# Insulin modulates the recovery of endothelial barrier function via Rac1 activation

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***Dedicated to:***

***.My family***

***Especially my parents for all the love, prayers and support.***

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| | |
|---|---|
| Approx. | Approximately |
| AJ | Adherens Junctions |
| ANOVA | Analysis of variance |
| APS | Ammonium per sulfate |
| ATP | Adenosine-5´-triphosphate |
| BSA | Bovine serum albumin |
| C | Control |
| °C | Degree Celsius |
| $Ca^{2+}$ | Calcium |
| $CaCl_2$ | Calcium chloride |
| cAMP | 3'-5'-cyclic adenosine monophosphate |
| cGMP | 3'-5'-cyclic guanosine monophosphate |
| cm/sec | Centimeters per second |
| $CO_2$ | Carbon dioxide |
| CPI-17 | PKC-potentiated inhibitor 17-kDa protein |
| DMSO | Dimethyl sulfoxide |
| DTT | 1,4-Dithiothreitol |
| EC | Endothelial cell |
| ECGS | Endothelial cell growth supplement |
| ECL | Enhanced chemiluminescence |
| MLCK | Myosin light chain kinase |
| MLCP | Myosin light chain phosphatase |
| EDTA | Ethylene diamine tetra acetic acid |
| eNOS | Endothelial nitric oxide synthase |
| F-actin | Filamentous actin |
| FCS | Fetal calf serum |
| G-actin | Globular actin |
| GTP | Guanosine-5´-triphosphate |
| GTPases | Guanosine triphosphate phosphohydrolyse |
| hrs | Hours |
| HBSS | Hanks' balanced salt solution |

| | |
|---|---|
| EGF | epidermal growth factor |
| HEPES | 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid |
| HUVECs | Human umbilical vein endothelial cells |
| HCl | Hydrochloric acid |
| IU | International unit |
| IR | Insulin receptor |
| IRS | Insulin receptor substrate |
| IgG | Immunoglobulin |
| IgM | Immunoglobulin M |
| I-R | Ischemia reperfusion |
| KCl | Potassium chloride |
| $KH_2PO_4$ | Potassium dihydrogen phosphate |
| kDa | Kilo Dalton |
| µM | Micromolar |
| $MgCl_2$ | Magnesium chloride |
| $MgSO_4$ | Magnesium sulphate |
| Min | Minutes |
| MLC | Myosin light chain |
| MLC~P | Phosphorylated myosin light chain |
| MLCK | Myosin light chain kinase |
| MLCP | Myosin light chain phosphatase |
| MAPK | Mitogen activated protein kinase |
| MYPT1 | Myosin phosphatase targeting subunit 1 |
| ML-7 | 1-(5-Iodonapthalene-1-sulfonyl) 1, 4-diazepine hydrochloride |
| mM | Millimolar |
| Nor | Normoxia |
| NO | Nitric oxide |
| n.s. | Not significantly different |
| $N_2$ | Nitrogen gas |
| NaCl | Sodium chloride |
| NaF | Sodium fluoride |
| $NaHCO_3$ | Sodium hydrogen carbonate |
| $Na_2HPO_4$ | Di-sodium hydrogen phosphate |

| | |
|---|---|
| $NaH_2PO_4$ | Sodium dihydrogen phosphate |
| Na-orthovanadate | Sodium orthovanadate |
| NP-40 | Nonidet P-40 |
| P | probability |
| PAK | p21-activated kinase |
| PBS | Phosphate-buffered saline |
| PI3K | Phosphatidylinositol-3-kinase |
| pH | Negative log of H+ concentration |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PMSF | Phenylmethylsulfonyl fluoride |
| PP1 | Protein phosphatase 1 |
| PFA | Paraformaldehyde |
| Rock | RhoA-dependent protein kinase |
| Ser19 | Serine 19 |
| SDS | Sodium dodecyl sulfate |
| SDS-PAGE | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| VSMC | Vascular smooth muscle cells |
| SEM | Standard error of means |
| Thr18 | Threonine 18 |
| Thr696 | Threonine 696 |
| Thr850 | Threonine 850 |
| TBS | Tris-buffered saline |
| TEMED | N, N, N', N',-tetramethylethylenediamine |
| Tris | Tris (hydroxymethyl) aminomethane |
| VE-cadherin | Vascular endothelial cadherin |
| % vol/vol | Volume by volume percentage |
| % wt/vol | Weight by volume percentage |

# 1. INTRODUCTION

## 1.1 Endothelial barrier function

Vascular endothelium lining the intima of blood vessels acts as a gatekeeper and actively controls the exchange /trafficking of water, small solutes, ions, blood cells and macromolecules across the vessel wall (Michel and curry, 1999; Mehta et al., 2004; Rao et al., 2005; Mehta and Malik, 2006). Failure of this endothelial cell (EC) barrier function results in an increased EC permeability, leakage of blood components and exudation of fluids to interstitium which may finally result in life threatening edema formation and may jeopardize survival of the tissue and causes organ dysfunction and may also contributes to pathogenesis of chronic cardiovascular diseases such as atherosclerosis and diabetes-associated vascular disease (Lum and Malik, 1996; van Hinsbergh 1997; Wojciak-Stothard et al., 1998, Libby et al., 2006; Pober and Sessa, 2007; Stocker and Keaney, 2004; van Hinsbergh and Van Nieuw Amerongen, 2002; Mehta and Malik, 2006; Bazzoni, 2006; Yuan et al., 2007; Kumar et al., 2009).

The maintenance of EC barrier integrity is crucial in maintaining physiological functions of different organs and is determined by the precise equilibrium between competing EC contractile forces generated by the actomyosin based EC contractile machinery and adhesive tethering forces generated by adhesive molecules located at endothelial cell-cell junctions and cell-matrix contacts (Garcia et al., 1995; Lum and Malik, 1996; Baldwin and Thurston 2001; Dudek and Garcia, 2001; Bogatcheva et al., 2002; Shen et al., 2009).

Inflammatory mediators such as thrombin, tumor necrosis factor alpha (TNF-α) and histamine disrupt the balance between contractile and adhesive forces, increase EC permeability via activation/stimulation of multiple signaling mechanisms, leading to activation of EC contractile machinery, weakening of cell adhesion structures, opening of gaps between adjacent cells and hence finally leading to EC barrier dysfunction (Schnittler et al., 1990; Garcia et al., 1995; 1996; Rabiet et al., 1996; Wojciak-Stothard et al., 1998; Gündüz et al., 2003; Birukova et al., 2004). Thrombin also inhibits the Rho GTPase Rac1 activation (Aslam et al., 2012) finally leading to disruption of EC barrier integrity, which is followed by slow recovery of the EC barrier function.

The mechanisms of EC barrier restoration subsequent to barrier disruption in response to the permeability increasing factors are essential for the maintenance of basal endothelial monolayer permeability and to prevent edema formation. However the precise mechanisms involved in this recovery of EC barrier function still remains to be elucidated. Comparatively little is known about the maneuvers that control the restoration of compromised EC barrier function.

Insulin is the principal hormone of metabolic homeostasis. Recent clinical findings demonstrate that intensive insulin therapy confers vasoprotective effects under inflammatory conditions and reduces diabetes associated major cardiovascular complications because of its anti-inflammatory and anti-atherosclerotic effects (Dandona et al., 2009; Kim et al., 2006; Nathan et al., 2005; Langouche et al., 2005).

The aim of the present study was to elucidate the molecular mechanism by which insulin may restore the EC barrier function in response to thrombin-induced hyperpermeability. Special emphasis was laid on the potential role of Rho GTPase Rac1. The study was carried out in a well-established *in vitro* model of Human umbilical vein endothelial cells (HUVECs) and a model of isolated saline perfused rat hearts.

## 1.2 Regulation of endothelial barrier function

### 1.2.1 Endothelial cell-cell junctions

Barrier function of vascular endothelium is mainly regulated by the dynamic opening and closure of gaps between intercellular junctions. These inter-endothelial junctions mediate cell-cell adhesion and facilitate communication between adjoining ECs and dynamically control the permeability of vessel wall endothelial barrier (Bazzoni and Dejana, 2004; Dejana, 2004 and 2008).

In ECs, adherens junctions (AJs) are of fundamental importance in mediating endothelial cell-cell adhesion and have a prominent role for regulating normal functioning of the endothelial barrier (Bazzoni and Dejana, 2004; Wallez and Huber, 2008; Dejana and Giampietro, 2012). AJs are predominantly composed of vascular

endothelial (VE)-cadherin linked to peripheral actin cytoskeleton present directly under the cell membrane. The interaction of AJs with the actin cytoskeleton is important for stabilization of junctions. VE-cadherin is present nearly in all vascular beds (Dejana et al., 1995; Lampugnani et al., 1995; Gao et al., 2000; Mehta and Malik, 2006; Vandenbroucke et al., 2008; Komarova and Malik, 2010; Giannotta et al., 2013). VE-cadherin is required for the proper assembly of AJs and plays a crucial role in the maintenance of normal EC barrier function. VE-cadherin glues the adjacent ECs together by homotypic binding with VE-cadherin on the neighboring cell's plasma membrane in calcium ($Ca^{2+}$)-dependent manner. VE-cadherin has extracellular and cytoplasmic domains and is intracellularly linked to the actin cytoskeleton via family of catenin (Lampugnani et al., 1995; Iyer et al., 2004; Mehta and Malik, 2006; Vandenbroucke et al., 2008; Dejana et al., 2008; Prasain and Stevens, 2009; Shen et al., 2009; Komarova and Malik, 2010). Catenins not only serve as a structural linkage between VE-cadherin and actin cytoskeleton but also transduce biochemical signals for cell-cell communications. AJs disassemble in response to inflammatory mediators leading to an increase in EC permeability (Mehta and Malik, 2006). Thrombin-induced phosphorylation of p120 catenin may also contribute to AJ disassembly (Konstantoulaki et al., 2003). The stability of the VE-cadherin, catenins and actin cytoskeleton complex is essential to maintain the AJ integrity and restrictiveness of EC barrier (Vincent et al., 2004; Sallee, 2006; Alcaide et al., 2008; Zebda et al., 2013).

### 1.2.2 Endothelial cell cytoskeleton

ECs have a functional cytoskeleton which is composed of three primary elements: actin filaments, intermediate filaments and microtubules which are in constant communication with one another (Dudek and Garcia 2001; Chang and Goldman, 2004; Revenu et al., 2004; Prasain and Stevens, 2009; Shen et al., 2009). Endothelial cytoskeleton is very important for cell motility, EC shape, endo-or exocytosis and cell- cell adhesion. Among these filamentous structures actin filaments are best characterized for its role in regulating EC permeability.

In ECs actin and myosin are major contractile components and together comprise of ~16% of total endothelial cellular proteins (Schnittler et al., 1990; Wong

and Gotlieb, 1990). Whereas actin alone represents about 5-15% of the total protein in ECs (Patterson and Lum, 2001) and exists in two different forms: in a monomeric globular form, known as G-actin and in a filamentous form, called F-actin (Tobacman and Korn, 1983). In general, the amount of F-actin and G-actin exists in an almost equal balance (Stossel et al., 1985).

Permeability increasing factors such as thrombin increases polymerization of actin filaments that leads to actin reshaping to form stress fibers (Goeckeler and Wysolmerski, 1995; Ehringer et al., 1999; van Nieuw Amerongen et al., 2000a; 2000b; Sandoval et al., 2001; Mehta et al., 2002). Stress fibers are composed of bundles of actomyosin that are necessary for inducing cell contraction (Hotulainen and Lappalainen, 2006) and consists of relatively short F-actin filaments (Brenner and Korn, 1979 and 1980; Cramer et al., 1997; Heimann et al., 1999) whereas cortical actin rim is comprised of long F-actin filaments (De Matteis and Morrow, 2000; Heimann et al., 1999). Stress fibers extends throughout the cytosol, they generate centripetally (inward) directed tension, promotes gaps formation between adjacent ECs and may therefore contribute to endothelial hyperpermeability (Vouret-Craviari et al., 1998; Dudek and Garcia, 2001; Hotulainen and Lappalainen, 2006).

The actin cytoskeleton undergoes depolymerization and polymerization based upon cellular demand. Both hyper-polymerization of F-actin by Jasplakinolide and depolymerization of F-actin-induced by cytochalasin D interfere with EC barrier function (Waschke et al., 2005) suggesting that polymerization state of actin cytoskeleton is critical for the maintenance of EC barrier functions. Furthermore, inhibition of actin polymerization prevented force generation (Kolodney and Wysolmerski, 1992) as well as endothelial monolayer hyperpermeability in response to permeability increasing factors (Vouret-Craviari et al., 1998; Mehta et al., 2002;). Thus actin cytoskeleton is a key player and an important actuator for the regulation of EC barrier function.

## 1.3 Endothelial contractile machinery

Endothelial contractile machinery is another important regulator of EC barrier function. It is well documented that mechano-chemical interaction between actin and myosin is primarily believed to be involved in driving EC contractile machinery and

thereby EC barrier integrity (Schnittler et al., 1990). It is well established that activation of the EC contractile machinery leads to an increase in permeability. The activation of EC contractile machinery is mainly controlled by the phosphorylation state of the regulatory myosin light chain (MLC) (Wysolmerski and Lagunoff, 1991) and the balanced activities of MLC kinase (MLCK) (Lazar and Garcia., 1999), and MLC phosphatase (MLCP) (Härtel et al., 2007; Knapp et al., 1999; Verin et al., 2000) determine the net phosphorylation state of the MLC and actin-myosin contractility (Shen et al., 2009 and 2010).The major components of EC contractile machinery are MLC, MLCK and MLCP and are discussed below.

MLC is a small protein of 20 kDa and phosphorylation of MLC induces a conformational change in the tertiary protein structure of myosin which increases actin-myosin interaction, this actin-myosin interaction generates a contractile force that retracts adjacent ECs apart from each other, leading to intercellular gap formation and finally loss of barrier function, whereas MLC dephosphorylation causes stabilization of endothelial barrier (Wysolmerski and Lagunoff, 1990; Sheldon et al., 1993; Garcia et al., 1995; Goeckeler and Wysolmerski, 1995; Moy et al., 1996 and 2002; Gündüz et al., 2003; Takashima, 2009; Kasseckert et al., 2009).

MLCK is a $Ca^{2+}$/calmodulin (CaM)-dependent kinase which phosphorylates MLC at Ser-19 and/or Thr-18 (Goeckeler and Wysolmerski, 1995; Kamm and stull 1986; Moy et al., 1993; Sheldon et al., 1993; Garcia et al., 1995; Amano et al., 1996; Hixenbaugh, 1997; Verin et al., 1998; Shen, 2010). It is well-established that MLCK becomes activated via $Ca^{2+}$ dependent mechanisms in response to inflammatory mediators like thrombin and histamine which leads to an increase in MLC phosphorylation, finally leading to increase in endothelial monolayer permeability and leaky barrier (Sheldon et al., 1993; Dudek and Garcia, 2001).

Initially in bovine pulmonary artery EC monolayers, the involvement of MLCK in the regulation of barrier permeability via MLC phosphorylation was discussed by Wysolmerski and Lagunoff (1990). Subsequent studies by using both *in vivo* and *in vitro* approaches/techniques have further elucidated the important role of MLCK in the regulation of permeability (Wainwright et al., 2003; Parker et al., 2000; Tinsely et al., 2000; Yuan et al., 1997; Khimenko et al., 1996; Garcia et al., 1995; Sheldon et al., 1993).

Besides MLCK, MLCP is also involved in controlling the phosphorylation state of MLC in ECs. Conversely, MLCP facilitates MLC dephosphorylation thus counteracts the MLCK activity, thereby terminating the tension, relaxes the actin cytoskeleton, and reduces the EC barrier permeability (Verin et al., 1995; Essler et al., 1998). The endothelial MLCP is a holoenzyme complex composed of three subunits, a regulatory myosin phosphatase targeting subunit (MYPT1, 130 kDa), a catalytic subunit, protein phosphatase 1 (PP1, 37-38 kDa) and a 20 kDa subunit of unknown function (Alessi et al., 1992; Shimizu et al., 1994; Shirazi et al., 1994; reviewed by Hartshorne et al., 1998; Verin et al., 2000; Goeckeler and Wysolmerski, 2005).

It is well-established that MLCP activation is regulated through phosphorylation of MYPT1 at its two main inhibitory phosphorylation sites, Thr696 and Thr850 (Kimura et al., 1996) or through direct inactivation of the catalytic subunit, PP1 by CPI 17 (an endogenous inhibitor of PP1). Several kinases have been described to phosphorylate MYPT1 at one or both of these sites. The major well-known kinase is RhoA-dependent kinase (Rock) that phosphorylates MYPT1 at both sites and inhibits MLCP activity (Härtel et al., 2007; Pandey et al., 2006; Goeckeler and Wysolmerski, 2005; Hartshorne, 1998; Fukata et al., 2001) thereby promoting MLC phosphorylation, actomyosin contraction and finally leading to increased EC permeability (van Nieuw Amerongen et al., 1998; Essler et al., 1998; Birukova et al., 2004).

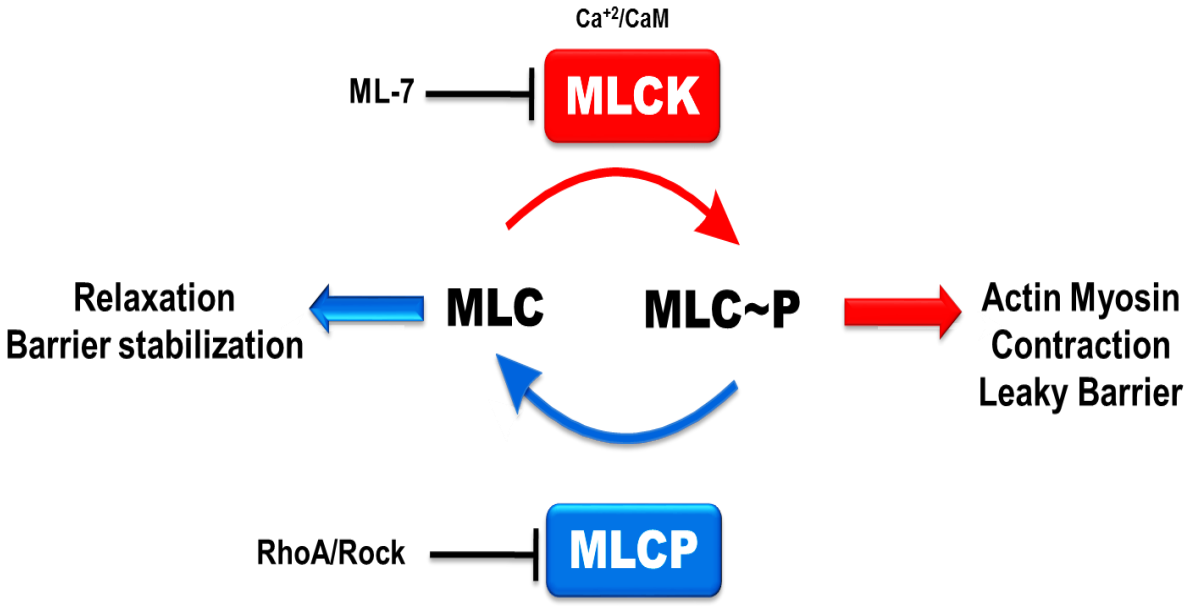



**Figure 1.1 Regulation of EC barrier function by EC contractile machinery**: Actin-myosin contraction is increased by MLC phosphorylation (MLC~P) and is an important step in the regulation of the activation of the EC contractile apparatus. MLC is phosphorylated by ($Ca^{2+}$/CaM)-dependent MLCK while dephosphorylated by MLCP. Activation of the EC contractile machinery leads to EC contraction and barrier dysfunction, whereas inactivation leads to relaxation and barrier stabilization. ML-7 is a specific inhibitor of MLCK while MLCP can be inactivated by RhoA/Rock.

## 1.4 Rho GTPases

The members of the Rho-family of monomeric GTPases (RhoA, Rac1 and Cdc42) have been well recognized to play an important role in regulating endothelial barrier integrity and have been implicated in controlling the endothelial actomyosin contractile machinery, actin cytoskeleton dynamics and integrity of cell adhesion structures (Wojciak-Stothard et al., 2001; Fukata and Kaibuchi, 2001; Wojciak-Stothard and Ridley, 2002). Two Rho GTPases, Rac1 and RhoA, act antagonistically; have emerged as key regulators of EC barrier function (Essler et al., 1998 and 1999; Hall, 1998; Wojciak-Stothard et al., 2001; Ridley, 2001; Braga, 2002; Etienne and Hall, 2002; Mehta and Malik, 2006; Vandenbroucke et al., 2008; Dejana et al., 2008; Aslam et al., 2011).

It is well-established that the Rho GTPase RhoA plays a vital role in regulating actomyosin contractile machinery in ECs and thereby is the key determinant of EC hyperpermeability (Carbajal and Schaeffer, 1999; Holinstat et al., 2003; Mehta et al., 2001; Van Nieuw Amerongen et al., 1998 and 2000). RhoA, through its specific downstream effector kinase, Rock. inactivates MLCP via phosphorylation of MYPT1 at its inhibitory sites which attenuates the phosphatase activity (Essler et al., 1998 and 1999; Totsukawa et al., 2000; Verin et al., 2001; Birukova et al., 2004; Vandenbroucke et al., 2008; Kumar et al., 2009; Härtel et al., 2007) thereby resulting in net increase in phosphorylated regulatory MLC and actomyosin contractility which facilitates disruption of the endothelial barrier. RhoA is also known to induce derangement of actin cytoskeleton in terms of stress fibers formation and disassembly of endothelial AJs in response to thrombin, histamine or bradykinin leading to increased EC permeability (Birukova et al., 2004a; Birukova et al., 2004d; Wojciak-Stothard et al., 2001; Wojciak-Stothard and Ridley, 2002; Essler et al., 1998 and 1999; Van Nieuw Amerongen, 2000).

In contrast to RhoA, Rac1 is required for the stability of VE-cadherin mediated endothelial AJs and thus plays a role in the stabilization of the EC barrier function (Wojciak-Stothard et al., 2001; Waschke et al., 2004). Rac1 activation reorganizes the actin cytoskeleton at the cell periphery and thus strengthens the establishment of endothelial AJs (Hall, 1998; Wojciak-Stothard et al., 2001; Wojciak-Stothard and Ridley, 2002; Kumar et al., 2009) and also reduces the macromolecule permeability

of EC monolayers (Waschke et al., 2006). The activation of Rac1 is regulated by GDP-GTP cycling, induced by specific guanine nucleotide exchange factors (GEFs), Tiam1 and TrioN, which control the transition of the inactive GDP-bound conformation to the active GTP-bound conformation (Gao et al., 2004). The activation of Rac1 can be inhibited by the specific pharmacological inhibitor NSC23766, which specifically inhibits Rac1 activity by blocking the binding of the Rac1 specific GEFs Tiam1 and TrioN (Gao et al., 2004). Even though, much is still a mystery about the role of Rho GTPases in EC permeability, the activation of Rac1 appears to be a suitable approach to stabilize or recover EC barrier function in inflammatory situations.

## 1.5 Endothelial barrier dysfunction: Endothelial hyperpermeability

Endothelial barrier dysfunction is a significant problem and resulting vascular leakage is implicated in the pathogenesis of various diseases including inflammation, trauma, sepsis, diabetes associated vascular disease, atherosclerosis and acute lung injury. Various inflammatory conditions are associated with the deterioration of EC barrier function and pathophysiology is characterized by leakage of fluid, plasma proteins or small molecules in the extra vascular space leading to tissue edema which finally results into severe, sometimes life threatening organ dysfunction.

There are three key mechanisms believed to be involved in inflammatory conditions that lead to endothelial hyperpermeability. (1) The cytoskeleton rearrangement: remodeling of cortical actin and generation of stress fibers, (2) activation of EC actomyosin based contractile machinery and (3) disassembly of AJs. All these elements together lead to formation of intercellular gaps, which perturb the normal EC barrier function (McDonald et al., 1999; Mehta and Malik, 2006).

Thrombin, a procoagulant serine protease, is a central vascular mediator of inflammation and hemostasis, generated in the vessels from pro-thrombin circulating in blood. Thrombin induces rise in intracellular $Ca^{2+}$ levels which phosphorylates MLC through activation of MLCK and RhoA/Rock signaling which downstream inhibits the MLCP finally leading to strong activation of EC contractile machinery and thereby contributes to thrombin-induced loss of EC barrier function (Lum and Malik, 1996; Dudek and Garcia, 2001; Coughlin, 2000; Bogatcheva et al., 2002). Thrombin-induced

increased vascular permeability has also been attributed to stress fiber formation. Thrombin also inhibits Rho-GTPase Rac1 activation (Aslam et al., 2012) that leads to disruption of endothelial AJs and thereby EC barrier integrity.

Ischemia-reperfusion (I-R) also induces loss of EC barrier function and the resulting myocardial edema jeopardizes the functional recovery of vital organs such as heart during reperfusion and may inhibit survival of myocardial tissue (Garcia-Dorado and Oliveras, 1993; Mehlhorn et al., 2001; Carden and Granger, 2000; Schäfer et al., 2003; Gündüz et al., 2006). I-R injury refers to edematous swelling of the reperfused tissue when the blood supply returns after an ischemic period (Dagenais et al., 1997; Eltzschig and Collard, 2004). This restoration of blood supply causes tissue inflammation due to neutrophil activation and release of cytokines. Previous reports indicate that reperfusion triggers the opening of intercellular gaps between ECs and $Ca^{2+}$ dependent activation of the EC contractile machinery contributes to the barrier failure under reperfusion (Schäfer et al., 2003). Previously, in saline-perfused hearts, a rapid post ischemic edematous swelling of the myocardium has also been observed (Noll et al., 1999; Di Napoli et al., 2001). Better insights into the molecular mechanisms related to endothelial hyperpermeability is required for developing effective therapeutic strategies.

## 1.6 Recovery of endothelial barrier function after barrier dysfunction

The mechanism of recovery of EC barrier function is critical for the maintenance of basal permeability and is important to prevent the potentially fatal increase in EC permeability. Failure to recover EC barrier function likely underlies the morbidity and mortality associated with vascular diseases. In contrast to mechanisms involved in regulating barrier disruption, detailed knowledge of the signaling pathways responsible for recovery of EC barrier function is, at present, lacking. Permeability increasing mediators usually induce a reversible increase in EC permeability (Tiruppathi et al., 1992; Moy et al., 1996; Mehta et al., 2002).

The process of barrier recovery requires the reannealing of previously opened gaps between the adjacent cells, suppression or inactivation of EC contraction (Garcia et al., 1995, Gündüz et al., 2003) and strengthening of cell-cell adhesion structures

(Mehta et al., 2002; Quadri et al., 2003; Kouklis, 2004). AJs disassemble in response to thrombin, increasing EC permeability; however, AJs also possess the ability to reassemble. This reannealing of AJs causes reversal of increase in endothelial monolayer permeability and restoration of vascular endothelial barrier integrity (Lum and Malik, 1996; Dudek and Garcia, 2001; Lampugnani et al., 1995; Kouklis et al., 2004; Broman et al., 2007; Dejana et al., 2008).

Studies have shown the crucial role of Rho GTPases (Rac1 and CDC42) in regulating recovery of EC barrier function. Both Rac1 and CDC42-induce reorganization of actin cytoskeleton, apposition of AJs at the plasma membrane and thus promotes reannealing of AJs (Kaibuchi et al., 1999). Rac1 is reported to be involved in actin cytoskeleton remodeling and lamellipodia formation (Hall, 1998 and 2005; Ehrlich et al., 2002). The mediators like oxidized lipids (Birukov et al., 2004), sphingosine 1- phosphate (S1P) (Singleton et al., 2005) ATP (Jacobson et al., 2006) and cAMP (Aslam et al., 2014) are known to restore EC barrier function by Rac1 activation.

Several signaling pathways have been shown to contribute in regulating recovery of EC barrier function: (a) p120 catenin (Iyer et al., 2004), p120 catenin participates in regulating endothelial barrier restoration by controlling the stability of VE-cadherin expression and, hence, AJs stability. (b) Activation of focal adhesion kinase (FAK) (Holinstat et al., 2006) also plays an important role in regulating barrier restoration. FAK suppress RhoA activity via P190RhoGAP activation which in turn inhibits EC contraction and thereby induces recovery of EC barrier function (Quadri et al., 2003; Holinstat et al., 2006). (c) Angiopoietin 1 activation of Tie2 receptor (Mehta and Malik 2006), (d) FoxM1 transcription factor (Zhao et al. 2006), (e) Sphingosine 1-phosphate (S1P) (Hla, 2003). S1P, a biologically active lipid secreted by red blood cells and activated platelets has also emerged as potent barrier stabilizing factor in cultured ECs and in intact microvessels (Mcverry and Garcia, 2004; Peters and Alewijnse, 2007). S1P mediated its effects via a number of downstream targets including Rac1 mediated cytoskeletal reorganization that promoted AJs assembly and EC barrier enhancement (Mehta et al., 2005; Singleton et al., 2005). (f) cAMP signaling also contributes to the recovery of EC barrier function after thrombin-induced hyperpermeability (Aslam et al., 2014). Evidence indicates that an increase in the

concentration of cAMP can be EC barrier protective. The cAMP downstream activates two effector proteins, the protein kinase A (PKA) and Epac (de Rooij et al., 1998; Aslam et al., 2010). Both PKA and Epac downstream activates Rac1 (Birukova et al., 2008 and 2010). Inhibition of these cAMP downstream effectors abrogates Rac1 activation, re-establishment of AJs and impedes the recovery of EC barrier function (Aslam et al., 2014). Better knowledge of the signals responsible for reversing the endothelial hyperpermeability may therefore lead to novel anti-inflammatory therapeutic targets capable of preventing inflammatory diseases manifested by vascular leak.

## 1.7 Insulin

Insulin is a key regulator and essential hormone of metabolic homeostasis. Based on previous findings there is convincing evidence that in addition to its well-known metabolic actions insulin also confers protective effects on the cardiovascular system and plays an important role in the normal functioning of the vasculature (Verma and McNeill, 1999; Mather et al., 2001).

Insulin biological actions are mediated by specific cell surface receptors. Like many other cell types ECs also possess insulin receptors (IR) (~ 40,000 IR per cell) (Zeng and Quon, 1996) with intrinsic tyrosine kinase activity. The insulin receptor is a heterotetrameric transmembrane glycoprotein composed of two α and β subunits (Kahn, 1985; Kahn and Crettaz 1985; Rosen et al., 1987). Insulin initiates its action by binding to specific receptors on the surface of the cell. The specific binding of insulin to its cell surface receptor leads to autophosphorylation on the IR- β subunit and tyrosine phosphorylation of intracellular substrates that include insulin receptor substrate (IRS) family members and other signaling molecules such as Shc. IRS is an essential component of insulin signaling pathway. Phosphorylated IRS serve as docking proteins for several downstream effectors of insulin signaling such as phosphatidylinositol 3-kinase (PI3K) and Grb2 (White, 2002; Gual et al., 2005). Phosphorylation of IRS proteins on multiple tyrosine residues by the activated IR initiates the activation of PI3K (Montagnani et al., 2002).

In response to insulin, two major signaling pathways are activated: the PI3K/Akt signaling pathway and mitogen activated protein kinase (MAPK) pathway (Myers and White, 2002; Taniguchi et al., 2006). Downstream from PI3K there is also an essential component of insulin signaling pathway called 3-phosphoinositide dependent protein kinase (PDK)-1. This kinase is crucial for the activation and phosphorylation of serine/threonine protein kinase Akt and atypical protein kinase C isoforms (Alessi et al., 1997; Vanhaesebroeck and Alessi, 2000). Protein kinase B (Akt) directly phosphorylates and activates endothelial NO synthase (eNOS) at Ser $^{1177}$, resulting in enhanced eNOS activity which leads to increased production of Nitric oxide (NO) within a matter of minutes (Alessi et al., 1997; Dimmeler et al., 1999; Dimmeler and Zeiher, 1999; Vanhaesebroeck and Alessi, 2000; Montagnani et al., 2001 and 2002; Kuboki et al., 2000; Zeng et al., 1996 and 2000).

PI3K/Akt signaling pathway has been termed as “survival pathway”. It has been identified to confer protective effect against reperfusion injury of the heart (Hausenloy and yellon, 2004). PI3K/Akt is a major component in insulin signaling pathway and inhibition of PI3K blocks NO production in response to insulin, demonstrating its essential role in insulin stimulated production of NO (Zeng and Quon 1996; Zeng et al., 2000; Fisslthaler et al., 2003; Hartell et al., 2005). *In vivo* administration of insulin is also known to attenuate reperfusion-induced cell death through PI3K/Akt and eNOS signaling pathway (Gao et al., 2002).

There is convincing evidence that insulin induces NO production in microvascular ECs and inhibition of eNOS not only prevented insulin-induced NO production but also in part antagonized insulin mediated microvascular EC barrier stabilization (Gündüz et al., 2010). Insulin also prevents oxidant-induced EC barrier dysfunction by inducing the release of NO (Rath et al., 2006). Insulin also acts as a vasodilatory hormone that mainly depend on endothelium derived NO (Steinberg et al., 1994; Scherrer et al., 1993; Laine et al., 2000; Sundell et al., 2002; Scott et al., 2002; Sundell and Knuuti, 2003; Vincent et al., 2006).

It is well known that insulin inhibits thrombin-induced vascular smooth muscle cell (VSMC) contraction (begum et al., 2000). Insulin-induces the relaxation of VSMCs via activation of myosin-bound phosphatase and inhibition of Rho kinase activity via NO/cGMP-dependent pathway (begum et al., 2000, Sandu et al., 2001). In VSMCs

insulin negatively regulates Rho signaling by preventing RhoA activation via the NO/cGMP signaling pathway which leads to myosin-bound phosphatase activation, disorganization of actin cytoskeleton and vasodilation (begum et al., 2002).

Previous reports indicate that insulin has profound protective effects in critical illness and in acute inflammatory conditions (Melin et al., 2002; Malmberg et al., 1995; Kinsley, 2004 Lewis et al., 2004; Furnary et al., 2004 Ritchie et al., 2004). Several reports have reported that insulin has direct protective effects on ECs (Aljada et al., 2000 and 2001; Jeschke et al., 2004; Fischer-Rasokat and Doenst, 2003).

Several clinical and experimental studies dealing with I-R could show that insulin dose at the time of reperfusion protects against the reperfusion-induced vascular injury in the heart (Melin et al., 2002; Malmberg et al., 1995; Ma et al., 2006). Insulin also protects cardiomyocytes against acute reoxygenation-induced hyper-contracture via activation of survival pathway consisting of PI3K, eNOS and cGMP-dependent protein kinase (PKG) (Abdallah et al., 2006).

Insulin increases myocardial blood flow and reduces coronary vascular resistance in a dose dependent manner. Moreover the mortality rate was reduced in critically ill patients who were more than 3 days in intensive care unit provided with intensive insulin therapy (Van den Berghe et al., 2001; Furnary et al., 2003; Van den Berghe, 2004; Lewis et al., 2004; Krinsley, 2004; Jeschke et al., 2004). Several clinical reports also show that intensive insulin therapy has vasoprotective effects under inflammatory conditions (Van der Berghe, 2001; Melin et al., 2002; Ritchie et al., 2004; Langouche et al., 2005; Dandona et al., 2009) and reduces major cardiovascular complications in diabetics (Nathan et al., 2005).

Previous reports indicate that in rat coronary microvascular endothelial monolayers, insulin not only stabilizes endothelial barrier under basal conditions but also antagonized the hyperpermeability-induced by inflammatory mediator, tumor necrosis factor alpha (TNFα) (Gündüz et al., 2010). This barrier stabilizing effect of insulin is mediated via PI3K/Akt and NO/cGMP-induced activation of Rac1 (Gündüz et al., 2010). However, the detailed mechanism of insulin mediated accelerated recovery of disrupted endothelial barrier integrity in response to permeability increasing factors is still elusive.

## 1.8 Aims and objectives of the study

The main aim of the present study was to broaden our knowledge on the contribution of insulin-mediated recovery of EC barrier function under patho-physiological conditions. Thrombin is used as a model inflammatory mediator, to activate EC barrier disrupting signaling or to induce EC monolayer hyperpermeability, followed by slow recovery of the EC barrier function. The mechanisms of EC barrier recovery are critical for the maintenance of basal monolayer permeability. Here the hypothesis was tested whether insulin can mediate the fast restoration/recovery of EC barrier function in response to thrombin-induced hyperpermeability. Moreover, a model of isolated saline perfused rat heart is applied to further prove the protective effects of insulin on endothelial barrier integrity in an intact coronary system.

The following questions were addressed in this thesis.

- Does PI3K/Akt pathway play a role in insulin-mediated restoration of EC barrier function?
- Does insulin has any effect on inactivation of EC contractile machinery and fast re-establishment of AJs in response to thrombin-induced EC barrier failure?
- Does RhoA/Rock signaling pathway play a role in insulin-mediated recovery of EC barrier function in response to thrombin challenge?
- Does eNOS/NO pathway play a role in insulin-mediated EC barrier recovery?
- Does Rho GTPase Rac1 play a role in insulin-mediated restoration of EC barrier function?
- Does insulin protect the heart against reperfusion-induced injury and imminent life threatening edema, in the intact isolated saline perfused rat heart?

To achieve these objectives a well-established *in vitro* model of HUVECs and a model of isolated saline-perfused rat hearts were used. Inflammatory mediator thrombin was present during the whole experimental period in order to imitate/emulate *in vivo* conditions.

The following experimental approaches were used to answer these questions.

- Macromolecule albumin permeability across HUVEC monolayers was used as functional assay to assess the impact of insulin on EC barrier restoration.
- Dynamics of activities of EC contractile machinery i.e. MLC and MYPT1 phosphorylation was analyzed by Western blot analysis.
- The effects of insulin on VE-cadherin based AJs were visualized by immunofluorescence confocal microscopy.
- Activation of Rac1 was analyzed by pull down assay.
- Myocardial water contents were determined by Langendorff perfusion system.

# 2. MATERIALS

## 2.1 Laboratory instruments

| | |
|---|---|
| Beckman Allegra 64R centrifuge | Beckman Coulter, USA |
| Beckman TL 100 ultracentrifuge | Beckman Coulter, USA |
| Electroblot chambers | Biotech-Fischer, Reiskirchen, Germany |
| Electrophoresis apparatus | Biometra, Goettingen, Germany |
| Gel documentation system (ChemiSmart 5000) | Peqlab, Erlangen, Germany |
| Glas coverslips | Menzel, Braunschweig |
| Glass ware | Schott, Mainz, Germany |
| Hamilton syringe | Hamilton, Bonaduz, Switzerland |
| Incubators | Heraeus, Hanau, Germany |
| Laminar flow hood | Heraeus, Hanau, Germany |
| LSM-510 Meta confocal microscope | Carl Zeiss, Jena |
| Magnet stirrer | Jahnke und Kunkel, Staufen, Germany |
| Neubauer chamber | Superior, Marienfeld, Germany |
| Phase contrast microscope | Olympus, Japan |
| PH-Meter | WTW-Weinheim, Germany |
| Photometer | Zeiss, Jena, Germany |
| Power supply | Biometra, Goettingen, Germany |
| Rocker | Biometra, Goettingen, Germany |
| Shaker | Biometra, Goettingen, Germany |
| Table top (centrifuge) | Eppendorf, Hamburg, Germany |
| Tubes | Eppendorf-Netheler-Hinz, Germany |
| Vortexer | Heidolph, Kelheim, Germany |
| Water bath | Julabo, Seelbach, Germany |
| Water demineralization unit | Millipore, Eschborn, Germany |

## 2.2 Chemicals and consumables

| | |
|---|---|
| Acrylamide/Bisacrylamide (1:19) Solution 40% | SERVA, Heidelberg, Germany |
| Ammonium persulfate | SERVA, Heidelberg, Germany |
| Benzonase® | Merck, Darmstadt, Germany |
| Bovine serum albumin | Sigma-Aldrich,Steinheim,Germany |
| Bromophenol blue | Sigma-Aldrich, Steinheim,Germany |
| Calcium chloride | Merck, Darmstadt, Germany |
| Collagenase II | PAA Labs., Pasching, Austria |
| Costar Transwell® filter membrane | Greiner bio-one, Frickenhausen, Germany |
| Complete® inhibitor cocktail | Roche, Mannheim, Germany |
| Culture dishes | BD, Heidelberg, Germany |
| Dimethyl sulfoxide | Sigma-Aldrich, Steinheim, Germany |
| Dithiothreitol (DTT) | Amersham Pharmacia, UK |
| EDTA | Carl Roth Karlsruhe, Germany |
| EC basal medium® kit | PromoCell®, Heidelberg, Germany |
| Eppendorf tubes (0.5, 1.5, 2 ml) | Eppendorf, Hamburg, Germany |
| Falcon tubes (50 ml, 12 ml) | BD, Heidelberg, Germany |
| FCS | PAA, Pasching, Austria |
| Filter papers | Biotech-Fischer, Reiskirchen, Germany |
| Gentamycin | Gibco BRL, Eggenstein, Germany |
| Glass cover slips | Menzel, Braunschweig, Germany |
| Glycerol (100%) | Sigma-Aldrich, Steinheim, Germany |
| Glycine | Carl Roth, Karlsruhe, Germany |
| HBSS | PAA, Pasching, Austria |
| HEPES | Sigma-ldrich,Steinheim,Germany |
| Insulin (human recombinant) | Sigma-Aldrich, Steinheim |
| Magnesium chloride | Fluka, Switzerland |
| Magnesium sulfate | Merck, Darmstadt, Germany |
| Manganese chloride | Merck, Darmstadt, Germany |
| β-mercaptoethanol | Merck, Darmstadt, Germany |

| | |
|---|---|
| Methanol | Merck, Darmstadt, Germany |
| Millipore water | Millipore, Eschborn, Germany |
| Nitrocellulose membrane | Schleicher und Schuell, Dassel, Germany |
| Non-fat milk powder | Applichem, Darmstadt, Germany |
| Nonidet P-40 | Sigma-Aldrich, Steinheim, Germany |
| Penicillin/streptomycin | Gibco BRL, Eggenstein, Germany |
| Paraformaldehyde | Merck, Darmstadt, Germany |
| Pipette tips | Eppendorf, Hamburg, Germany |
| Pipettes | Eppendorf, Hamburg, Germany |
| PMSF | Sigma-Aldrich, Steinheim, Germany |
| Ponceau S solution | SERVA, Heidelberg, Germany |
| Potassium chloride | Merck, Darmstadt, Germany |
| Potassium dihydrogen phosphate | Merck, Darmstadt, Germany |
| Page ruler pre-stained protein ladder | Thermo Scientific, Braunschweig, Germany |
| Rubber policeman | BD, Heidelberg, Germany |
| Scalpal (disposble) | Feather, Osaka, Japan |
| Sodium azide | Merck, Darmstadt, Germany |
| Sodium bicarbonate | Carl Roth, Karlsruhe, Germany |
| Sodium chloride | Carl Roth, Karlsruhe, Germany |
| Sodium di-hydrogen phosphate | Carl Roth, Karlsruhe, Germany |
| Sodium dodecyl sulfate | SERVA, Heidelberg, Germany |
| Sodium fluoride | Sigma-Aldrich, Steinheim, Germany |
| Sodium hydroxide | Carl Roth, Karlsruhe, Germany |
| Sodium orthovanadate | Sigma-Aldrich, Steinheim, Germany |
| Sterile filters (0.22 µm) | Sartorius, Goettingen, Germany |
| Sterile pipettes | BD, Heidelberg, Germany |
| Super signal-west® (ECL solution) | Fischer scientific, Niederlassung Nidderau, Germany |
| Syringes (20 ml, 2 ml) | BD, Heidelberg, Germany |
| TEMED | Sigma-Aldrich, Steinheim, Germany |
| Thrombin | Sigma-Aldrich, Steinheim, Germany |
| Tris base | Carl Roth, Karlsruhe, Germany |

| | |
|---|---|
| Triton X-100 | SERVA, Heidelberg, Germany |
| Trypan blue | Sigma-ldrich,Steinheim,Germany |
| Trypsin-EDTA solution | Biochrom AG, Berlin |
| Tween 20 | Amersham Pharmacia, UK |
| Whatman® 3 MM filter paper | Millipore, Eschborn, Germany |

## 2.3 Antibodies

**Primary antibodies:**

| **Antibodies** | **Source** |
|---|---|
| Anti-phospho Akt (Mouse IgG) | Cell Signaling Technology, USA |
| Anti-phospho MLC (Rabbit IgG) | Cell Signaling Technology, USA |
| Anti-phospho MYPT1 (Thr850) (Rabbit IgG) | Merck Millipore, Schwalbach, Germany |
| Anti-Rac1-GTP (Rabbit polyclonal) | Cytoskeleton Inc., Denver USA |
| Anti-VE-cadherin (Mouse IgG) | Beckman Coulter, Krefeld, Germany |
| Anti-GAPDH | Cell Signaling Technology, USA |

**Secondary antibodies:**

| **Antibody** | **Source** |
|---|---|
| Anti-Mouse IgG HRP-conjugated | Amersham Biosciences, Heidelberg, Germany |
| Anti-Rabbit IgG HRP-conjugated | Amersham Biosciences, Heidelberg, Germany |
| Anti-mouse IgG Alexa-Flour 488-conjugated | Invitrogen, Karlsruhe, Germany |

## 2.4 Kits

| | |
|---|---|
| Rac1-Activation-Assay Kit | Cytoskeleton Inc., USA |

## 2.5 Inhibitors

| | |
|---|---|
| S961 | Sigma, Steinheim, Germany |

| | |
|---|---|
| Wortmannin | Calbiochem, Darmstadt, Germany |
| L-NAME *(Nω-Nitro-L-arginine methyl ester* Hydrochloride) | Sigma, Steinheim, Germany |
| ML-7 Hydrochloride | Enzo, Lörrach Germany |
| Y27632 | Calbiochem,Darmstadt, Germany |
| NSC23766 *(N-[N-(3, 5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester (DAPT))* | Calbiochem, Darmstadt, Germany |

## 2.6 Buffers

**2x-SDS sample buffer**

| | |
|---|---|
| Tris-HCl (pH 6.8) | 250 mM |
| Glycerol | 20 % |
| SDS | 4 % (wt/vol) |
| DTT | 1 mM (wt/vol) |
| β-mercaptoethanol | 5% (vol/vol) |
| Bromophenol Blue | 0.001% (wt/vol) |
| NaF | 20 mM |
| Na-orthovanadate | 1.5 mM |

**10x TBS (pH 7.4)**

| | |
|---|---|
| Tris-HCl | 100 mM |
| NaCl | 1.6 M |

**TBS Tween (TBST)**

| | |
|---|---|
| 1x TBS | 1000 ml |
| Tween 20 | 1 ml (0.1%, vol/vol) |

**10X PBS (pH 7.4)**

| | |
|---|---|
| NaCl | 1.37 M |
| KCl | 27 mM |
| $Na_2HPO_4$ | 0.1 M |
| $KH_2PO_4$ | 17 mM |

**Rac1 pull down assay**

**Lysis buffer**

| | |
|---|---|
| HEPES | 25 mM |
| NaCl | 150 mM |
| EDTA | 1 mM |
| $MgCl_2$ | 5 mM |
| Na-orthovanadate | 2 mM |
| DTT | 5 mM |
| NaF | 10 mM |
| Triton-X-100 | 0.5% |
| PMSF | 0.5 mM |
| Glycerol | 2% |

Supplemented with complete® protease inhibitor cocktail

**Wash buffer**

| | |
|---|---|
| Tris-HCl (pH 7.4) | 25 mM |
| NaCl | 150 mM |
| $MgCl_2$ | 10 mm |
| Triton X-100 | 1% |
| PMSF | 0.5 mM |

Supplemented with complete® protease inhibitor cocktail

## 2.7 Softwares

| | |
|---|---|
| Microsoft Word 2007 | Microsoft Corp., USA |
| Microsoft Excel 2007 | Microsoft Corp., USA |
| Microsoft Power Point 2007 | Microsoft Corp., USA |
| Microsoft Windows XP Professional | Microsoft Corp., USA |
| Quantity one analysis software | Bio Rad, Hercules, USA |
| LSM 510 | Carl-Zeiss, Jena, Germany |

# 3. METHODS

## 3.1 Cell culture

### Preparation of human umbilical vein endothelial cells (HUVECs)

### Media, sera, buffers and growth supplements

| | |
|---|---|
| **Collagenase solution:** | |
| HBSS (Hank´s balanced salt solution) | x ml |
| Collagenase II, 293 IU/mg (wt/vol) | 0.025% |
| $CaCl_2$ | 1.5 mM |
| $MgCl_2$ | 0.5 mM |

| | |
|---|---|
| **Endothelial cell culture medium** | |
| EC basal medium (PromoCell®) supplemented with | |
| Fetal calf serum (FCS; vol/vol) | 10% |
| EC growth supplement/Heparin (wt/vol) | 0.4% |
| Hydrocortisone (wt/vol) | 0.1% |
| Basic fibroblast factor (wt/vol) | 1 ng/ml |
| Epidermal growth factor (wt/vol) | 0.1 ng/ml |
| Penicillin/streptomycin (vol/vol) | 2% |
| Trypsin/EDTA | 0.05% |

**Procedure:** Human umbilical cords were obtained from University Hospital Giessen after approval from hospital ethics committee. HUVECs were prepared from freshly collected umbilical cords according to Jaffe et al., 1973, with some changes. After cleaning, the umbilical vein was cannulated and rinsed with HBSS to clear away the traces of blood. To detach the HUVECs from the vessel wall the lumen of the vein was filled with collagenase solution and incubated for 30 min at 37°C. After incubation the collagenase solution containing the primary HUVECs, was gently flushed from the vein by perfusion with 30 ml of HBSS containing 3% (vol/vol) FCS, to inactivate the collagenase activity. The effluent was collected in a 50 ml tube and centrifuged at 250 × g for 5 min at room temperature. The supernatant was discarded and the cell pellet was resuspended in EC culture medium containing 0.1% (vol/vol) gentamycin. Thereafter, the cell suspension in EC culture medium was seeded in 1-3, 10 cm cell culture dishes and incubated at 37°C with 5% $CO_2$ for 3 hrs. Afterwards, cells were washed with HBSS to clear away the cell debris, non-adherent and non-ECs and were incubated with EC culture medium supplemented with 0.1% (vol/vol) gentamycin with 5% $CO_2$ at 37°C. After 24 hours the EC culture medium was replaced with fresh EC culture medium and HUVECs were grown to confluence.

### 3.2 Sub- culturing of HUVECs

After reaching confluency, primary HUVECs were trypsinized in phosphate-buffered saline (PBS) [composition: 137 mM NaCl, 2.7 mM KCl, 1.7 mM $KH_2PO_4$, and 10 mM $Na_2HPO_4$; pH 7.4, supplemented with 0.05% (wt/vol) trypsin, and 0.02% (wt/vol) EDTA] for approximately 1-5 min. at 37°C to allow the majority of the cells to detach from the cell culture dish and the digestion was stopped by adding FCS. Trypsinated cells were centrifuged at 250 × g for 10 min and resuspended into EC culture medium and seeded at a density of $2\times10^5$ cells/$cm^2$ on Transwell® filters (for permeability) or on 35 mm or 60 mm dishes (for Western blot analysis and pull down assay). For immunostaining and Confocal microscopy HUVECs were seeded on 25 mm glass coverslips. The experiments were performed with confluent HUVEC monolayers of passage 1-2.

## 3.3 General experimental protocol

The basal medium used to perform experiments was HBSS supplemented freshly with 1.2 mM $MgCl_2$ and 1.3 mM $CaCl_2$. After an initial equilibration period of 30 min on heating plates at 37°C prior to addition of drugs, agents were added as indicated. Stock solutions of insulin, thrombin, S961, ML-7, Y27632, L-NAME and wortmannin were prepared immediately before use. Stock solution of insulin was in 25 mM HEPES and stock solutions of L-NAME, thrombin, S961 and Y27632 were prepared with basal medium. Stock solutions of wortmannin and ML-7 were prepared with dimethyl sulfoxide (DMSO). HUVECs were incubated with appropriate volumes of these solutions yielding final solvent concentrations < 0.1% (vol/vol). The same final concentrations of basal medium, DMSO or HEPES were also added in all respective control experiments. In those experiments where pharmacological inhibitors were used, the cells were preincubated with inhibitors for 30 min before addition of insulin as mentioned in figure legends. In a set of pilot experiments, the optimal effective concentration of the drugs used in this study were determined. The agents were used in their optimal effective concentrations as follows: insulin 1 IU/ml, thrombin (0.2 - 0.3 IU/ml), S961 (1 μM), wortmannin (0.01 μM), ML-7 (10 μM), Y27632 (1 μM), L-NAME (100 μM) and NSC23766 (50 μM.)

## 3.4 Protein analysis

### 3.4.1 Preparation of protein samples

HUVECs were lysed in 150 μl 2x SDS sample buffer [Buffer composition: 250 mM Tris/HCl; pH 6.8, 4% (wt/vol) SDS, 20% (vol/vol) glycerol, 20 mM NaF, Na-orthovanadate 1.5 mM, 0.001% (wt/vol) bromophenol blue, and 10 mM DTT (added freshly before use)]. Afterwards, 50 IU/ml Benzonase® and 2 mM $MgCl_2$ was added freshly before use and lysate was collected in a 1.5 ml Eppendorf tube by scraping with the help of a rubber policeman**.** Samples were denatured at 95°C for 3 min and used immediately for electrophoresis or stored at -20°C for future use.

### 3.4.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

| | | |
|---|---|---|
| Resolving gel buffer: | Tris/HCl; pH 8.8 | 120 mM |
| Stacking gel buffer: | Tris/HCl; pH 6.8 | 120 mM |

**10x Gel running buffer**

| | |
|---|---|
| Tris | 250 mM |
| Glycine | 2.0 M |
| SDS (wt/vol) | 10% |

## SDS gels

The composition of gels of different percentages is given below:

| **Gels** | **Resolving Gels (For 2 gels)** | | | **Stacking Gels** |
|---|---|---|---|---|
| **Solutions** | **5 %** | **10%** | **12.5%** | **6%** |
| **Acrylamide/Bisacrylamide (1:19) 40% (wt./vol)** | 2.25 ml | 4.50 ml | 5.63 ml | 0.75 ml |
| **Resolving gel buffer** | 6.75 ml | 6.75 ml | 6.75 ml | ----- |
| **Stacking gel buffer** | ----- | ----- | ----- | 0.75 ml |
| **Millipore water** | 8.70 ml | 6.45 | 5.33 ml | 4.43 ml |
| **SDS 10% (wt./vol)** | 180 µl | 180 µl | 180 µl | 60 µl |
| **APS 10% (wt./vol)** | 150 µl | 150 µl | 150 µl | 37.5 µl |
| **TEMED** | 15 µl | 15 µl | 15 µl | 7.5 µl |

**Procedure:** Glass plates, spacers and combs were cleaned with water and ethanol, then gel apparatus was assembled and the resolving gel solution was poured and layered with ethanol or water. The gel was left to polymerize for 1 hour at room temperature. Once the gel was polymerized the top of the gel was completely rinsed with water. The layer of water was removed. The stacking gel solution was prepared immediately before use and was poured on top of the resolving gel cautiously so that there was no bubble. The comb was inserted and the stacking gel was left to polymerize at room temperature for 30 min. After removing the comb carefully (not to damage the wells) 1x running gel buffer was added to the chamber and the sample wells were cleaned to remove the unpolymerized acrylamide with the same buffer using a syringe. Protein samples were loaded into the wells and the gel was run at 120 volts for 3 hrs. The run was stopped when bromophenol blue had passed through the gel. The molecular weight of proteins was determined by comparison with PageRuler™ prestained protein ladder.

## 3.5 Western blot analysis

Proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane by semi-dry blotting method. Later on specific proteins were immunodetected by using specific antibodies.

### Materials and solutions

- Blotting chamber
- Nitrocellulose membrane,
- Whatman® 3 MM filter paper,
- Blotting buffer 150 mM Glycine; 25 mM Tris/HCl, pH 8.3 and 10% (vol/vol) methanol
- Millipore water

**Procedure:** Nitrocellulose transfer membrane and six pieces of Whatman® 3 MM filter papers, cut to the same size and dimensions as the gel. The graphite blotting chamber was set up as follows: Three sheets of filter paper (Whatman® 3 MM) soaked in blotting buffer, were placed in the center of the graphite anode of the blotting chamber. Nitrocellulose membrane equilibrated in blotting buffer for 10-15 min placed on top of these sheets of filter paper. After briefly equilibrating with blotting buffer, the SDS-gel (only resolving gel) was layered on top of the nitrocellulose membrane, carefully avoiding air bubbles. Three sheets of filter paper, presoaked in blotting buffer, were placed on top of the gel followed by the graphite cathode of the blotting chamber. Transfer was achieved by application of 0.8 mA/cm$^2$ current for approximately 1 hour.

### 3.5.1 Ponceau staining of transferred proteins

After blotting, the membrane was stained with Ponceau-S solution to observe the efficiency of protein transfer. This stain is reversible and gives pink bands on a light background. The nitrocellulose membrane was washed with Millipore water for 1 min, incubated in Ponceau-S solution for 2-3 min with constant shaking at room temperature. Afterwards the membrane was destained by washing in Millipore water to the desired contrast and photographed. To destain completely, the membrane was washed with 1x Tris-buffered saline (TBS) plus 0.1% Tween 20 under constant shaking at room temperature.

### 3.5.2 Immunodetection of proteins

**Solutions:**

**10x (TBS)**

| | |
|---|---|
| Tris/HCl (pH 7.4) | 100 mM |
| NaCl | 1.6 M |

**TBS Tween (TBST)**

1x TBS

0.1% (vol/vol) Tween 20

**Blocking-buffer and antibody-dilution buffer**

After a brief washing with Millipore water and 1x TBST and before the immunological detection of relevant proteins, the binding sites of unspecific proteins were blocked by 5% (wt/vol) non-fat dried milk powder in 1x TBST (Milk) or 5% (wt/vol) BSA in 1x TBST for 1 hour at room temperature with constant rocking

**Primary Antibodies**

| **Antibody** | **Dilution** | **Dilution buffer** |
|---|---|---|
| Anti-phospho Akt (Mouse IgG) | 1:1000 | 3% BSA |
| Anti-phospho MLC (Rabbit IgG) | 1:1000 | 3% BSA |
| Anti-phospho MYPT1 (Thr850) (Rabbit IgG) | 1:1000 | 3% BSA |
| Anti-Rac1 (Rabbit IgG) | 1:1000 | 3% BSA |
| Anti-GAPDH | 1:2000 | 3% BSA |

**Secondary antibodies, horseradish peroxidase (HRP)-labeled**

| **Antibody** | **Dilution** | **Dilution buffer** |
|---|---|---|
| Anti-rabbit IgG | 1:1000 | 5% milk powder |
| Anti-mouse IgG | 1:1000 | 5% milk powder |
| Anti-mouse IgM | 1:2000 | 5% milk powder |

**Procedure:** After blocking, the membrane was incubated overnight with primary antibody at 4°C with gentle shaking. The membrane was then washed at room temperature with 1x TBST 3-4 times for 5-10 min each with constant rocking and incubated with HRP- conjugated secondary antibody for 1 hour at room temperature.

### 3.5.3 Enhanced chemiluminescence (ECL)

After incubation with the secondary antibody, membrane was then washed 2-3 times as stated before and then incubated with enhanced chemiluminescence (ECL) solution (30 seconds to 1 minute). The appearing luminescence was detected and recorded with Peqlab, ChemiSmart gel documentation system as per manufacturer´s instructions and images were analyzed by using Quantity One software (Bio-Rad).

## 3.6 Measurement of endothelial monolayer permeability

The permeability of trypan blue-labelled albumin across HUVEC monolayer was measured as described by Noll et al., 1999, using a two compartment system. The system consists of “luminal” (upper) and “abluminal” (lower) compartments. The compartments were separated by a filter membrane (with pore size 0.4 µm, 6.5 mm diameter). The cells were cultured in luminal compartment on the membrane of the Transwell® filters till confluence. HBSS supplemented with 1.2 mM $MgCl_2$ 1.3 mM $CaCl_2$ and 2% (vol/vol) FCS used as a basal medium was added in both compartments. The luminal compartment containing the HUVEC monolayer had 2.5 ml volume of this medium while the abluminal compartment contained 6.6 ml of the medium. The fluid in the "abluminal" compartment was constantly stirred using magnetic stirrers. This system measures transendothelial flux from luminal to abluminal compartments in the absence of hydrostatic pressure gradients. In the luminal compartment final concentration of 60 µM of the trypan blue-labelled albumin was added. The appearance of trypan blue-labelled albumin from the luminal to the abluminal compartment was monitored continuously after every 60 seconds by pumping the liquid through a spectrophotometer (Specord 10, Zeiss Jena, Germany).

To avoid measurement artifacts a two-wavelength measurement mode was used (control 700 nm versus trypan blue 600 nm).

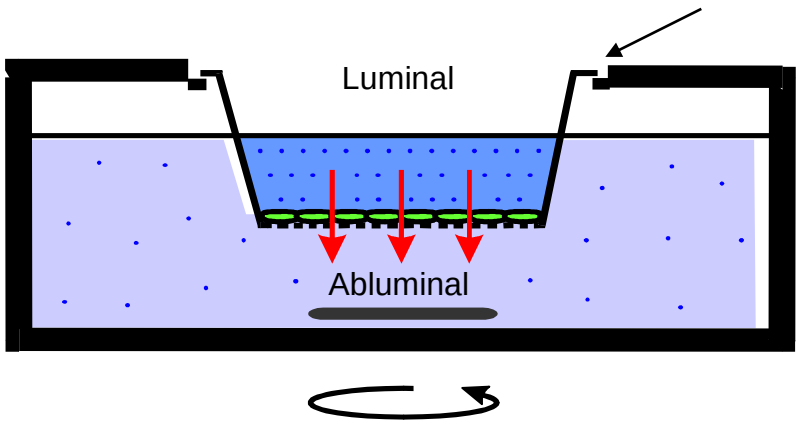
The albumin flux (F, measured in mol/ (sec x $cm^2$) across HUVEC monolayer surface area (S) was calculated as the increase in albumin concentration ($d[A]_2$) during the time interval (dt) in the lower compartment with the volume (V) as follows:

$$F = \frac{d\,[A]_2 / dt \times V}{S} \qquad (1)$$

The combined permeability coefficient (P [cm/sec]) of both EC monolayer and filter membrane was calculated as:

$$P = \frac{F}{([A]_1 - [A]_2)} \qquad (2)$$

Where $[A]_1$ and $[A]_2$ are the albumin concentrations in the luminal and abluminal compartments, respectively.




Twocompartmentsystem

**Figure 3.1.** Two compartment system of measuring trypan blue- labelled albumin permeability across the HUVEC monolayer

## 3.7 Immunofluorescence microscopy

**Solutions and materials:**

Blocking buffer consists of 5% (vol/vol) FCS and 5% (wt/vol) BSA in 1x PBS

**Primary Antibodies**

| **Antibody** | **Dilution** | **Dilution buffer** |
|---|---|---|
| Anti-VE-cadherin (Mouse IgG) | 1:100 | blocking buffer |

**Secondary Antibodies**

| **Antibody** | **Dilution** | **Dilution buffer** |
|---|---|---|
| Anti-mouse IgG (Alexa fluor 488) | 1:400 | PBS |

**Protocol:** HUVECs were grown on glass cover slips in 35 $mm^2$ culture dishes until confluence. After treatments, cells were washed thrice with *1x* PBS (pH 7.4) and then fixed with 4% paraformaldehyde for 20 min at room temperature or ice cold 100% methanol for 20 min at -20°C. Afterwards cells were washed three times for 10 min each with 1x PBS and permeabilized with 1x PBS containing 0.2% (vol/vol) Triton X-100 at 37°C for 20 min. The cells were then washed 2-3 times with 1x PBS. Non-specific binding was blocked by incubating cells with blocking solution (5% (wt/vol) BSA and 5% (vol/vol) FCS in 1x PBS) for 45 min. Cells were incubated with respective primary antibody in a dilution of 1:100 in blocking solution overnight at 4°C, washed three times with 1x PBS for 10 min each and subsequently incubated with secondary antibody tagged with Alexa fluor 488 in a dilution of 1: 400 for 1 hour at room temperature followed by 2-3 times gently washed with 1x PBS.

The coverslips were finally mounted onto glass objective slides with a drop of commercial mounting solution. Confocal images were obtained and analyzed by using laser scanning microscopy (Carl Zeiss LSM 510, Jena, Germany). Fluorophores were excited by using argon (492 nm) and He-Ne (545 nm) lasers. Image acquisition and analysis was performed using software provided with the confocal microscope.

## 3.8 Detection of activated Rac1

Rac1 belongs to Rho family of small GTPases. The activation state of Rac1 was assessed by pull down assay. The assay is based on the principle that only the interaction of active form of the GTPase with its specific downstream effectors. The assay was performed according to the manufacturer's instructions using the Rac1 p21 binding domain (PBD) of p21-activated protein kinase 1 (PAK1), which leads to its activation, therefore, the p21 binding domain (PBD) of PAK 1 can be used as a probe to specifically isolate activated GTP-bound Rac1.

**Procedure:** Confluent HUVEC monolayers were stimulated with insulin and thrombin as described in respective figures. Subsequently the cells were washed with ice-cold PBS and lysed with 600 µl of lysis buffer (composition of the buffer: 25 mM Hepes; pH 7.4, 150 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 2 mM Na-orthovanadate, 10 mM NaF, 5 mM DTT, 0.5 mM PMSF, 0.5% (vol/vol) Triton X-100, 2% (vol/vol) glycerol and supplemented with Complete® protease inhibitor cocktail) on ice for 10 min. The cells were harvested by cell scraper, lysate were transferred to pre-labelled sample tubes on ice. The lysate was centrifuged for 1 minute at 14000 x g at 4°C. 600 µg of cell lysates were incubated with 10 µg of GST-PAK beads (Cytoskeleton Inc.) at 4°C for 40 min. The beads were washed four times with wash buffer (composition of the wash buffer: 25 mM Tris-HCl; pH 7.4, 10 mM $MgCl_2$, 1% (vol/vol) Triton X-100, 0.5 mM PMSF, 150 mM NaCl, and Complete® protease inhibitor cocktail), The pellet containing the beads with active Rac1 was eluted in 40-50 µl of Laemmli sample buffer, boiled at 95°C for 5 min and loaded on 12.5% SDS gel. Bound Rac1 protein was then detected by Western blot using specific polyclonal antibodies against Rac1 (Cytoskeleton Inc; Denver). For the cross comparison of Rac1 activation (level of GTP-bound Rac1) the total amount of Rac1 in cell lysates was used as a control.

## 3.9 Determination of myocardial water content

All experiments were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the German law of animal welfare. Hearts from male Wistar rats with an average weight of 250-g were excised rapidly, and were mounted immediately after isolation on a Langendorff perfusion system in a temperature-controlled chamber (37°C), as previously described by (Noll et al., 1999) with some modifications. Hearts were then perfused with Krebs-Henseleit buffer (composition of the buffer in mM: (NaCl 140, $NaHCO_3$ 24.0, $MgSO_4$ 1.0, $KH_2PO_4$ 0.4, KCl 2.7, $CaCl_2$ 1.8 and glucose 5.0) for 30 min (10 ml/min) prior to each experiment. The buffer pH was maintained 7.4 by gassing with carbogen (95% $O_2$ and 5% $CO_2$) and filtrated with 0.45 μM diameter membrane filters. Hearts were then exposed to one of the following protocols: (a) Normoxic (Nor) conditions for 90 min (b) hypoxia for 60 min followed by 30 min of reperfusion (Rep) (c) hypoxia for 60 min followed by 30 min of reperfusion with insulin 0.1 IU/ml, during first 10 minutes of reperfusion, (d) 60 min of hypoxia followed by 30 min of reperfusion in which NSC23766, a specific Rac1 inhibitor, was added to the perfusion medium during last 20 min of hypoxia and first 10 min of reperfusion in the presence of insulin (during first 10 min of reperfusion only).The normoxic perfusion (10 ml/min) was with Krebs-Henseleit buffer gassed with 95% $O_2$ (vol/vol)/5% $CO_2$ (vol/vol), the thermostatic heart chamber was flushed with humidified air, and hypoxic perfusion with Krebs-Henseleit buffer with humidified 95% $N_2$ (vol/vol)/5% $CO_2$ (vol/vol). At the end of each experiment, wet weight and after 24 hrs. dry weight of the perfused rat hearts were measured. Myocardial water content was calculated as follows:

Myocardial water content = wet weight- dry weight

$$\text{Myocardial water content per 100 g heart weight} = \frac{\text{Wet weight - dry weight}}{\text{Dry weight}} \times 100$$

## 3.10 Statistical analysis

Data are expressed as means ± SEM of 3-5 experiments from 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 analysis. Probability (P) values of less than 0.05 ($P< 0.05$) were assumed significant.

# 4. RESULTS

## 4.1 Insulin enhances the recovery of EC barrier function in response to thrombin challenge

In the first step the effect of insulin on recovery of EC barrier function disrupted by inflammatory agent thrombin was analyzed. As shown in Figure 4.1 exposure of ECs to thrombin (0.2 IU/ml) caused a robust increase in EC permeability which peaked within ~10-15 min in response to thrombin challenge and slowly returned to basal level approx. within 2 hrs. In the presence of insulin the thrombin-induced hyperpermeability was significantly reduced which was observed at all concentrations of insulin. Furthermore insulin-induced a fast restoration of EC barrier function in response to thrombin challenge, as was reflected by more rapid decrease in permeability to albumin towards basal level, and remained at lower level for the whole period of observation. The time required for the 50% recovery of EC monolayer permeability from the maximum increase of permeability was significantly less in the presence of insulin (~25 min vs. ~ 51 min, for insulin (1 IU/ml) plus thrombin and thrombin alone, respectively). Thus insulin accelerated restoration of EC barrier function, as was characterized by more rapid recovery of endothelial permeability to the basal level. This restoration of EC barrier function by insulin was concentration-dependent and was significant already at 0.01 IU/ml concentration of insulin (equivalent to ~ 50nM). Furthermore, the maximum reduction in macromolecule permeability was at 1 IU/ml of insulin concentration as shown in Figure 4.1. Therefore, this concentration of insulin was used for all subsequent experiments.

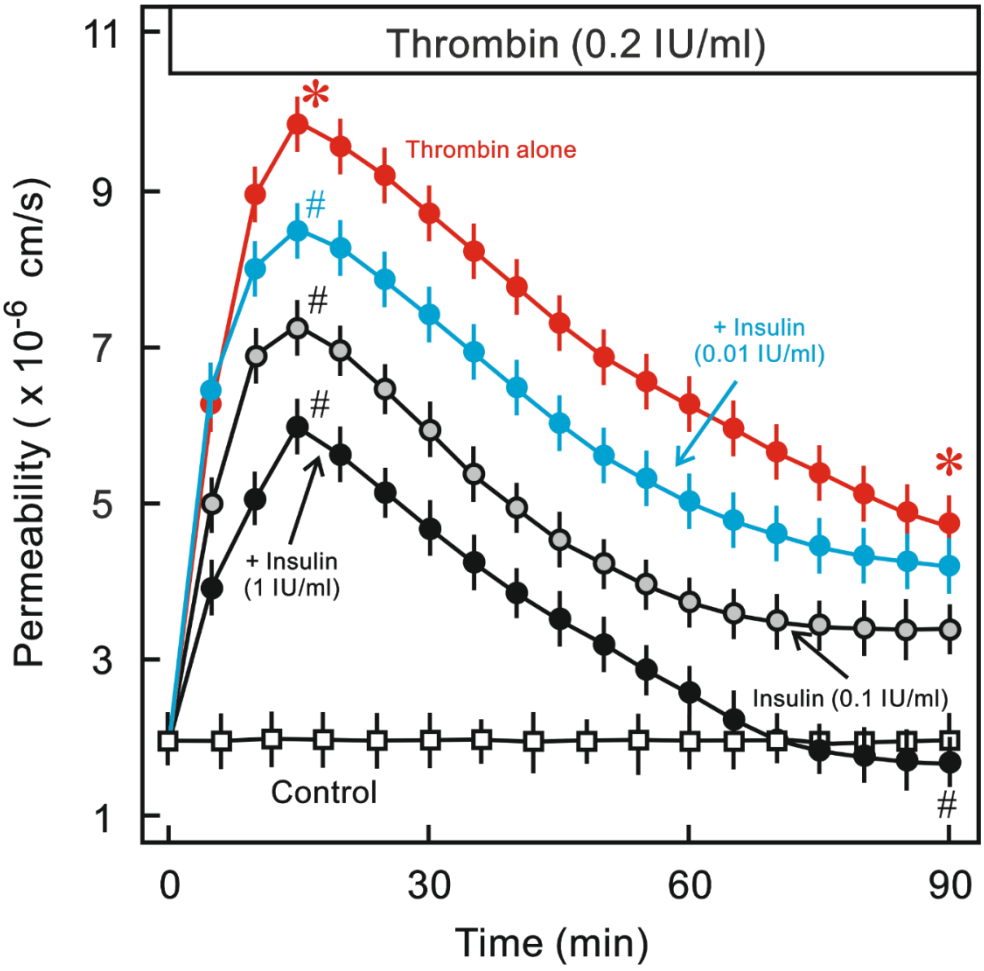



**Figure 4.1 Effect of insulin on thrombin-induced hyperpermeability.**

HUVEC monolayers were exposed to thrombin (0.2 IU/ml) in the presence or absence of different concentrations of insulin (0.01, 0.1, and 1 IU/ml) or vehicle (control) as indicated. Data are mean ± SEM of 5 separate experiments of independent cell preparations; $^{*}P<0.05$ versus control; $^{\#}P<0.05$ versus thrombin alone.

## 4.2 Effect of insulin on VE-cadherin mediated endothelial adherens junctions in response to thrombin challenge

One of the major regulators of EC barrier integrity is the actin cytoskeleton anchored AJs consisting of VE-cadherin, which together with associated family of catenins seals the adjoining cells together and thereby limits the passage of macromolecules across the vessel wall (Dejana et al., 2008; Lampugnani et al., 1995). Therefore, the effect of insulin on VE-cadherin dynamics at cell-cell junctions after thrombin challenge was analyzed.

VE-cadherin was decorated at the cell-cell junctions under basal conditions (Figure 4.2, 0 min). However, exposure of HUVEC monolayers to thrombin (0.3 IU/ml) resulted in drastic disappearance of VE-cadherin from cell borders within 10 min, leading to intercellular gap formation and an increase in permeability. Thrombin effect was very rapid and is followed by slow reappearance of VE-cadherin at cell-cell junctions. This thrombin effect was attenuated in the presence of insulin (1 IU/ml). Furthermore in thrombin challenged cells, insulin promoted fast re-appearance of VE-cadherin at cell-cell junctions within 10 min and was even more pronounced at 30 min, showing protective effect of insulin against thrombin-induced loss of AJs, indicating that insulin strengthens as well as promotes quick re-establishment of cell-cell adhesion structures, thus accelerates recovery of EC barrier function after thrombin-induced EC barrier breakdown.

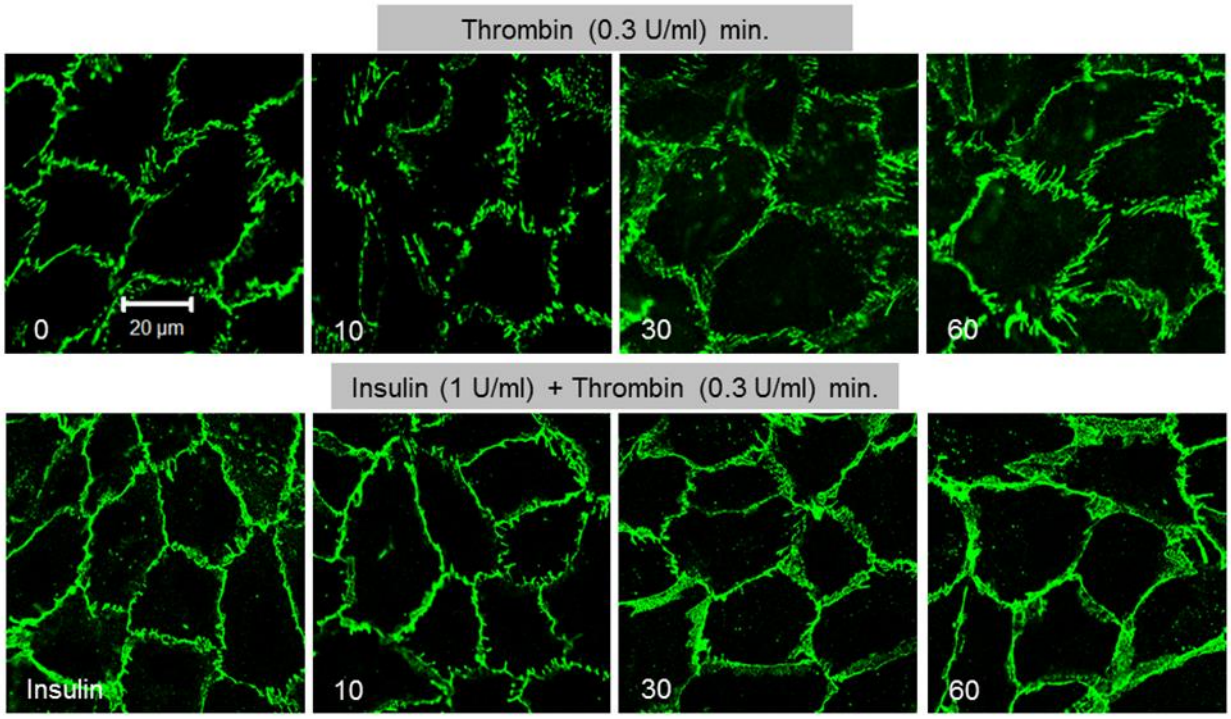



**Figure 4.2 Effect of insulin on thrombin-induced VE-cadherin localization and EC monolayer disruption.** HUVECs were grown to confluence on glass cover slips and were exposed to thrombin (0.3 IU/ml) in the absence or presence of insulin (1 IU/ml for 10 min) for indicated time periods. Methanol fixed and immunostained for VE-cadherin. Scale bar 20 µm; shown are representative figures of VE-cadherin immunostaining of five separate experiments with independent cell preparations.

### 4.3 Role of insulin receptors in insulin-mediated endothelial barrier recovery

Like many other cell types ECs also express insulin receptors (Zeng et al., 2000; Nitert et al., 2005). Therefore in the next step the involvement of insulin receptors in the EC barrier restoration effects of insulin was analyzed. In these experiments S961, a highly specific insulin receptor antagonist (Schaffer et al., 2008) was applied to block insulin receptors. Preincubation of ECs for 30 min with S961 (1 µM) abolished the barrier recovery effect of insulin in response to thrombin-induced hyperpermeability as shown in Figure 4.3, indicating a receptor-mediated effect.

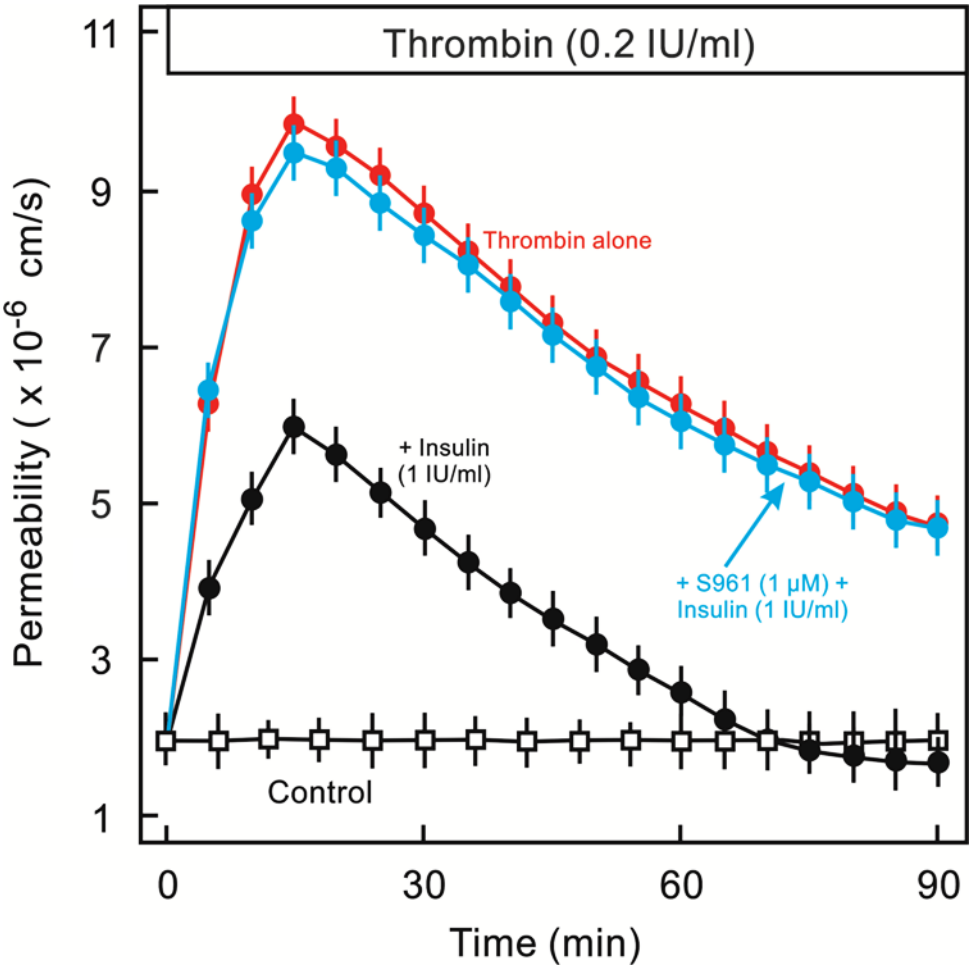


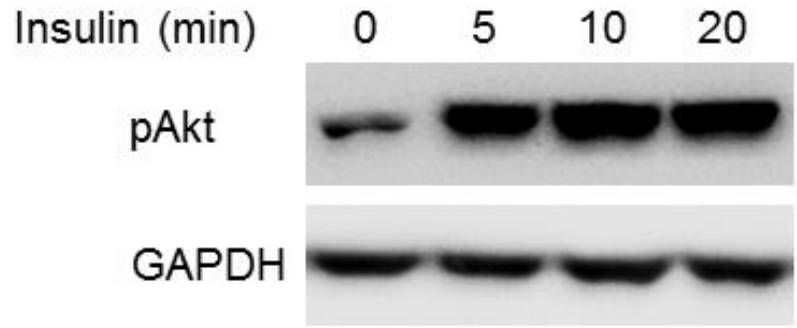

**Figure 4.3 Role of insulin receptors on insulin-mediated endothelial barrier recovery.** HUVEC monolayers were exposed to thrombin (0.2 IU/ml) in the presence or absence of insulin (1 IU/ml) or vehicle (control) as indicated. In a set of experiments ECs were exposed to thrombin in the presence of S961 (1 µM) plus insulin. Data are mean ± SEM of five separate experiments of independent cell preparations; $^{*}P<0.05$ versus control; $^{\#}P<0.05$ versus thrombin alone; n.s.: not significantly different from thrombin alone.

## 4.4 Role of PI3K/Akt pathway in insulin-mediated barrier recovery in HUVECs.

There is convincing evidence that in ECs insulin mediates most of its effects via activation of PI3K/Akt pathway (Zeng et al., 2000; Hermann et al., 2000). Protein kinase B (Akt) is a downstream effector of PI3K, therefore, the effect of insulin on Akt activation (phosphorylation) was analyzed by Western blot analysis using a phospho-specific antibody against Akt. Insulin-induced a rapid increase in Akt phosphorylation in HUVECs as shown in Figure 4.4A. Phosphorylation of Akt reached its maximal within 5 min and sustained over the maximum indicated periods of observation as shown in Figure 4.4A

In order to determine the contribution of PI3K in insulin-mediated EC barrier recovery in response to thrombin-induced hyperpermeability, the effect of specific PI3K inhibitor wortmannin was investigated. ECs were pre-incubated with specific PI3K inhibitor wortmannin (Wort: 0.1 μM) for 30 min. As shown in Figure 4.4B inhibition of PI3K with wortmannin completely abolished the insulin-mediated restoration of EC barrier function.

**A**


Insulin (min)
0
5
10
20
pAkt
GAPDH

**B**

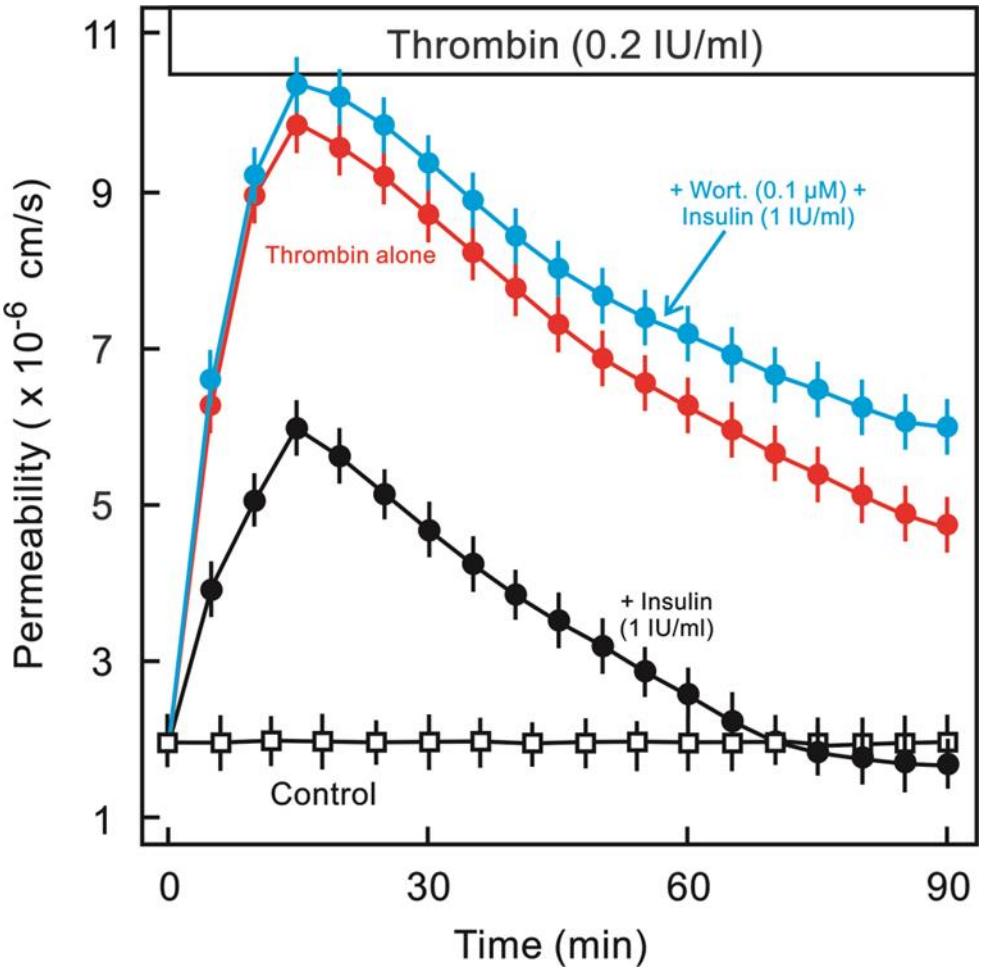

Thrombin (0.2 IU/ml)
Permeability ( x 10-6 cm/s)
11
9
7
5
3
1
0
30
60
90
Time (min)
Thrombin alone
+ Wort. (0.1 μM) +
Insulin (1 IU/ml)
+ Insulin
(1 IU/ml)
Control

**Figure 4.4 Role of PI3K/Akt pathway in insulin-mediated EC barrier restoration**

Effect of Insulin on Akt phosphorylation (pAkt). **(A)** Representative Western blots of Akt phosphorylation. HUVECs were incubated with insulin (1 IU/ml) for different time intervals (min) as indicated. Western blotting was performed with an anti phospho-Akt antibody. The same membranes were reprobed with anti-GAPDH antibody as loading control. The Western blots are representative of 3 different experiments using independent cell preparations.

**(B)** HUVEC monolayers were challenged with thrombin (0.2 IU/ml) in the absence or presence of insulin (1 IU/ml) or preincubated with PI3K inhibitor wortmannin (Wort; 0.1 μM) for 30 min plus insulin plus thrombin or vehicle (control) as indicated. Data are mean ± SEM of five separate experiments of independent cell preparations. $^{*}P<0.05$ versus control; $^{\#}P<0.05$ versus thrombin alone; n.s.: not significantly different from thrombin alone.

## 4.5 The effect of eNOS inhibition on insulin-mediated EC barrier restoration

Insulin activates EC nitric oxide synthase (eNOS) and hence induces NO production (Zeng et al., 2000; Montagnani et al., 2001 and 2002) and thereby may stabilize microvascular EC barrier function (Gündüz et al., 2010). Therefore, the involvement of eNOS in insulin-mediated recovery of EC barrier function in response to thrombin-induced hyperpermeability was analyzed by using a specific well-established eNOS inhibitor L-NAME. In these experiments endothelial monolayers were preincubated with L-NAME (100 μM) for 30 minutes. As shown in Fig. 4.5, inhibition of eNOS had no significant effect on insulin-mediated EC barrier restoration.

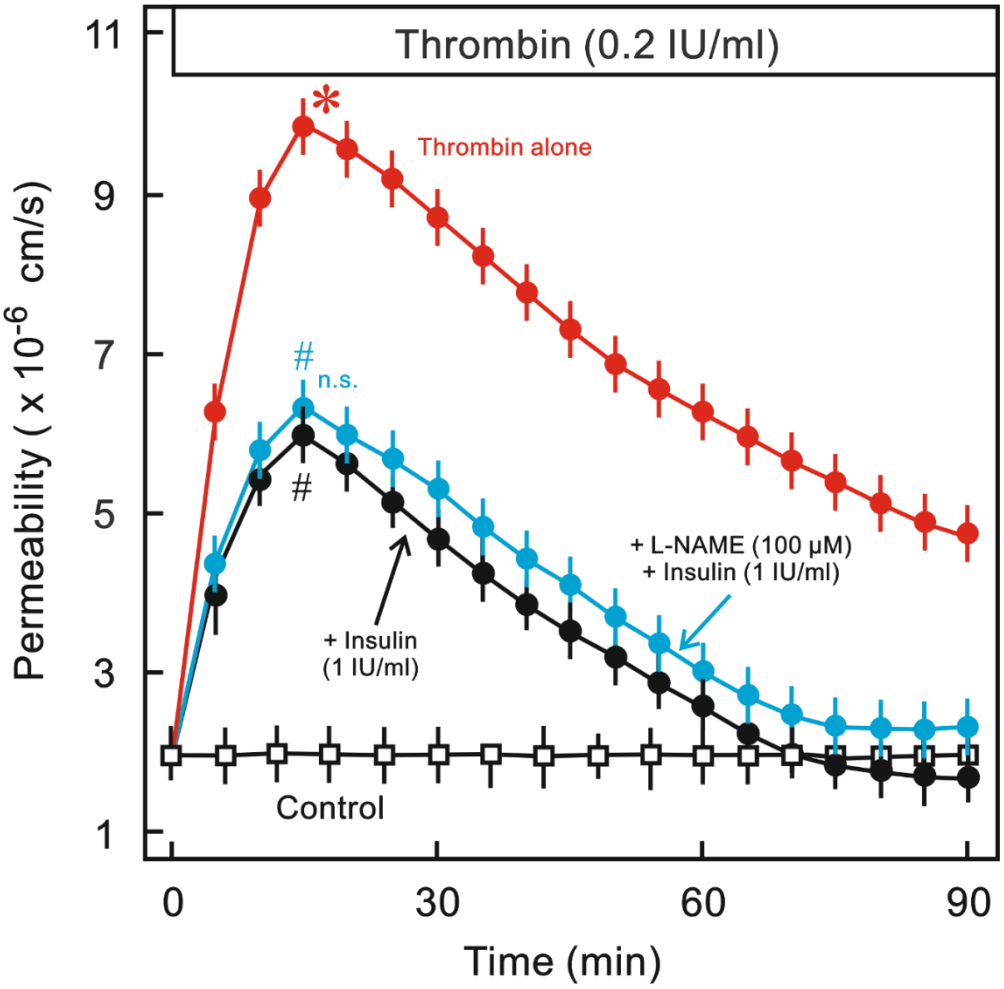



**Figure 4.5 Effect of eNOS inhibition on insulin-mediated restoration of EC barrier function in response to thrombin-induced hyperpermeability.** HUVEC monolayers were exposed to thrombin (0.2 IU/ml) in the presence or absence of insulin (1 IU/ml) or in the presence of specific eNOS inhibitor L-NAME (100 µM) plus insulin or vehicle (control) as indicated. Data are mean ± SEM of 5 separate experiments of independent cell preparations. *P<0.05 versus control; #P<0.05 versus thrombin alone; n.s.: not significantly different from insulin plus thrombin.

## 4.6 Effect of MLCK inhibition on insulin-mediated EC barrier restoration

It is well established that EC barrier function is regulated by balanced activity of two important enzymes, MLCK and MLCP. Previously, the involvement of both MLCK activation and RhoA/Rock-mediated MLCP inhibition in thrombin-induced EC barrier dysfunction has been demonstrated (Garcia et al., 1996; Dudek and Garcia, 2001; Velasco et al., 2002). In order to understand which of these two plays role in insulin-mediated EC barrier restoration in response to thrombin challenge, the activity of these enzymes was inhibited by using specific pharmacological inhibitor. Therefore in the next step the role of MLCK on insulin-mediated recovery of EC barrier function after thrombin challenge was assessed. (ML-7 is a specific pharmacological inhibitor of MLCK added at optimum concentration to block MLCK activity). MLCK is a $Ca^{2+}$ /calmodulin (CaM)-dependent kinase that functions principally to phosphorylate regulatory MLC at Ser19 and subsequently at Thr18 (Shen, 2010; Garcia et al., 1995; Goeckeler and Wysolmerski, 1995, Hixenbaugh et al., 1997; Moy et al., 2002; Verin et al., 1998). It is well established that inflammatory agents like thrombin causes activation of MLCK which leads to EC retraction and results in barrier disruption (Sheldon et al., 1993; Dudek and Garcia, 2001).To elucidate the role of this kinase on macromolecular permeability, HUVECs were exposed to thrombin (0.2 IU/ml) in the presence or absence of insulin (1 IU/ml) or in the presence of pharmacological inhibitor ML-7 (10 μM) or ML-7 plus insulin.

As shown in Figure 4.6 pharmacological inhibition of MLCK with ML-7 could neither significantly attenuate the thrombin-induced hyperpermeability nor had significant additive effect on insulin-mediated accelerated reduction of EC barrier permeability, suggesting that MLCK does not play any significant role in insulin-mediated recovery of EC barrier function.

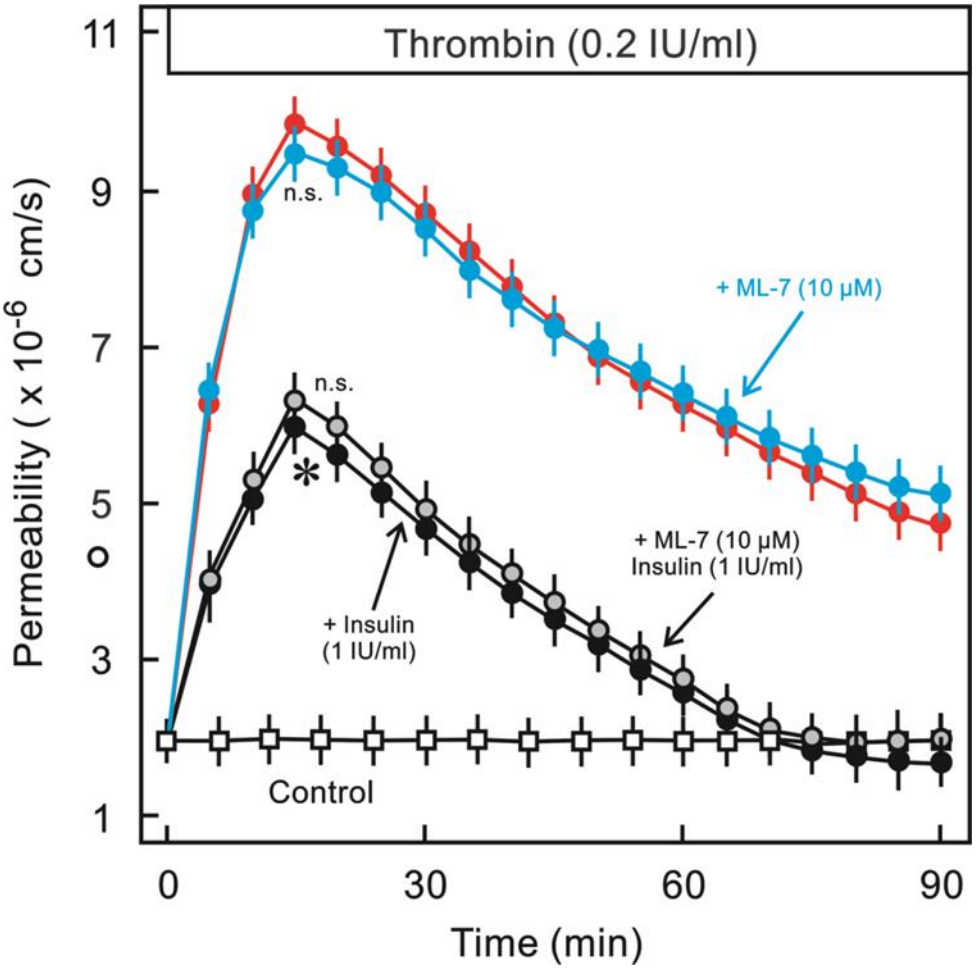



**Figure 4.6 Effect of inhibition of MLCK on insulin-mediated EC barrier restoration**

HUVEC monolayers were exposed to thrombin (0.2 IU/ml) in the presence or absence of insulin (1 IU/ml) or in the presence of specific MLCK inhibitor ML-7 (10 µM), or ML-7 plus insulin, or vehicle (control) as indicated. Data are mean ± SEM of 5 separate experiments of independent cell preparations. $^{*}P< 0.05$ versus control; $^{\#}P< 0.05$ versus thrombin alone; n.s.: not significantly different from insulin plus thrombin.

## 4.7 Effect of insulin on EC contractile machinery and dynamics of RhoA/Rock activity

Endothelial actin-myosin based contractile machinery is an important regulator of EC barrier function (Garcia et al., 1995). Since, phosphorylation state of the regulatory MLC precisely controls the activation of EC contractile machinery, changes in this biochemical parameter of endothelial contractile activation was examined. ECs were exposed to thrombin (0.2 IU/ml) for different time points (min) in the presence or absence of insulin (1 IU/ml) as indicated. MLC phosphorylation was analyzed by Western blot using a phospho-specific antibody directed against Ser-19/Thr-18 of MLC. As shown in Figure 4.7A exposure of ECs to thrombin caused a rapid rise in MLC phosphorylation within 2 min, reaching maximum in 5 min and remained highly phosphorylated for 20 min followed by a gradual decline towards baseline. In the presence of insulin, the thrombin-induced effect on MLC phosphorylation was attenuated and dephosphorylation of MLC was faster in insulin treated ECs suggesting that insulin is capable to antagonize thrombin-induced activation of EC contractile machinery.

Since phosphorylation of MLC is also known to be mediated via RhoA/Rock signalling pathway in ECs (Feng et al., 1999; Velasco et al., 2002), therefore, activation of RhoA/Rock signalling pathway was analyzed by measuring the phosphorylation state of MYPT1 at Threonine 850 (Thr-850) which is directly phosphorylated by Rock. ECs were stimulated with thrombin (0.2 IU/ml) for indicated time periods in the presence or absence of insulin (1 IU/ml) and MYPT1 phosphorylation was analyzed by Western blot.

As shown in Figure 4.7A thrombin caused a robust increase in MYPT1 phosphorylation as early as 2 min and remained phosphorylated on nearly same level until 20 min followed by gradual declined towards the base line which was evident at 60 minutes of thrombin stimulation, however even at 60 minutes, MYPT1 phosphorylation level remained elevated in thrombin challenged cells as compared to unstimulated control ECs. Insulin effect on thrombin-induced MYPT1 phosphorylation was rather delayed as compared to its effect on MLC phosphorylation and a significant effect was seen only after 30 min.

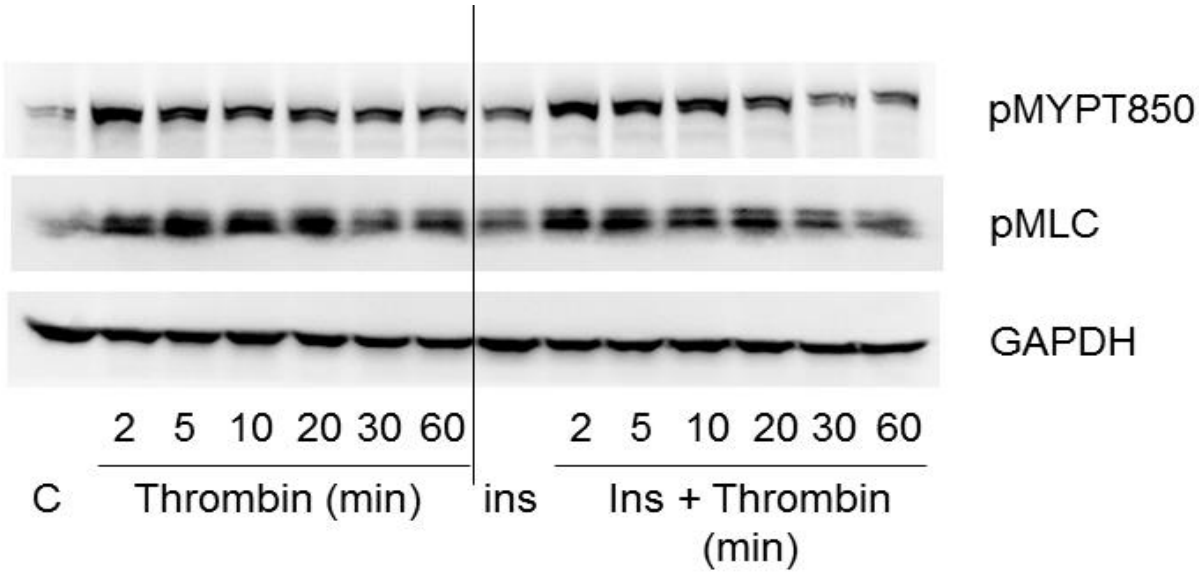



**Figure 4.7A Effect of insulin on thrombin-induced MLC and MYPT1 phosphorylation in HUVECs.** Representative Western blots of MYPT1 and MLC phosphorylation. Confluent HUVEC monolayers were exposed to thrombin (0.2 IU/ml) in the presence or absence of insulin (Ins, 1 IU/ml) for different time periods (min) or insulin alone or vehicle (C, control) as indicated. MYPT1 phosphorylation at Thr850 and MLC phosphorylation at Ser19/Thr18 was analyzed by using phospho-specific antibodies. GAPDH was used as loading control. Western blots are representative of 3 different experiments of independent cell preparations.

In the next step the role of Rho effector, Rock; in insulin-mediated recovery of EC barrier function in response to thrombin challenge was examined, by measuring the flux of trypan blue-labelled albumin across HUVEC monolayers. ECs were exposed to thrombin (0.2 IU/ml) in the presence or absence of insulin (1 IU/ml) or preincubated (30 min) with the low concentration of Rock inhibitor Y27632 (1 μM) in the presence or absence of insulin. As shown in Figure 4.7B preincubation of ECs with Y27632 (1 μM) significantly reduced thrombin-induced hyperpermeability both in the presence or absence of insulin. However in the presence of insulin the additive effect on EC barrier recovery was observed. Interestingly the time required for the 50% recovery of EC permeability from the maximum increase of permeability was less in the presence of Rock inhibitor Y27632 (~ 25 min vs. ~ 20 min for insulin plus thrombin and insulin plus thrombin in the presence of Y27632, respectively). These data indicate that inhibition of Rock has additive effect on insulin-mediated recovery of failed barrier.

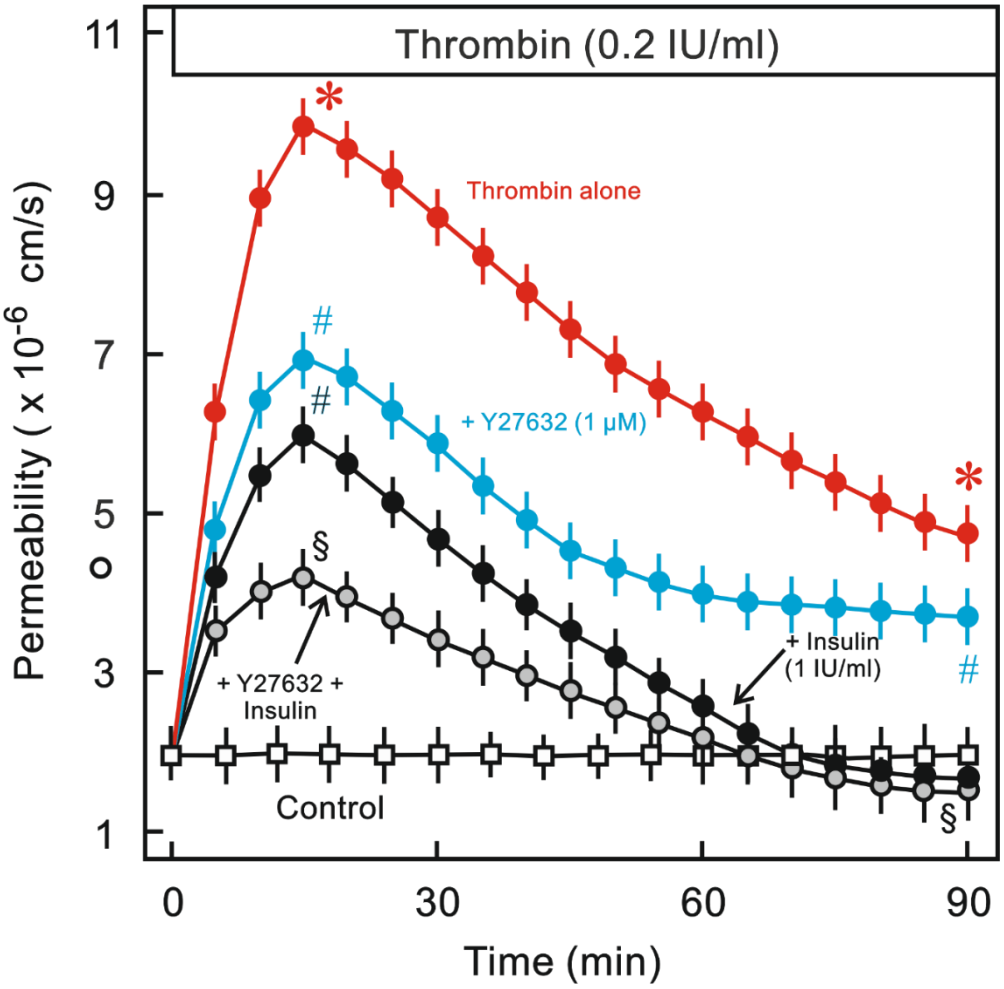



**Figure 4.7B Effect of Rho kinase inhibition on insulin-mediated EC barrier restoration.** HUVEC monolayers were exposed to thrombin (0.2 IU/ml) in the absence or presence of insulin (1 IU/ml) or preincubated with specific Rock inhibitor Y27632 (1 μM) for 30 min or Y27632 plus insulin or vehicle (control) as indicated and albumin flux (permeability) across HUVEC monolayer was measured. Data are mean ± SEM of 5 separate experiments using independent cell preparations.*P< 0.05 versus control; #P<0.05 versus thrombin alone; §P<0.05 versus insulin plus thrombin

## 4.8 Effect of insulin on Rho GTPase Rac1 activation and its role in insulin-mediated EC barrier restoration.

Rho GTPase Rac1 is a well characterized regulator of peripheral actin dynamics and is required for the stability of endothelial AJs and thus stabilizes EC barrier integrity (Hall, 1998; Wojciak-stothard et al., 2001; Waschke et al., 2004 and 2006). In the next step, the involvement of Rac1 in insulin-mediated restoration of EC barrier function was examined.

The effect of insulin on Rac1 activity was measured by pull down assay and the pull downs were analyzed for the presence of activated Rac1 (Rac1-GTP) by Western blotting. HUVECs were exposed to thrombin (0.2 IU/ml) in the presence or absence of insulin (1 IU/ml) and a change in Rac1 activity was analyzed for different time periods (min) as indicted. As shown in Figure 4.8A exposure of ECs to thrombin resulted in a significant reduction in Rac1 activity. In the absence of insulin delayed activation of Rac1 was observed and a significant effect was seen only after 30 min. On the other hand, in the presence of insulin a robust activation of Rac1 within first 15 min was observed suggesting the potential role of insulin in Rac1 activation.

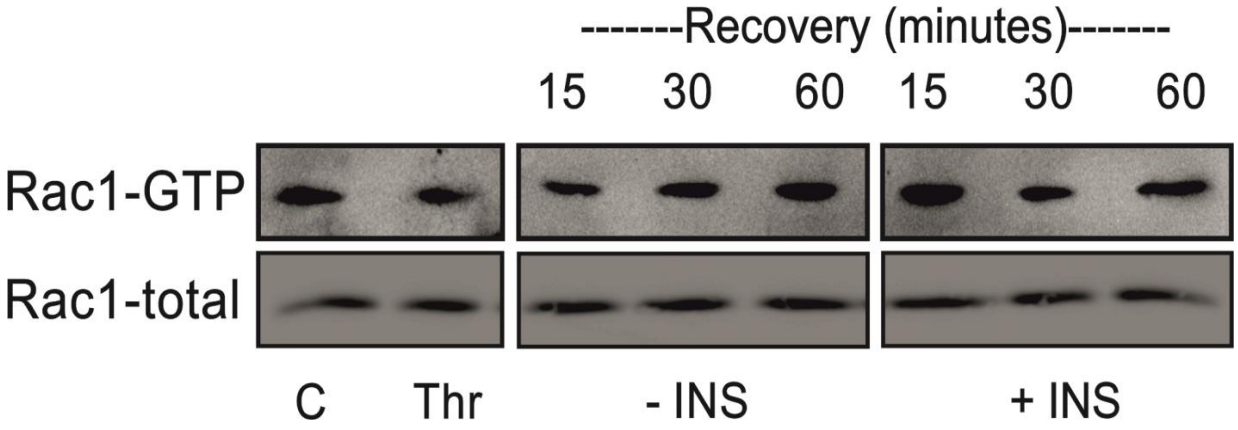



**Figure 4.8A Effect of insulin on Rac1 activation.** Representative Western blots of Rac1-GTP for different time points (min) and Rac1-total as indicated. HUVEC monolayers were treated with thrombin (0.2 IU/ml) in the presence or absence of insulin (1 IU/ml) for different time periods or vehicle (control: C) as indicated. The active Rac1 (Rac1-GTP) was analyzed by pull down assay. Whole cell lysate was used to demonstrate equal loading. Data are mean ± SEM of 5 separate experiments of independent cell preparations.

In the next step the role of Rac1 activation in insulin-mediated EC barrier restoration after thrombin-induced hyperpermeability was further confirmed by using the specific pharmacological Rac1 inhibitor NSC23766 (50 μM) (Gao et al., 2004). ECs were exposed to thrombin (0.2 IU/ml) in the presence or absence of insulin (1 IU/ml) or preincubated with NSC23766 for 30 min in the presence of insulin and albumin flux across HUVEC monolayer was analyzed. As shown in Figure 4.8B pre-incubation of HUVECs with NSC23766 completely abolished the recovery effect of insulin on EC barrier permeability further indicating that insulin-mediated restoration of EC barrier function is via Rac1-dependent manner.

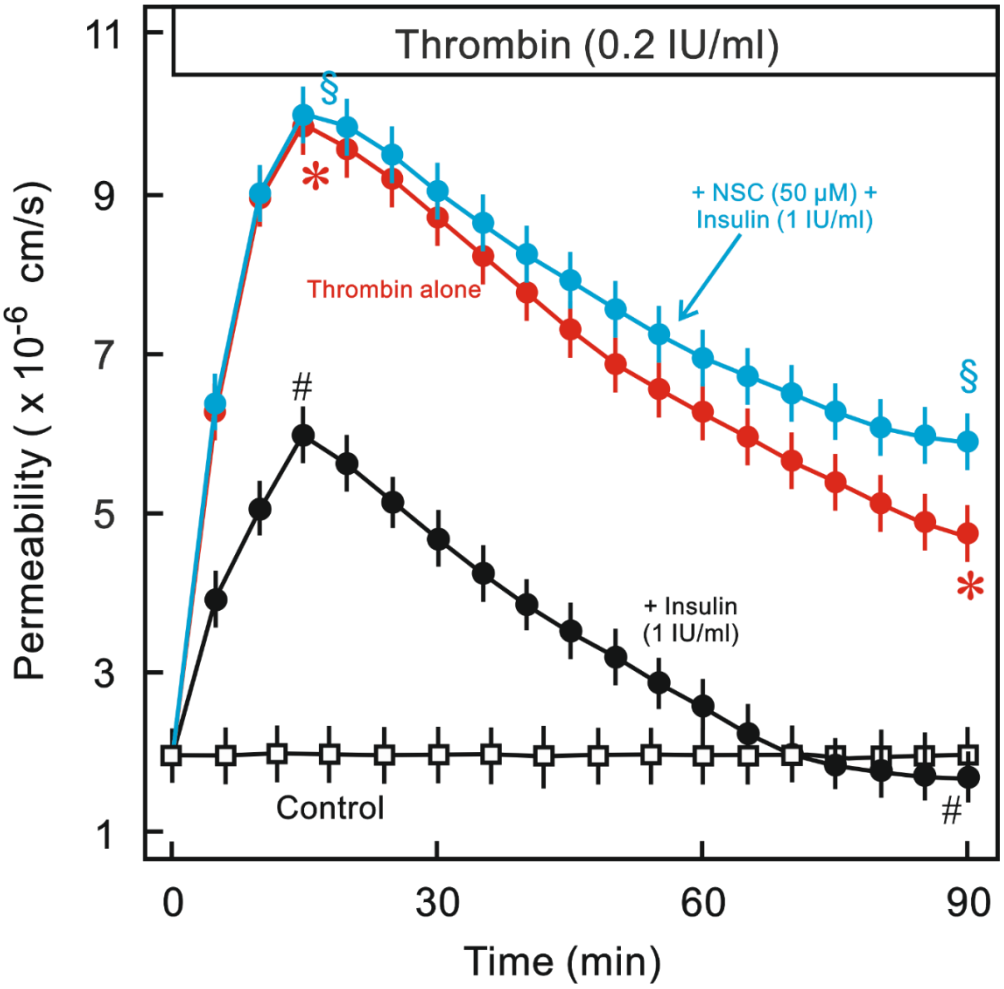



**Figure 4.8B Effect of Rac1 inhibition on insulin-mediated EC barrier restoration.** HUVEC monolayers were exposed to thrombin (0.2 IU/ml) in the presence or absence of insulin (1 IU/ml), or specific Rac1 inhibitor NSC23766 (50 µM) plus insulin, or vehicle (control) as indicated and albumin flux (permeability) across HUVEC monolayer was measured. Data are mean ± SEM of five separate experiments of independent cell preparations. $^{*}$P< 0.05 versus control; $^{\#}$P< 0.05 versus thrombin alone; $^{\S}$P< 0.05 versus insulin plus thrombin.

## 4.9 Effect of insulin on ischemia-reperfusion-induced myocardial water contents

Finally a series of experiments were performed in a well-established isolated saline perfused rat heart model (Noll et al., 1999), to further verify the EC barrier recovery effects of insulin in an intact coronary system. Myocardial water content was defined as an index for capillary leakage and tissue edema formation. To analyze this, isolated perfused rat hearts were exposed to ischemia for one hour followed by 30 min of reperfusion (Rep) and myocardial water content was determined as described in methods section. Under control normoxic conditions (Nor), the mean myocardial water content of the normoxic perfused rat hearts was 455 mL/100g dry weight after 90 min as shown in Figure 4.9.

Exposure of the isolated perfused rat hearts to ischemia followed by reperfusion caused a significant increase in myocardial water content to 554 mL/100 g dry weight. To analyze whether insulin can abolish ischemia-reperfusion-induced increase in myocardial water contents, reperfusion medium was supplemented with 0.1 IU/ml insulin (a concentration only one-tenth of that used in cell culture model), during the first 10 min of reperfusion markedly reduced the reperfusion-induced increase in myocardial water content. In one set of experiments hearts were perfused with insulin in the presence of specific Rac1 inhibitor NSC23766 (50 μM), added 20 min before the start of reperfusion and during the first 10 min of reperfusion. This manoeuvre abolished the protective effect of insulin on reperfusion-induced increase in myocardial water content clearly indicating that insulin-induced barrier recovery is via Rac1 dependent pathway.

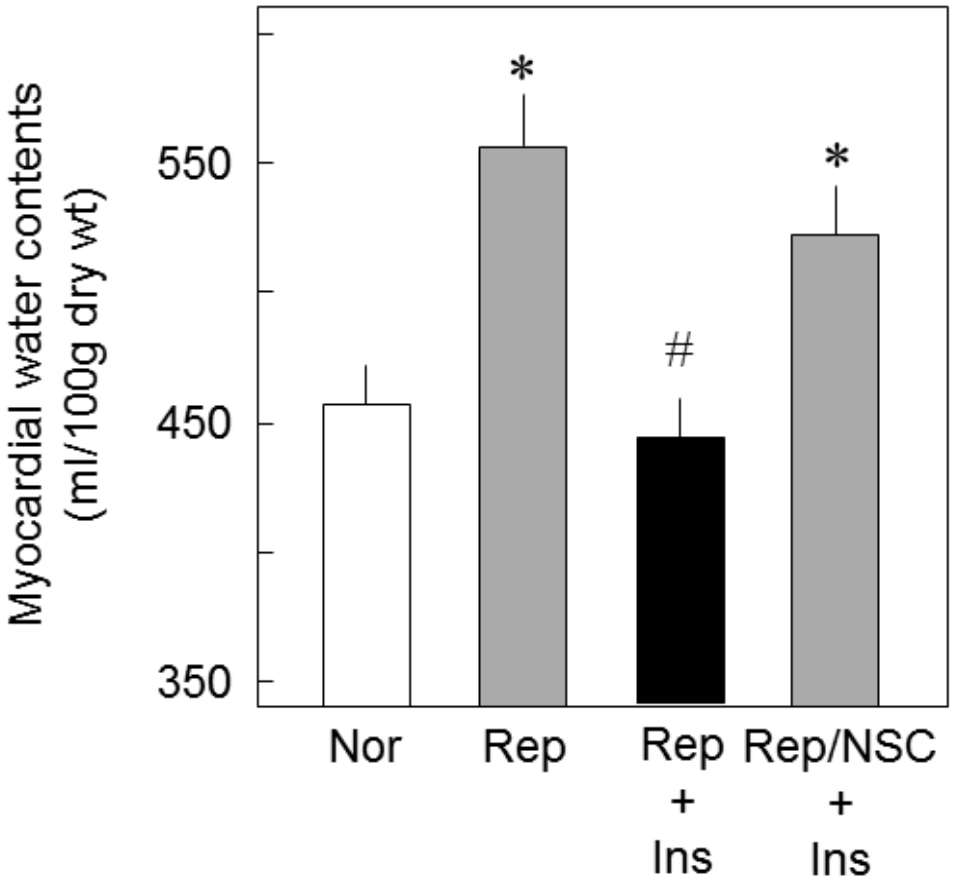



**Figure 4.9 Effect of insulin on myocardial water content of the isolated saline perfused rat hearts after ischemia-reperfusion.** Hearts were exposed for 60 min to ischemia followed by 30 min of reperfusion (Rep) or 90 min of normoxia (Nor). Insulin (Ins; 0.1 IU/ml) was added at the start of reperfusion during the first 10 min. In one set of experiments hearts were perfused in the presence of insulin and Rac1 inhibitor NSC23766 (50 μM), which was added 20 min before the start of reperfusion and during the first 10 min of reperfusion. Data are mean ± SEM of 5 separate experiments with independent organ preparations. $^{*}P<0.05$ versus Nor; $^{\#}P<0.05$ versus Rep.

# 5. DISCUSSION

## 5.1 Main Findings

The precise regulation of semi-permeable barrier function of vascular endothelium lining intima of blood vessels is very important for the exchange of water, small solutes, ions and macromolecules (Michel and curry, 1999; Mehta and Malik, 2006). Disruption of this EC barrier function is often the underlying cause of vascular leakage and life threatening edema formation during pathophysiological conditions like inflammation and ischemia-reperfusion injury (Bazzoni, 2006; Yuan et al., 2007; Kumar et al., 2009).

It is well-established that disruption of endothelial AJs and activation of the EC contractile machinery are the key steps towards opening of intercellular gaps and finally leading to EC barrier failure under conditions of hyperpermeability which is a characteristic response to inflammatory agents such as thrombin (Gündüz et al., 2003; Birukova et al., 2004; Aslam et al., 2012 and 2014). Inflammatory agent thrombin disrupts EC barrier integrity, followed by a slow recovery of the EC barrier function within 2 hrs.

The mechanisms regulating recovery of EC barrier function are essential to regain vascular integrity and successful resolution of edema. However; the precise molecular mechanisms involved in this recovery/restoration of EC barrier function subsequent to EC barrier disruption have not yet been well understood. Comparatively little is known about the maneuvers that can enhance/facilitate the restoration of compromised EC barrier function.

It is well known that intensive insulin therapy confers vasoprotective effects and reduces major cardiovascular complications in diabetics, because of its anti-inflammatory and anti-atherosclerotic effects (Nathan et al., 2005; Langouche et al., 2005; Kim et al., 2006; Dandona et al., 2009). Hyperpermeability of vasculature is the hallmark of aforementioned disease states.

The present study was carried out to address the potential role of insulin in the recovery of EC barrier function in response to thrombin-induced hyperpermeability. Special focus was laid on the dynamics of endothelial contractile activation, disassembly and re-assembly of AJs and dynamic activities and involvement of Rac1. Our data demonstrate that insulin accelerates recovery of EC barrier function in response to thrombin challenge in a well-established *in vitro* model of HUVECs. Moreover, it also abolished the ischemia-reperfusion-induced vascular leakage and edema formation in the intact isolated-perfused rat hearts.

The main and novel findings of the present study are as follows

1. Insulin enhances the recovery of EC barrier function in response to thrombin-induced EC hyperpermeability in a receptor-dependent manner.
2. Insulin fastens re-establishment of VE-cadherin dependent AJs after thrombin challenge.
3. Insulin-induced recovery of EC barrier function is via PI3K/Akt-dependent pathway.
4. eNOS/NO signaling plays no role in insulin-mediated recovery of EC barrier function.
5. Inhibition of MLCK does not play any role in insulin-mediated restoration of EC barrier function.
6. Insulin partly antagonized thrombin-induced increase in MLC and MYPT1 phosphorylation.
7. Insulin-mediated restoration of EC barrier function is via Rho GTPase Rac1 activation.
8. Insulin abolished the ischemia-reperfusion-induced increase in myocardial water content via Rac1 activation.

## 5.2 Insulin accelerates the recovery of EC barrier function in response to thrombin-induced hyperpermeability in a receptor-dependent manner

Consistent with previous studies the data of the present study show that inflammatory mediator thrombin disrupted the permeability of HUVEC monolayer followed by slow recovery towards basal permeability level. Treatment with insulin significantly abolishes thrombin effects from permeability-increasing to a barrier-recovery response in ECs under study. Remarkably, the time required for the half maximal recovery of monolayer permeability was significantly less in the presence of insulin suggesting that insulin accelerated EC barrier restoration. Like other many cell types ECs also express insulin receptors (Zeng et al., 2000; Nitert et al., 2005). Inhibition of insulin receptors abolished the EC barrier recovery effect of insulin which demonstrating barrier restoration effect of insulin is receptor-mediated which is in line with our previous report (Gündüz et al., 2010) showing that insulin stabilized microvascular EC barrier function in a receptor-dependent manner.

## 5.3 Insulin induces fast reassembly of AJs after thrombin challenge.

Endothelial actin cytoskeleton anchored AJs composed of VE-cadherin are one of the key regulators of EC barrier function. Reassembly of AJs precedes the recovery of vascular endothelial barrier integrity and is integral for the maintenance of normal state of EC barrier function (Bazzoni and Dejana, 2004; Wallez and Huber, 2008). Thrombin induces EC hyperpermeability via disassembly of AJs (Garcia et al., 1986) leading to drastic disappearance of VE-cadherin from cell-cell junctions, and intercellular gap formation. This is followed by slow reappearance of VE-cadherin at cell-cell junctions. The changes in VE-cadherin localization at cell-cell junctions are well coordinated with the dynamics of increase and decrease in albumin permeability. Insulin attenuated this thrombin effect on endothelial AJs and promoted fast re-appearance of VE-cadherin at cell-cell junctions thereby strengthened AJs. Insulin-induced fast re-establishment of AJs is mediated via translocation of VE-cadherin to cell-cell junctions.

## 5.4 Insulin-induced EC barrier restoration is via PI3K/Akt-dependent pathway

A classical signaling pathway activated by insulin is PI3K/Akt signaling which mediates most of insulin effects in variety of cell types including ECs (Gündüz et al., 2010; Lee and Ragolia, 2006; Zeng et al., 2000; Hermann et al., 2000). The current study further establishes that the insulin-dependent activation of the PI3K/Akt signaling pathway is required for insulin-mediated EC barrier restoration process. The activation of the PI3K/Akt signaling pathway by insulin in ECs under study is demonstrated by Akt phosphorylation. Insulin-induced a robust increase in Akt phosphorylation and Akt phosphorylation is sustained over the maximum indicated periods of time. Accordingly, inhibition of PI3K completely abolished the insulin-mediated recovery of EC barrier function in response to thrombin-induced EC hyperpermeability, further supporting that insulin-mediated recovery of EC barrier function is via activation of PI3K/Akt pathway.

## 5.5 Effect of eNOS inhibition on insulin-mediated EC barrier recovery

In ECs insulin induces NO production via PI3K/Akt-dependent activation of eNOS (Zeng et al., 2000; Federici et al., 2002; Montagnani et al., 2001 and 2002 ;) which has been shown to stabilize in part microvascular EC barrier function (Gündüz et al., 2010). Therefore, the effect of L-NAME mediated eNOS inhibition on the EC barrier recovery effect of insulin in response to thrombin challenge was investigated.

In contrast to our previous report (Gündüz et al., 2010) on rat coronary microvascular ECs, inhibition of eNOS in HUVECs was unable to abrogate EC barrier stabilizing effects of insulin. This difference may be due to different vascular beds investigated in these studies or even may be due to species differences.

## 5.6 Role of endothelial contractile machinery.

Actin-myosin based EC contractile machinery is one of the important determinants of EC barrier function (Garcia et al., 1995). MLC is a regulatory component of the EC contractile machinery and its phosphorylation plays a pivotal role in controlling the activation of EC contractile machinery, this parameter of contractile activation was also explored in this study

MLC phosphorylation is regulated by antagonistic but balanced activities of MLCP (Härtel et al., 2007; Knapp et al., 1999; Verin et al., 1995 and 2000) and MLCK (Verin et al., 1998; Lazar and Garcia., 1999). It is well established that thrombin causes inhibition of MLCP which results in EC contraction and finally leading to EC barrier disruption (Sheldon et al., 1993; Hixenbaugh et al., 1997; Verin et al., 1998; Goeckeler and Wysolmerski, 2005). The results of the present study demonstrate that thrombin caused a rapid rise in MLC phosphorylation. Insulin effectively attenuated this effect of thrombin in ECs under study and MLC dephosphorylation was faster in the presence of insulin, suggesting that insulin is capable to antagonize thrombin-induced activation of EC contractile machinery and may accelerate restoration of thrombin-induced failed barrier.

Inhibition of MLCK with ML-7 (a specific inhibitor of MLCK) could neither significantly attenuate the thrombin-induced EC hyperpermeability nor have any additive effect on insulin mediated recovery of EC barrier function suggesting that MLCK does not play role in insulin-mediated EC barrier restoration process. A similar report recently in ECs showed that, ML-7 could neither attenuate the thrombin-induced EC hyperpermeability nor augment the EC barrier recovery (Aslam et al., 2014). Even though previous reports indicate that ML-7 antagonized hypoxia/reoxygenation induced EC hyperpermeability (Aslam et al., 2013).

However, in contrast to the present study, Moy et al. showed that ML-7 blocked thrombin-induced hyperpermeability in HUVECs (Moy et al., 2002). The discordance between Moy group and present study is possibly due to high concentrations of drugs used by them. They used thrombin at a very high concentration (7 IU/ml) which is 35-fold higher compared to use in the present study and ML-7 was used at concentration of 100 μM (10-fold higher used in the present study). At high concentration ML-7 may

also block PKC (Odani et al., 2003) and these inhibitory effects presented by Moy group are perhaps due to inhibition of PKC and not due to MLCK.

It is well-demonstrated that RhoA/Rock signaling pathway plays an important role in regulating EC barrier function and pharmacological inhibition of Rock with specific cell permeant inhibitor (Y27632, 10 μM) not only antagonized thrombin-induced contractile activation (Aslam et al., 2010) but also reduced thrombin-induced hyperpermeability (Aslam et al., 2014). Therefore, in the context of the study in the next step the effect of inhibition of RhoA/Rock signaling in insulin mediated EC barrier restoration was observed. Indeed, inhibition of Rock with 10 fold low concentration of Y27632 (1 μM) restores HUVEC monolayer permeability in both insulin and thrombin stimulated conditions. However, inhibition of the RhoA/Rock pathway in the presence of insulin has additive effect on recovery of failed barrier. This suggests the existence of Rock-independent pathway mediating EC barrier restoration.

A second mechanism of MLC phosphorylation is via activation of RhoA/Rock signaling pathway. The endothelial MLCP holoenzyme is a heterotrimeric enzyme composed of a regulatory subunit, (MYPT1; an endogenous direct substrate of Rock) (Aslam et al., 2010) and a catalytic subunit (PP1). RhoA/Rock signaling pathway is one of the most important intracellular phosphorylation trigger of MYPT1 at its inhibitory sites (Kimura et al., 1996). MYPT1 phosphorylation at Thr-850 led to inhibition of MLCP, activation of EC contractile machinery and thereby barrier malfunction (Feng et al., 1999; Velasco et al., 2002; Birukova et al., 2004). Therefore, in the context of the study the question was examined whether insulin can counteract thrombin-induced MYPT1 phosphorylation. The data of the present study demonstrate that thrombin caused a robust increase in MYPT1 phosphorylation which is in consistent with the assumption that thrombin-induced activation of contractile machinery is dependent on inhibition of MLCP. Insulin decreases thrombin-induced MYPT1 phosphorylation. Insulin effect on MYPT1 dephosphorylation is rather delayed as compared to its effect on MLC dephosphorylation. The fast dephosphorylation of MLC but delayed dephosphorylation of MYPT1 in the presence of insulin suggesting that in addition to MLCP activation, other signaling mechanisms are involved in the control of contractile inactivation and EC barrier restoration and needs further investigations.

## 5.7 Effect of Insulin on Rho GTPase, Rac1 activity and its role in insulin-mediated EC barrier recovery.

Rac1, a member of Rho family of GTPases, have been well recognized as an important regulators of endothelial actin cytoskeleton dynamics and stimulates the formation of AJs and that it plays a critical role for the maintenance of EC barrier integrity (Wojciak-stothard et al., 2001 and 2006; Waschke et al., 2004 and 2006; Vandenbroucke et al., 2008; Gündüz et al., 2010; Aslam et al., 2011, 2013 and 2014; Schnittler et al., 2014). Therefore in the next step the dynamics of changes in the Rac1 activation were analyzed.

The results of the present study clearly show that insulin activates Rac1 in ECs under study and protects against thrombin-induced Rac1 inactivation. Even though the precise mechanism of Rac1 activation is beyond the scope of this study. Rac1 activation could be mediated via activation of the Rac1-specific guanine nucleotide exchange factors (GEFs) Tiam1 and TrioN, Insulin can activate TrioN and Tiam1, the GEFs of Rac1, because pharmacological inhibition of these Rac1-GEFs with the specific inhibitor, NSC23766, abolished the EC barrier recovery effect of insulin on macromolecular permeability, indicating an essential role of Rac1 in insulin-mediated restoration of EC barrier function. Nevertheless, these findings strongly emphasize the important and decisive role of Rac1 in insulin-mediated recovery of EC barrier function.

## 5.8 Insulin reduces ischemia-reperfusion-induced increased myocardial water content via Rac1.

Ischemia-reperfusion disrupts the barrier function of vascular endothelium, leading to myocardial edema formation which jeopardizes the functional recovery of the heart during reperfusion (Garcia and Oliveras, 1993; Rubboli et al., 1994; Mehlhorn et al., 2001; Dongaonka et al., 2012). The barrier recovery effect of insulin against ischemia-reperfusion-induced endothelial barrier failure was also further supported by our data obtained in the intact coronary system of the isolated saline perfused rat heat model (Noll et .al., 1999). Insulin, when applied at the onset of reperfusion for only a short period of time strongly reduced the reperfusion-induced increase in myocardial water content. These data depict that insulin plays a beneficial role in the stabilization

of vascular barrier permeability in the intact coronary system, indicating that insulin application during the reperfusion can protect the heart against reperfusion-induced injury and an imminent life threatening edema. However in the intact coronary system this protective effect of insulin was abolished if the heart was perfused in the presence of insulin with specific Rac1 inhibitor NSC23766, clearly indicating that insulin-mediated EC barrier protection/restoration is via Rac1 dependent.

## 5.9 Conclusion

Collectively, the results of the present study conclude that insulin accelerates restoration of EC barrier function in response to thrombin-induced hyperpermeability of HUVEC monolayers via enhancement of cell-cell adhesions and inactivation of the EC contractile machinery. Moreover, insulin also abolished reperfusion-induced vascular leakage in isolated-saline perfused rat hearts. These EC barrier restoration effects of insulin are mediated via PI3K/Akt and Rho GTPase, Rac1 activation which plays a decisive role in insulin-mediated EC barrier restoration in cultured EC monolayers as well as in intact coronary system of isolated-saline perfused rat heart. Furthermore thrombin-induced activation of RhoA/Rock is an important trigger in inducing EC barrier disruption, inhibition of RhoA/Rock signaling enhances the rate of endothelial barrier recovery in the presence of insulin. Importantly, inhibition of eNOS and MLCK do not play any role in insulin-mediated endothelial barrier recovery. Taken together, these findings may warrant further evaluation of the therapeutic potential of insulin on accelerated recovery of failed EC barrier function and reduction of vascular leakage under inflammatory situations and beyond.

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# 7. SUMMARY

The vascular endothelium forms a semi-permeable barrier between blood and interstitium controlling the exchange of water, small solutes, ions, macromolecules and blood cells across the vessel wall. Loss of EC barrier function results in leakage of blood components to interstitium and finally life threatening edema formation during pathophysiological conditions like inflammation and ischemia-reperfusion. Thus, restoration of EC barrier function is important to regain vascular barrier integrity and to prevent edema formation. However, little is known about the mediators and mechanisms involved in the recovery of compromised EC barrier function.

The maintenance of EC barrier integrity is highly dependent on the VE-cadherin-based AJs and actomyosin-based EC contractile machinery. It is now well-established that the disruption of endothelial AJs and activation of EC contractile machinery are the key steps leading towards EC barrier failure under hyper-permeable conditions, a typical EC response to inflammatory mediators such as thrombin.

Insulin is an essential hormone and a key regulator of metabolism. Additionally, it also confers protective effects on the cardiovascular system. Inflammatory mediators like thrombin disrupts EC barrier function, which is recovered slowly. Here, the hypothesis was addressed whether insulin can mediate a faster restoration of EC barrier function and the underlying signaling mechanism by which insulin recovers the EC barrier function was elucidated.

Our data demonstrate that thrombin-induced hyperpermeability of HUVEC monolayers has accelerated recovery in the presence of insulin in a concentration-dependent manner with maximal effect at 1 IU/ml of insulin concentration. This barrier restoration effect of insulin in response to thrombin-induced hyperpermeability was blocked by a specific insulin receptor antagonist. Insulin also abolished reperfusion-induced vascular leakage in isolated-saline perfused rat hearts. Insulin-induced a rapid increase in Akt phosphorylation in HUVECs. Accordingly, inhibition of PI3K with wortmannin completely abolished the insulin-mediated EC barrier restoration, further supporting that the EC barrier recovery function of Insulin is via activation of PI3K/Akt pathway. However, MLCK and eNOS/NO signaling pathways are not involved in

insulin-mediated recovery of EC barrier function. Insulin attenuated thrombin-induced increase in MLC and MYPT1 phosphorylation and also fastens the re-establishment of VE-cadherin dependent endothelial AJs in response to thrombin challenge indicating a contractile inactivation and stabilization of cell-cell adhesion structures. Remarkably, inhibition of Rock has additive effect on insulin-mediated accelerated restoration of failed barrier. Insulin activated Rho GTPase Rac1 and pharmacological inhibition of Rac1 activity by using a specific inhibitor (NSC23766) abrogated the EC barrier recovery effect of insulin on cultured EC monolayers, as well as on reperfusion-induced vascular leakage in intact isolated-saline perfused rat heart, suggesting a Rac1-dependent phenomenon.

In conclusion, insulin accelerates restoration of EC barrier function in response to thrombin-induced EC barrier disruption via enhancement of cell-cell adhesion structures and inactivation of the EC contractile machinery. Insulin also abolished reperfusion-induced vascular leakage in isolated saline perfused rat hearts. These EC barrier restoration effects of insulin are mediated via PI3K/Akt and Rho GTPase-Rac1 activation which plays a decisive role in insulin-mediated recovery of EC barrier function both in HUVEC monolayers and in isolated perfused rat hearts.

# 8. ZUSAMMENFASSUNG

Das vaskuläre Endothel stellt eine semipermeable Barriere zwischen Blut und Interstitium dar. Es kontrolliert den Austausch von Wasser, gelösten Substanzen, Ionen, Makromolekülen und Blutzellen durch die Gefäßwand. Unter verschiedenen pathophysiologischen Bedingungen, beispielsweise bei Entzündung und Ischämie-Reperfusion, kann der Verlust dieser Schrankenfunktion zu einer Leckage der Blutkomponenten ins Interstitium und damit zu einem lebensbedrohlichen Ödem führen. Die Wiederherstellung der endothelialen Schrankenfunktion ist wichtig, um die vaskuläre Integrität zu erhalten und die Entwicklung von Ödemen zu verhindern. Allerdings ist bisher wenig über Mediatoren und Mechanismen bekannt, welche in die Erholung der Barrierenfunktion involviert sind. Der Erhalt der endothelialen Barrierenintegrität hängt einerseits von interzellulären Adhäsionsstrukturen (AJ) ab, die auf VE-Cadherin basieren, andererseits vom endothelialen kontraktilen Apparat, der auf Actin-Myosin basiert. Es ist bekannt, dass eine Zerstörung der endothelialen AJs und eine Aktivierung der endothelialen kontraktilen Elemente die Schlüsselschritte sind, die zum Verlust der Schrankenfunktion führen.

Das essentielle Hormon Insulin ist ein wichtiger Stoffwechselregulator und hat zudem protektive Effekte im kardiovaskulären System. Entzündungsmediatoren wie Thrombin stören die endotheliale Schrankenfunktion, die sich dann nur langsam erholt. In dieser Studie wurde die Hypothese geprüft, ob Insulin eine beschleunigte Regeneration der endothelialen Barrierefunktion vermitteln kann und welche insulinvermittelten Signalwege hier zugrunde liegen.

Unsere Ergebnisse zeigen, dass sich in Anwesenheit von Insulin die Thrombin-induzierte Hyperpermeabilität von HUVEC-Monolayern schneller regeneriert. Dieser Effekt ist konzentrationsabhängig. Die durch Insulin induzierte Erholung kann durch den spezifischen Insulinrezeptor-Inhibitor S961 blockiert werden. Des Weiteren kann durch Insulin die Reperfusions-induzierte vaskuläre Leckage in isolierten, salin perfundierten Rattenherzen aufgehoben werden. Insulin löst einen schnellen Anstieg der Akt-Phosphorylierung in HUVEC aus. Die Wiederherstellung der endothelialen Barrierefunktion ist hierbei abhängig vom PI3K/Akt-Signalweg. MLCK- und eNOS/NO-

Signalwege sind nicht involviert. Insulin hemmt den Thrombin-induzierten Anstieg von MLC- und MYPT1-Phosporylierung und beschleunigt die Wiederherstellung der VE-Cadherin-abhängigen endothelialen AJs. Dies weist auf eine Inaktivierung des kontraktilen Apparates und auf eine Stabilisierung von Zell-Zell-Adhäsionsstrukturen hin. Eine ROCK-Hemmung hat einen additiven protektiven Effekt. Insulin aktiviert die Rho GTPase Rac1. Die pharmakologische Hemmung von Rac1 mithilfe spezifischer Inhibitoren (NSC23766) hebt bei Reperfusions-induzierter vaskulärer Leckage die Erholung der endothelialen Schrankenfunktion in isolierten EC Monoschichten von isolierten, salin perfundierten Rattenherzen durch Insulin auf. Dieser Effekt ist ein Hinweis für einen Rac-1 abhängigen Mechanismus.

Schlussfolgernd ist zu konstatieren, dass Insulin die Erholung der EC Schrankenfunktion nach Thrombin-induziertem Schaden durch eine Verstärkung der Zell-Zell-Adhäsion und eine Inaktivierung des kontraktilen Apparates beschleunigt. Insulin hemmt zudem die vaskuläre Leckage von isolierten, salin perfundierten Rattenherzen. Diese protektiven Effekte werden über einen PI3K/Akt- und Rho GTPase-Rac1 Signalweg vermittelt und spielen eine entscheidende Rolle bei der insulinvermittelten Erholung der endothelialen Schrankenfunktion sowohl in HUVEC als auch in isolierten, salin perfundierten Rattenherzen.

# 9. Erklärung zur Dissertation

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nichtveröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten sowie ethische, datenschutzrechtliche und tierschutzrechtliche Grundsätze befolgt. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, oder habe diese nachstehend spezifiziert. Die vorgelegte Arbeit wurde weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt und indirekt an der Entstehung der vorliegenden Arbeit beteiligt waren. Mit der Überprüfung meiner Arbeit durch eine Plagiatserkennungssoftware bzw. ein internetbasiertes Softwareprogramm erkläre ich mich einverstanden.“

Gießen, 17.12.2015 *Imran Hussain*

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1. Gündüz D, Thom J, **Hussain I**, Lopez D, Härtel FV, Erdogan A, Grebe M, Sedding D, Piper HM, Tillmanns H, Noll T, Aslam M. Insulin Stabilizes Microvascular Endothelial Barrier Function via Phosphatidylinositol 3-Kinase/Akt-Mediated Rac1 Activation. ***Arterioscler Thromb Vasc Biol,*** Mar 25, 2010.

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