Intermedin induces loss of coronary microvascular endothelial barrier via derangement of actin cytoskeleton: role of RhoA and Rac1

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Aims

Intermedin (IMD) is a novel member of the calcitonin gene-related peptide family, which acts via calcitonin receptor-like receptors (CLRs), mediating activation of cAMP signalling. The main objective of the present study was to analyse the molecular mechanisms of the differential effects of IMD on the macromolecule permeability of endothelial cells of different vascular beds.

Methods and results

Here we demonstrate that IMD increases permeability of rat coronary microvascular endothelial cells (RCECs) and reduces permeability of human umbilical vein endothelial cells (HUVECs) and rat aortic endothelial cells via CLRs and cAMP. Intermedin causes a derangement of the actin cytoskeleton accompanied by loss of vascular endothelial cadherin (VE-cadherin) in RCECs, while it causes a rearrangement of the actin cytoskeleton and VE-cadherin at cell–cell junctions in HUVECs. Intermedin inactivates the RhoA/Rho-kinase (Rock) pathway in both cell types; however, it inactivates Rac1 in RCECs but not in HUVECs. Inhibition and rescue experiments demonstrate that both RhoA and Rac1 are required for the RCEC barrier stability, while in HUVECs the inhibition of RhoA/Rock signalling does not interfere with basal permeability.

Conclusion

The opposite effects of IMD on permeability of RCECs and HUVECs are due to differential regulation of actin cytoskeleton dynamics via RhoA and Rac1. Moreover, Rac1 activity is regulated by the RhoA/Rock pathway in RCECs but not in HUVECs.

Keywords

Intermedin • RhoA • Rac1 • Permeability • Actin cytoskeleton

1. Introduction

Vascular endothelium forms a selective barrier and regulates the trafficking of macromolecules and blood cells across the vessel wall.1 The integrity of the endothelial barrier is highly dependent on actin cytoskeleton-mediated adherens junctions consisting of vascular endothelial cadherin (VE-cadherin) and associated catenins, which link the VE-cadherin to the actin cytoskeleton.2 Changes in actin cytoskeleton dynamics affect the stability of these adherens junctions.2 It is widely accepted that the Rho-family of GTPases (RhoA, Rac1, and cdc42) are important regulators of the actin cytoskeleton and adherens junctions and hence play a crucial role in the maintenance of the endothelial barrier.3–7 Perturbed activities of these Rho-GTPases in different pathological conditions lead to derangement of the actin cytoskeleton and loss of adherens junctions, leading to endothelial barrier failure.3,6

Intermedin (IMD) is a novel member of the calcitonin gene-related peptide (CGRP) superfamily, having 33% sequence homology with adrenomedullin (AM).8 Intermedin is expressed in a variety of tissues and organs, including brain, heart and endothelium.9 The biological effects of IMD and adrenomedullin are mediated via G-protein-coupled receptors, calcitonin receptor-like receptors (CLRs) in association with one of the receptor activity-modifying proteins (RAMP1, 2, or 3).8,10 Calcitonin receptor-like receptors in association with RAMP1 form CGRP receptors,10,11 which are sensitive to αCGRP8-37.11 Calcitonin receptor-like receptors in

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combination with RAMP2 and 3 constitute adrenomedullin 1 (AM1) and adrenomedullin 2 (AM2) receptors, respectively, which have a low affinity for αCGRP8–37 but are highly sensitive to AM12–52.10,12,13 Binding of IMD with its receptors leads to activation of adenyl cyclase and elevation of intracellular cAMP.14,15 However, it has been well documented that cAMP has differential effects on the endothelial barrier of different vascular beds. In aortic and pulmonary endothelial cells, it has been shown to reduce the permeability of cultured endothelial monolayers.14–17 In line with these previous reports, we have recently shown that IMD, acting via cAMP/protein kinase A (PKA), has barrier-stabilizing effects in isolated murine lungs and human pulmonary endothelial cells.18 Conversely, in isolated coronary endothelial monolayers,14,19,20 isolated coronary microvessels,21 hamster cheek pouch,22 and in retinal vessels in vivo,23–26 it causes an increase in endothelial permeability. Recently, it was reported that the expression of IMD and its receptors in heart is increased in different pathological conditions, such as congestive heart failure and hypertension.27,28 The pathophysiological role of IMD in the regulation of the coronary microvascular barrier has not been investigated previously. The present study was designed to analyse the effects and mechanisms of IMD on the endothelial barrier of coronary microvasculature.

In the present study, we used well-established rat coronary microvascular endothelial cell (RCEC) and human umbilical vein endothelial cell (HUVEC) models to compare the IMD effects on macromolecule permeability of these two cell models and mechanistically dissected the differential effects of IMD on monolayer permeability. The main emphasis was laid on the regulation of the Rho-GTPases RhoA and Rac1, the two main regulators of endothelial actin dynamics and adherens junctions. αCGRP8–37 and AM12–52 were used as CGRP and AM receptor antagonists, respectively.8,12 Activity of RhoA was inhibited by a cell-permeable bacterial toxin, C3-transferase, and Rho-kinase (Rock) was inhibited by specific pharmacological inhibitors, Y27632 and H1077. We show that IMD increases macromolecule permeability of RCECs monolayers and reduces the permeability of HUVEC monolayers. This differential effect is mediated via differential regulation of Rac1 activity.

2. Methods

2.1 Materials

For detailed Materials, see Supplementary material online.

2.2 Cell culture

The study conforms to the principles outlined in the ‘Declaration of Helsinki’ (Cardiovascular Research 1997;352–3). The study protocols were approved by the local animal ethics committee of the University of Giessen, and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85–23, revised 1996). Male Wistar rats (200–250 g) were anaesthetized with CO2 and killed by cervical dislocation. Hearts were removed, and coronary microvascular endothelial cells (RCECs) were isolated and cultured as described previously.29 Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords derived from normal healthy, uncomplicated pregnancies obtained from university hospital Giessen after approval from the hospital ethics committee and informed consent from patients, and were cultured as described before.30 Briefly, the umbilical cords were washed with phosphate-buffered saline, filled with collageenase solution and incubated at 37°C for 30 min. The solution was then removed, centrifuged at 500g, and the cell pellet was resuspended in culture medium. All the experiments were performed with passage 1 cells. Rat aortic endothelial cells were purchased from CELL applications Inc. (San Diego, CA, USA) in passage 1 and cultured according to the instructions of the supplier.

2.3 Assay of cAMP

Levels of cAMP were measured using a cAMP colorimetric kit (Assay designs, Loerach, Germany) according to the manufacturer’s protocol.

2.4 Macromolecule permeability measurement

The macromolecule permeability of endothelial monolayers cultured on polycarbonate filters was measured as described previously.18 Briefly, both the ‘luminal’ and ‘abluminal’ compartments contained as the basal medium modified Tyrode solution. The flux of labelled albumin from the luminal to the abluminal compartment was continuously measured online by a spectrophotometer (Specord 10; Zeiss, Jena, Germany).

2.5 Myosin phosphatase targeting subunit phosphorylation and RhoA activity

Myosin phosphatase targeting subunit (MYPT) phosphorylation at Thr850 was measured by immunoblotting with a specific anti-phospho-Thr850 antibody, and activation of RhoA was measured by ELISA-based assay.

2.6 Rac1 pulldown assays

The activation of Rac1 was assessed using a pull-down assay kit (cytoskeleton, Denver, CO, USA) according to the manufacturer’s instructions. For details, see Supplementary material online.

2.7 RhoA plasmid and transfection

Constitutive active RhoA (pEF-BOS-RhoA14)31 was kindly provided by Professor Kozo Kaibuchi (Nagoya University, Japan) and over-expressed in RCECs using a neon-electroporation system (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

2.8 Immunofluorescence microscopy

The endothelial monolayers cultured on glass coverslips were fixed with methanol or paraformaldehyde, immunostained with respective antibodies and were examined using a Zeiss LSM-510M inverted microscope. For details please see Supplementary material online.

2.9 Statistical analysis

Data are given as means ± SEM of three to five experiments using independent cell preparations. The comparison of means between groups was performed by one-way analysis of variance (ANOVA) followed by a Student–Newman–Keuls post hoc test. Changes in parameters within the same group were assessed by multiple ANOVA. Probability (P) values of less than 0.05 were considered significant.

3. Results

3.1 Intermedin activates the cAMP/PKA pathway in RCECs and HUVECs

Activation of the cAMP/PKA pathway was analysed both by direct measurement of intracellular cAMP concentrations and by analysing the phosphorylation state of vasodilator-stimulated phosphoprotein (VASP), an established endogenous substrate of PKA, at PKA specific site Ser157.32 RCECs produced cAMP in a concentration-dependent manner when exposed to increasing concentrations of IMD. Concentrations of 100 pM gave a twofold increase in cAMP compared with basal levels in serum-free medium, with an EC50 of ~1 mM (Figure 1A). Moreover, the increase in cAMP production was time
Figure 1 Intermedin (IMD) activates the cAMP/protein kinase A (PKA) pathway. (A) Intermedin induces cAMP production in rat coronary microvascular endothelial cells (RCECs). The RCECs were treated with increasing concentrations of IMD for 30 min, and the cAMP concentrations were measured by a colorimetric method. (B) The RCECs were exposed to IMD (10 nM) for the indicated time periods. n = 3; *p < 0.05 vs. control. (C) Effect of IMD, αCGRP8–37 and PKI on VASP phosphorylation at Ser157. Representative western blots of VASP phosphorylation. The RCECs were exposed to IMD (10 nM), αCGRP8–37 (1 μM), αCGRP8–37 plus IMD, PKI (20 μM), and PKI plus IMD or vehicle (C; control) for 30 min. The cells were incubated with PKI for 60 min or αCGRP8–37 for 30 min before IMD was added. The western blots are representative of three separate experiments with independent cell preparations.

A similar level of activation of cAMP/PKA was also seen in HUVEC monolayers (see Supplementary material online, Figure S1).

3.2 Intermedin has differential effects on RCEC and HUVEC monolayer permeability

The effect of IMD on endothelial barrier function was analysed by measuring the flux of trypan blue-labelled albumin across endothelial cell monolayers. As shown in Figure 2A and B, IMD increased macro-molecule permeability of RCEC monolayers in a concentration-dependent manner, producing half-maximal effect at ≈1 nM and maximal effect at 10 nM. This concentration of IMD was used for all further experiments. This increase in permeability produced by IMD was likewise evoked by a direct activator of adenyl cyclase, forskolin (FSK; 5 μM).

This IMD-induced increase in permeability was blunted by αCGRP8–37 in a concentration-dependent manner, producing maximal inhibition with 1 μM (Figure 2C and D). αCGRP8–37 had no effect on FSK-induced hyperpermeability (see Supplementary material online, Figure S3A). To further analyse whether this effect is mediated via activation of the cAMP/PKA pathway, RCECs were pre-incubated with PKI, a specific PKA inhibitor. As shown in Figure 2E, PKI significantly reduced the IMD-induced increase in macromolecule permeability. As cAMP can activate both PKA and Epac (exchange protein directly activated by cAMP), three cAMP analogues, 6-Bnz-cAMP and 8-CPT-cAMP, which specifically activate PKA and Epac, respectively, were used. As shown in Figure 2F, specific activation of PKA but not Epac led to an increase in RCEC monolayer permeability.

In contrast, exposure of HUVEC monolayers to IMD (10 nM) resulted in a reduction in macromolecule permeability (Figure 2G), which was comparable to that seen with FSK (5 μM). The AM receptor antagonist, hAM22–52, abolished the IMD- (Figure 2G) but not the FSK-mediated reduction in permeability (see Supplementary material online, Figure S3B). αCGRP8–37 attenuated effect of IMD much more weakly in HUVECs (data not shown). Moreover, the IMD-mediated reduction in permeability was significantly attenuated by PKI (Figure 2H). Likewise, IMD and FSK reduced the albumin permeability of rat aortic endothelial cell and porcine aortic endothelial cell monolayers (see Supplementary material online, Figure S2).

3.3 Intermedin has differential effects on VE-cadherin and actin cytoskeleton in RCEC and HUVEC monolayers

The endothelial barrier is largely dependent upon adherens junctions and actin cytoskeleton dynamics. To investigate the effect of IMD on adherens junctions, we examined the organization of VE-cadherin at cell borders in the presence or absence of IMD. VE-cadherin was distributed at the cell periphery in basal conditions. However, exposure of RCEC monolayers to IMD resulted in a drastic loss of VE-cadherin from cell borders, leading to intercellular gap formation within 30 min (Figure 3A). These effects of IMD were completely blocked by pre-incubating RCECs with αCGRP8–37. Similar effects were seen with FSK. The stability of adherens junctions is mainly dependent on peripheral actin cytoskeleton dynamics. Therefore, the effect of IMD on the actin cytoskeleton was analysed. In basal conditions, RCECs showed a greater number of stress fibres and a thin line of peripheral actin. Exposure of RCEC monolayers to IMD resulted in complete derangement of the actin cytoskeleton, which appeared as scrambled

dependent, reaching maximal in 30 min (Figure 1B). As shown in Figure 1C, phosphorylation of VASP was significantly enhanced in cells treated with IMD in comparison with untreated cells. Intermedin-induced VASP phosphorylation was completely blocked by pre-incubating RCECs either with the CGRP-receptor antagonist, αCGRP8–37, or with PKI, a cell-permeable PKA inhibitory peptide.
Figure 2. Effect of IMD on RCECs permeability. (A) RCEC monolayers were treated with IMD (0.1, 1, and 10 nM), FSK (5 μM), or vehicle (control) as indicated. Data are means ± SD of five separate experiments with independent cell preparations. *P < 0.05 vs. control. (B) Concentration–response curve of the effect of IMD as in A on permeability after 30 min. The RCEC monolayers were exposed to IMD as in A or vehicle (control) as indicated. n = 5; *P < 0.05 vs. control. (C) Effect of CGRP receptor inhibition on the IMD-induced increase in permeability. The RCEC monolayers were exposed to IMD (10 nM), αCGRP8–37 (1 μM) plus IMD, or vehicle (control) as indicated. n = 3; *P < 0.05 vs. control; #P < 0.05 vs. IMD alone. (D) Concentration–response curve of the effect of αCGRP8–37 on IMD-induced hyperpermeability. The RCEC monolayers were exposed to IMD (10 nM), in the absence or presence of increasing concentrations of αCGRP8–37 or vehicle (control) as indicated. n = 3; *P < 0.05 vs. IMD alone. (E) Effect of PKA inhibition on IMD-induced hyperpermeability. The RCEC monolayers were treated with IMD (10 nM), PKI (40 μM) plus IMD, or vehicle (control), as indicated. n = 3; *P < 0.05 vs. control; #P < 0.05 vs. IMD alone. (F) Effect of cAMP analogues on RCEC monolayer permeability. The RCEC monolayers were treated with the PKA activator, 6-Brz-cAMP (50 μM), the Epac activator, 8-CPT-cAMP (200 μM), or vehicle (control) as indicated. n = 3; *P < 0.05 vs. control. (G) Human umbilical vein endothelial cell (HUVEC) monolayers were exposed to IMD (10 nM), hAM22–52 (1 μM; a specific AM-receptor antagonist) plus IMD, FSK (5 μM), or vehicle (control) as indicated. n = 3; *P < 0.05 vs. control; #P < 0.05 vs. IMD alone. (H) The HUVEC monolayers were treated with IMD (10 nM), PKI (20 μM), PKI plus IMD, or vehicle (control; C), as indicated. n = 3; *P < 0.05 vs. control; #P < 0.05 vs. IMD alone.
Figure 3  Effect of IMD on VE-cadherin and actin cytoskeleton. (A, B) RCEC monolayers were exposed to IMD (10 nM), αCGRP₈₋₃₇ (1 μM) plus IMD, FSK (5 μM), or vehicle (control) for 30 min and immunostained for VE-cadherin (A) and F-actin (B; labelled with tetramethyl rhodamine isothiocyanate (TRITC)-phalloidin), and nuclei were labelled with nuclear specific dye TOPRO®. Scale bar represents 20 μm. (C, D) HUVEC monolayers were exposed to IMD (10 nM) or vehicle (control) for 15 min and immunostained for VE-cadherin (C) and F-actin (D; with TRITC-phalloidin). Representative immunostainings of three experiments using independent cell preparations are shown. (E–G) Effect of IMD on RhoA/Rock activity. (E) RCECs were exposed to IMD (10 nM) or FSK (5 μM), and active RhoA was detected by an ELISA-based assay as described in the Methods. The levels of total RhoA in the lysates were analysed by immunoblot and were used to normalize for loading. The active RhoA is given as x-fold of control. n = 3; *P < 0.05 vs. control. (F) Effect of IMD on MYPT1 phosphorylation at Thr850 in RCECs. Upper panel shows representative western blots of MYPT1 phosphorylation at Thr850 and vinculin (as loading control). The RCECs were treated with IMD (10 nM), forskolin (FSK; 10 μM), Y27632 (Rho-kinase inhibitor; 10 μM), or vehicle (control; C) for 30 min. Lower panel shows densitometric analysis of the western blots. n = 3; *P < 0.05 vs. control. (G) HUVEC monolayers were exposed to IMD (10 nM) or FSK (5 μM), and active RhoA was detected by an ELISA-based assay. The levels of total RhoA in the lysates were analysed by immunoblot and were used to normalize for loading. The active RhoA is given as x-fold of control. n = 3; *P < 0.05 vs. control.
Figure 4 Effect of IMD on Rac1 activity. (A) Upper panel shows representative western blots of Rac1-GTP and total Rac1. The RCEC monolayers were treated with IMD (10 nM), FSK (5 μM), or vehicle (control; C) for 30 min. Lower panel shows densitometric analysis of the western blots. n = 3; *P < 0.05 vs. control. (B) Upper panel shows representative western blots of Rac1-GTP and total Rac1. HUVEC monolayers were treated with IMD (10 nM), FSK (5 μM), or vehicle (control; C) for 30 min. Lower panel shows densitometric analysis of the western blots. n = 3; *P < 0.05 vs. control. (C) Effect of inhibition of RhoA/Rock signalling on Rac1 activity in RCECs. Upper panel shows representative western blots of Rac1-GTP and total Rac1. The RCEC monolayers were treated with RhoA inhibitor C3-transferase (C3T; 2 μg/mL; 60 min), Rock inhibitor Y27632 (10 μM for 30 min), IMD, Y27632 plus IMD, or vehicle (control; C). Lower panel shows densitometric analysis of the western blots. n = 3; *P < 0.05 vs. control. (D) HUVEC monolayers were treated with Y27632 (10 μM; 30 min) or vehicle (control; C). Lower panel shows densitometric analysis of the western blots. n = 3; *P < 0.05 vs. control. (E) Effect of Rac1 inactivation on RhoA activity. The RCEC or HUVEC monolayers were treated with Rac1 inhibitor NSC23766 (NSC; 100 μM) or vehicle (control; C), and active RhoA was detected by an ELISA-based assay as described in the Methods. The levels of total RhoA in the lysates were analysed by immunoblot and were used to normalize for loading. The active RhoA is given as x-fold of control. n = 3; *P < 0.05 vs. control. (F) Active Rac1. The RCECs were treated with NSC23766 (NSC; 100 μM) or vehicle (control; C).
knots, resulting in loss of cell demarcation (Figure 3B); however, these effects of IMD were reversible. Washing off the IMD and replacement with normal medium resulted in complete recovery of the actin cytoskeleton and cell shape (data not shown). These effects of IMD were completely abolished by pre-incubation of RCECs with a CGRP8–37 (Figure 3A and B) and PKI (see Supplementary material online, Figure S4B).

In contrast to RCECs, in HUVEC monolayers IMD induced a recruitment of VE-cadherin at adherens junctions and tightened the cell–cell contacts (Figure 3C). Like RCECs, IMD also induced a loss of actin stress fibres in HUVECs; however, IMD induced a shift of actin from stress fibres towards peripheral actin (Figure 3D), preserving the cellular architecture. These effects were significantly attenuated by PKI (see Supplementary material online, Figure S4B).

### 3.4 Intermedin inactivates the RhoA/Rho kinase pathway in both RCECs and HUVECs

The Rho family of GTPases, particularly RhoA and Rac1, are well-known regulators of actin cytoskeleton and hence the dynamics of adherens junctions. RhoA controls the formation of actin stress fibres running across the cell. Therefore, the activation state of RhoA was analysed by ELISA-based pulldown assay. The basal RhoA activity in RCECs was relatively high compared with that of HUVECs (data not shown), which corresponds to a higher number of stress fibres in these cells. RhoA activity was significantly abrogated by treatment with both IMD and FSK (Figure 3E). RhoA produces most of its effects via activation of its downstream effector, Rho-kinase (Rock). Therefore, the effect of IMD on Rock activity was
Figure 6 Effect of activation of RhoA and Rac1 on RCEC actin cytoskeleton and permeability. (A) RCECs were transfected with constitutive active RhoA (V14RhoA) or empty vector for 48 h and treated with IMD (10 nM; 30 min) or vehicle, and actin was stained with TRITC-phalloidin. (B) Western blot showing expression of RhoA in transfected cells. (C) Western blot showing Rac1 activity. RCECs were exposed to sodium orthovanadate (100 μM) or vehicle. (D) Actin and VE-cadherin staining. RCECs were exposed to IMD or Y27632 in the presence or absence of sodium orthovanadate (100 μM). (E) RCECs were exposed to IMD, vanadate plus IMD, or vehicle (control), and permeability was measured. n = 3; *P < 0.05 vs. control. (F) Schematic presentation of findings of the present study. Abbreviations: AJ, adherens junctions; Rock, Rho-kinase.
analysed. The activation state of Rock was measured by analysing the phosphorylation state at Thr850 of myosin phosphatase targeting subunit (MYPT1), a well-known endogenous direct substrate of Rock. Exposure of RCEC monolayers to IMD resulted in a significant reduction in phosphorylation of MYPT1 (Figure 3F). A similar effect was observed with FSK and a specific Rock inhibitor, Y27632. Likewise, exposure of HUVECs to IMD resulted in inactivation of RhoA (Figure 3G) to a similar extent as in RCECs, which corresponds to the loss of stress fibres in these cells, as shown in Figure 3D.

3.5 Intermedin has differential effects on Rac1 activity in RCECs and HUVECs

Rac1 controls the actin polymerization at the cell periphery and thus promotes VE-cadherin-mediated adherens junctions. Therefore, the effect of IMD on Rac1 activity was measured by pull-down assay. Incubating RCEC monolayers with IMD and FSK resulted in a significant loss in Rac1 activity (Figure 4A). This observation was in contrast to the effect of IMD seen in HUVEC monolayers. Exposure of HUVEC monolayers to IMD and FSK caused an activation of Rac1 (Figure 4B). To analyse whether the RhoA/Rock pathway interacts with Rac1 activity, we used specific inhibitors of RhoA and Rock. Inhibition of either RhoA by the specific cell-permeable inhibitor, C3-transferase (C3T), or downstream Rock by the specific inhibitor, Y27632, resulted in a significant inhibition of Rac1 activity in RCECs (Figure 4C) but not in HUVECs (Figure 4D). Furthermore, RhoA inhibition did not affect IMD-mediated Rac1 activation in HUVECs (Figure 4D). In the next step, we analysed whether Rock activity is also dependent on Rac1 activity. Exposure of either HUVEC or RCEC monolayers to NSC23766, a specific Rac1 inhibitor, did not affect RhoA activity (Figure 4E), although this concentration of NSC23766 completely blocked Rac1 activity (Figure 4F).

3.6 Inhibition of the RhoA/Rock pathway causes derangement of actin cytoskeleton and barrier failure in RCECs but not in HUVECs

It has previously been shown that IMD causes RhoA/Rock-dependent inactivation of Rac1 in RCECs, accompanied by derangement of the actin cytoskeleton and barrier failure. Therefore, it was analysed whether inhibition of RhoA or Rock exerts similar effects on the endothelial actin cytoskeleton and permeability in RCECs. These parameters were analysed in the presence of RhoA and Rock inhibitors. As shown in Figure 5A, exposure of RCECs to either C3T or Y27632 resulted in derangement of the actin cytoskeleton, as seen with IMD. Accordingly, Y27632 robustly increased endothelial macromolecule permeability (Figure 5B). The C3T effect was slow in progression, but the maximal effect was comparable to that of Y27632. The delayed effect of C3T is due to its slow membrane permeability. A structurally different Rho-kinase inhibitor, H1077, had a similar effect (data not shown).

Conversely, when HUVEC monolayers were exposed to a Rock inhibitor, it resulted only in loss of actin stress fibres without disturbing cortical actin and basal permeability (Figure 5C and D).

3.7 Activation of either RhoA or Rac1 protects RCECs against effects of IMD

To analyse the role of RhoA and Rac1 in the regulation of RCEC permeability, a set of rescue experiments was performed. Over-expression of a constitutively active RhoA abolished IMD-induced actin cytoskeleton derangement (Figure 6A). Rac1 was pharmacologically activated using sodium orthovanadate, which is known to activate Rac1 by an unknown mechanism. As shown in Figure 6C, vanadate activated Rac1 in RCECs, and when co-incubated with IMD or Y27632, it induced a shift of depolymerized actin induced by these agents towards cortical actin and relocated VE-cadherin at the cell borders (Figure 6D). Likewise, pre-incubation of RCECs with vanadate abolished the IMD-induced increase in permeability, and when vanadate was added to pre-stimulated RCEC monolayers with IMD, the permeability immediately returned to the basal level (Figure 6E).

4. Discussion

The aim of the present study was to analyse the molecular mechanisms of differential effects of IMD on the barrier function of endothelial cells from rat coronary microvasculature (RCECs) and HUVECs. The major and novel findings are as follows: (1) IMD increases macromolecule permeability of RCEC monolayers, while it reduces the permeability of macrovascular endothelial cell (rat aortic and HUVEC) monolayers in a receptor-dependent manner; (2) IMD inactivates both RhoA/Rock and Rac1 signalling in RCECs, while in HUVECs it activates Rac1 signalling; this results in (3) complete breakdown of actin architecture and adherens junctions in RCECs while preserving it in HUVECs; and (4) Rac1 activity lies downstream to RhoA/Rock signalling in RCECs but not in HUVECs.

Intermedin is a newly discovered member of the CGRP family and, like adrenomedullin, acts via the CLR/RAMP receptor complex through increased production of intracellular cAMP in a variety of systems, including heart. In line with these reports, the present study shows that IMD activates the cAMP/PKA pathway in both endothelial cell types under study in a receptor-dependent manner. The effects of IMD in RCECs were sensitive to αCGRP8–37, which indicates the presence of Cgrp receptor, while in HUVECs the effects of IMD were sensitive to hAM22–52, which indicates the presence of AM receptors.

Endothelial permeability is a dynamic process and is not uniform throughout the body; it is also differentially affected by physiological mediators such as cAMP-elevating agents. We have recently shown that IMD stabilizes the endothelial barrier in isolated mouse lungs and human pulmonary endothelial cells in a cAMP/PKA-dependent manner, which is in line with several other studies which report barrier-stabilizing effects of cAMP, and also with the present study, where we show that IMD reduces HUVEC and aortic endothelial cell permeability. In contrast, we and others have previously reported the barrier-disrupting effect of cAMP-enhancing agents in coronary endothelial cells, as well as in isolated coronary vessels. In congruence with these previous reports, here we show that IMD increases RCEC permeability. The dependence of this IMD effect on cAMP/PKA are well explained by the experiments using the specific PKA inhibitor, PKI. The dependence of this barrier-disrupting effect of cAMP on PKA was demonstrated by using a cAMP analogue, 6-Bnz-cAMP, which activates PKA specifically. Apart from PKA, cAMP may also activate Epac. The involvement of Epac in cAMP-induced barrier failure of RCEC monolayers was excluded by using the specific activator of Epac, 8-PT-cAMP. In HUVECs, however, the IMD-mediated reduction in permeability was partly blunted by PKA inhibition. This is in agreement with a recent publication by us, and others, where we show that cAMP signalling...
(FSK)-mediated barrier protection in HUVECs is partly dependent on PKA and Epac via two different but interacting signalling pathways. Why Epac signalling can reduce the permeability in HUVECs and has no effect on RCECs needs to be investigated further.

The integrity of the endothelial barrier is highly dependent on the endothelial actin cytoskeleton-mediated adherens junctions consisting of VE-cadherin, which together with other actin-binding proteins, seals the adjoining cells and thereby limits the passage of macromolecules across the microvasculature.2,3 In the present study, we demonstrate that IMD, as well as FSK, induced a loss of VE-cadherin from cell–cell borders in RCEC monolayers, while in contrast it enhanced VE-cadherin localization at cell–cell contacts in HUVEC monolayers. The localization of VE-cadherin at cell–cell junctions is highly dependent on actin cytoskeleton dynamics.4,37 The actin cytoskeleton, particularly the cortical actin, plays a crucial role in the assembly and organization of adherens junctions.1,4,37 Treatment of endothelial cells with the actin depolymerizing agent, cytochalasin, leads to loss of VE-cadherin from cell borders,4 indicating that an intact actin cytoskeleton is required for the maintenance of endothelial cell–cell adhesion structures. Here we show that IMD caused a complete derangement of actin cytoskeleton in RCEC monolayers in a reversible manner; however, opposite to this phenomenon, in HUVECs there was a rearrangement of the actin cytoskeleton, keeping the cellular morphology intact. The destruction of actin cytoskeleton architecture in RCEC monolayers indicates a strong activation of cellular actin depolymerizing and/or inhibition of actin polymerizing machinery by IMD.

Members of the Rho family of GTPases, RhoA and Rac1, are well-known regulators of the endothelial actin cytoskeleton and hence cell–cell junctions.5,6 RhoA, via its downstream Rock, regulates the formation and arrangement of actin stress fibres running across the cell, while Rac1 controls the assembly of peripheral actin and stimulates the formation of adherens junctions.4,6,37 Inhibition of RhoA alone would result in depolymerization of actin stress fibres, which would shift the balance towards peripheral actin cytoskeleton, thus enhancing the peripheral band. This phenomenon was seen in HUVECs. Intermedin caused an inhibition of RhoA and activation of Rac1, thus shifting the balance towards cortical actin and strengthening the VE-cadherin-mediated adherens junctions. Conversely, in RCECs IMD inhibits the activity of both RhoA/Rock and Rac1, resulting in complete depolymerization of the actin cytoskeleton and loss of cellular architecture. The requirement of Rac1 in maintenance of the barrier function of RCEC monolayers and isolated rat hearts has recently been demonstrated by us38 and in another cell type by others.39

As both RhoA and Rac1 were inhibited in IMD-treated RCECs, it was analysed whether activation of these GTPases is interdependent. We show for the first time that inhibition of either RhoA or downstream Rock with specific inhibitors resulted in inactivation of Rac1, demonstrating that the activity of Rac1 is regulated downstream to RhoA/Rock signalling in RCECs. However, it is still elusive how Rock regulates the Rac1 activity. One possible mechanism could be Rock-mediated phosphorylation of one of the Rac1 guanine nucleotide exchange factors (GEFs), as described recently for the phosphorylation of sif and Tiam1-like exchange factor (STEF), a Rac1 GEF, by Rock in COS-7 cells.40 Another possibility could be differential compartmentalization of cAMP/PKA activity, because it has recently been described that activation of soluble but not membrane adenyl cyclase can disrupt the endothelial barrier.41

Inhibition of Rock in HUVECs had no effect on Rac1 activity, indicating an independent regulation of RhoA and Rac1 activity. Inhibition of Rac1 with a specific inhibitor did not result in any change in RhoA activity in either cell type. If inhibition of RhoA or Rock results in inactivation of Rac1 in RCECs, it should also result in loss of the actin cytoskeleton and barrier function. This is clearly demonstrated in the experiment using RhoA and Rock inhibitors. Inhibition of either RhoA or Rock in RCECs resulted in derangement of the actin architecture and an increase in permeability comparable to that induced by IMD. In HUVEC monolayers, inhibition of RhoA/Rock signalling results only in loss of stress fibres while preserving the peripheral actin and cell shape and has no significant effect on basal permeability. Finally, the dependence of IMD-induced RCEC actin derangement on RhoA activity is demonstrated with rescue experiments. Overexpression of constitutively active RhoA abolished the IMD-induced derangement of the actin cytoskeleton. Sodium orthovanadate has been shown to activate Rac134 by an unknown mechanism and to modulate actin dynamics42 in endothelial cells. Sodium orthovanadate activated Rac1 in RCECs and induced the relocalization of IMD- and Y27632-induced depolymerized actin at the cellular periphery. It did not activate RhoA (data not shown), which is also evident from actin relocation only at the cell periphery and not at stress fibres. Along with actin, the VE-cadherin also relocated at cellular junctions. Likewise, vanadate reversed the IMD-induced increase in RCEC permeability.

In summary, we explain for the first time the molecular mechanism of differential regulation of the endothelial barrier of RCEC and HUVEC monolayers by cAMP/PKA signalling via differential regulation of Rac1 activity. Furthermore, we show for the first time that Rac1 activity in RCECs is regulated by the RhoA/Rock pathway. Figure 6f summarizes the findings of the present study and our previous reports.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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