

Much less is known about the function of other, AM-derived peptides. Although e.g. PAMP was also shown to induce vasodilatation (147, 148), it may bind to distinct receptors (149, 150), and it may counteract some AM actions (151). AM fragments like AM1-25 or AM26-52, which both were detected in human tissues, may act as naturally occurring antagonists of AM (56, 57). Further investigations are needed to dissect the role of AM, and its related peptides on the regulation of endothelial barrier function in particular and the regulation of the vascular system in general.

Acknowledgements

The authors apologize for not citing more original manuscripts due to space restrictions and hope that the cited reviews will provide more detail.

References


