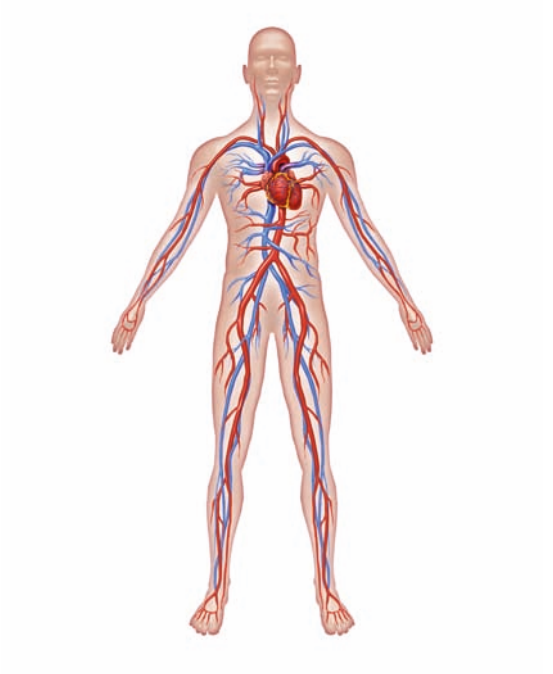
Role of CPI-17 in ischemia-reperfusion induced barrier failure

Sabiha Nazli



INAUGURAL DISSERTATION
submitted to the Faculty of Medicine
in partial fulfilment of the requirements
for the PhD-degree
of the Faculties of Veterinary Medicine and Medicine
of the Justus Liebig University Giessen, Germany

édition scientifique
VVB LAUFERSWEILER VERLAG

1. Auflage 2013

1st Edition 2013

Printed in Germany

édition scientifique
VVB LAUFERSWEILER VERLAG

STAUFENBERGRING 15, D-35396 GIESSEN
Tel: 0641-5599888 Fax: 0641-5599890
email: redaktion@doktorverlag.de

www.doktorverlag.de

Role of CPI-17 in ischemia-reperfusion induced barrier failure

INAUGURAL DISSERTATION

submitted to the Faculty of Medicine
in partial fulfillment of the requirements
for the PhD-Degree
of the Faculties of Veterinary Medicine and
Medicine of the Justus Liebig University Giessen

by

Sabiha Nazli

of

Vehari, Pakistan

Giessen 2013

From the Institute of Physiology
Director/Chairman: **Prof. Dr. Rainer Schulz**
of the Faculty of Medicine of the Justus Liebig University Giessen

First Supervisor and Committee Member: **Prof. Dr. Thomas Noll**
Second Supervisor and Committee Member: **Prof. Dr. Rudolf Schubert**
Committee Members: **Prof. Dr. Wolfgang Kummer, Prof. Dr. Dr. Stefan Arnhold**

Date of Doctoral Defense: 01.07.2013

DEDICATED TO

MY BELOVED HUSBAND

SHAHID RASHID

TABLE OF CONTENTS

ABBREVIATIONS

APS	Ammonium per sulfate
ATP	Adenosine-5-triphosphate
bFGF	Basic fibroblast growth factor
BIM	Bisindolylmaleimide
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CPI-17	PKC-potentiated inhibitor 17-kDa protein
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EC	Endothelial cell
ECGS	Endothelial cell growth supplement
ECL	Enhanced chemiluminescence
EC-MLCK	Endothelial cell myosin light chain kinase
EDTA	Ethylene diamine tetraacetic acid tetraacetic acid
F-actin	Filamentous actin
FCS	Fetal calf serum
G-actin	Globular actin
HBSS	Hanks' balanced salt solution
hEGF	Human epidermal growth factor
HEPES	4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
kDa	Kilo dalton
$MgCl_2$	Magnesium chloride
min	Minutes
MLC	Myosin light chain
MLC~P	Phosphorylated myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
ML-7	1-(5-Iodonapthalene-1-sulfonyl)homopiperazine

MYPT1	Myosin phosphatase targeting subunit 1
NaCl	Sodium chloride
NaF	Sodium fluoride
Na_2HPO_4	Di-sodium hydrogen phosphate
NaH_2PO_4	Sodium dihydrogen phosphate
Na-orthovanadate	Sodium orthovanadate
Neg siRNA	Negative small interfering RNA
NO	Nitric oxide
NP-40	Nonidet P-40
P_i	Phosphate
PAEC	Porcine aortic endothelial cells
PBS	Phosphate-buffered saline
pH	Negative log of H^+ concentration
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol-12-myristate-13-acetate
PMSF	Phenylmethylsulfonyl fluoride
PP1	Protein phosphatase 1
ROCK	RhoA-associated protein kinase
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
Ser19	Serine 19
siRNA	Small interfering RNA
Thr18	Threonine 18
Thr38	Threonine 38
Thr696	Threonine 696
Thr850	Threonine 850
TBS	Tris-buffered saline
TEMED	N, N, N’, N’,-tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
U	Unit
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
% vol/vol	Volume by volume percentage

% wt/vol	Weight by volume percentage
Y-27632	(1R,4r)-4-((R)-1aminoethyl)-N-(pyridine-4 yl)cyclohexanecarboxamide dihydrochloride
ZO-1	Zona-occluden-1

1 INTRODUCTION

1.1 Vascular Endothelium

The vascular endothelium originates from the mesoderm and lines the inner surfaces of all blood vessels and the cavities of the heart. Blood vessels are composed of three layers. The thick outer layer, the tunica adventitia, is made up of connective tissue. The middle layer, the tunica media, contains smooth muscle cells, which contract and dilate to maintain blood pressure. The interior lining, the tunica intima, is formed by a thin layer of endothelial cells (EC), denoted as endothelium. The endothelium fulfils a multitude of physiological functions. It is a selective “tissue-blood barrier” between the vascular lumen and the interstitial space, maintains an antithrombotic and anticoagulant balance in the blood stream, and plays an important role in vasodilation/constriction. Endothelial dysfunction leads to the development of pathological processes such as leukocyte adherence, platelet activation, impaired coagulation pro-oxidation, mitogenesis, vascular inflammation, thrombosis, and atherosclerosis (Verma and Anderson, 2002). Therefore, the study of endothelial function during ischemia-reperfusion is an important tool to analyze the cause of cardiovascular diseases.

1.2 Endothelium Permeability

The maintenance of a semi-permeable barrier by the endothelium is important for controlling the movement of ions, solutes, and macromolecules across the vessel wall. The elements regulating the integrity of the endothelium and endothelial permeability are: (1) cell-surface carrier proteins, (2) interendothelial junctions, (3) the electrostatic charge of endothelial membranes, and (4) the structure of the basal membrane. Among these, interendothelial junctions are one of the most important elements, which establish sites of physical attachment between two adjacent cell membranes. The transport of fluid and molecules across the endothelium occurs via transcellular and paracellular pathways. The transcellular pathway is involved in the transportation of macromolecular plasma proteins such as albumin by caveolae and vesiculo-vacuolar organelle (Feng et al., 1996), while small molecules such as glucose and plasma fluids pass the barrier through small gaps between EC on a

paracellular rout (Lewalle et al., 1997). Three types of interendothelial junctions have been found in EC: adherens junctions (Rubin, 1992), tight junctions (Gumbiner, 1993; Anderson et al., 1993), and gap junctions (Beyer, 1993).

Adherens junctions participate in the formation and stabilization of cell-cell adhesion and reorganization of the actin cytoskeleton. These junctions are formed by the members of transmembrane proteins family, cadherin that are linked to intracellular cytoskeletal and signaling molecules. The endothelial adherens junctions are formed from specific and non-specific types of cadherins: vascular endothelial (VE)-cadherin (Dejana et al., 1995), which is not found in other cells like blood cells or hematopoietic stem cells (Lampugnani et al., 1995) and neuronal (N)-cadherin, which is also present in other cell types, including neural cells and smooth muscle cells. The comparable significant amount of N-cadherin has been found in endothelium (Salomon et al., 1992). Other non-cell-type-specific cadherins, such as P-cadherin expression was detected by PCR analysis but could not be seen with antibody staining and T-cadherin was also expressed in different types of endothelial cells (Ivanov et al., 2001).

VE-cadherin makes a complexe through its cytoplasmic tail to the catenins (α, β, and γ-catenin or plakoglobin) (Lampugnani et al., 1995). β-catenin and plakoglobin are closely related and bind to α-catenin, which interacts with actin binding proteins, such as α-actinin and zonula occludens-1 (ZO-1) (Weis and Nelson, 2006). The binding of VE-cadherin with catenins is important to perform their adhesive function. The extracellular domain of VE-cadherin is necessary to make the first contact and clustering of the molecule while the association of cytoplasmic domain with actin cytoskeleton through catenins is important for the strength of the complex and control of vascular permeability (Navarro et al., 1995). Tyrosine phosphorylation of adherens junction proteins such as VE-cadherin and its linker catenin is associated with weak junctions and impaired vascular permeability. The vascular permeability increasing factors such as tumor necrosis factor-α (Angelini et al., 2006), histamine (Andriopoulou et al., 1999; Shasby et al., 2002), platelet-activating factor (Hudry-Clergeon et al., 2005), and VEGF (Esser et al., 1998) provoke the tyrosine phosphorylation of VE-cadherin and catenins. The tyrosine phosphorylation of VE-cadherin is also induced by the adhesion of leukocytes to endothelial cells via

intercellular adhesion molecule-1 (ICAM1) (Allingham et al., 2007; Turowski et al., 2008).

Tight junctions are formed by the transmembrane and intracellular molecules such as occludin, the claudins, and junctional adhesion protein ZO-1 (Mitic and Anderson, 1998). On the other hand, ZO-1 plays an important role in signal transduction and to make a contact between occludin and the actin cytoskeleton (Fanning et al., 1998).

Furthermore, another type of junction, the gap junction, is formed by connexins (Cx). Three types of connexins (i.e., Cx43, Cx40, and Cx37) have been found in the endothelium. Usually, connexins are arranged in connexons, which serve as channels for the intercellular passage of ions and small molecules (Simon and Goodenough, 1998).

Contiguous to cell-cell adhesion structures, endothelial cells possess an actin-myosin-based contractile machinery, which is an important determinant of endothelial barrier integrity. It maintains the retractile tension of the EC, and is involved in the dynamic changes of the actin cytoskeleton in response to external mechanical forces like shear stress. Its activity is triggered by the phosphorylation state of the myosin light chain (MLC), a small regulatory subunit of myosin (Wysolmerski and Lagunoff, 1991). The imbalance between adhesive and contractile force causes gap formation leading to endothelial barrier failure.

1.3 Endothelium Dysfunction

Endothelial dysfunction is an impairment of normal endothelial functions and was first described in human hypertension in 1990 (Panza et al., 1990). Afterwards, impaired vasodilation in hypertension has been reported by others including small resistance vessels (Park et al., 2001; Schiffrin et al., 2000). Impaired endothelium dependent vasodilation has also been described in type 1 (Beckman et al., 2003) and type 2 diabetes (Rizzoni et al., 2001; Schofield et al., 2002; Endemann et al., 2004), coronary artery disease (Monnink et al., 2002), congestive heart failure (Landmesser et al., 2002), and chronic renal failure (Bolton et al., 2001; Thambyrajah et al., 2000).

EC release vasodilator mediators such as nitric oxide (NO), Prostaglandin I2

and endothelium-derived hyperpolarizing factor in response to chemical and mechanical stimuli, including thrombin, bradykinin, blood pressure, and shear stress, respectively. EC also release vasoconstrictor mediators such as endothelin-1, angiotensin II, thromboxane A2, reactive oxygen species (ROS), and prostaglandins H_2 to control blood pressure.

In endothelial dysfunction, there are reduced vasodilatory responses such as NO generation, oxidative stress, and reduced production of hyperpolarizing factor. Upregulation of ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), E-selectin, generation of chemokines such as monocyte chemoattractant protein-1, and production of plasminogen activator inhibitor-1 participate in the initiation of the inflammatory process (Taddei and Salvetti, 2002). Furthermore, the free radicals are produced during reperfusion to activate the adhesion of neutrophils to endothelial cells (Thiagarajan et al., 1997). The decreased production of NO and the increased production of free radicals will emphasize the sticking of neutrophils to endothelial cells to develop neutrophil-mediated endothelial injury. Endothelial dysfunction after reperfusion may cause platelet aggregation and it increases the risk of thrombosis (Laude et al., 2001). The insufficient production of NO is also involved in the development of heart failure. The coronary endothelial vasodilator dysfunction leads to atherosclerotic disease progression. Therefore, the coronary endothelial vasoreactivity measurement is an important diagnostic tool to detect coronary heart disease (Schachinger et al., 2000). The endothelial dysfunction increases the release of vasoconstrictors, decreases the production of vasorelaxing agents, and the synthesis of dysfunctional coagulation cascades, results in the pathophysiology of pre-eclampsia (Roberts et al., 1989).

Vasoactive mediators such as thrombin and histamine have long been known to cause interendothelial gap formation and to increase endothelial permeability *in vivo* (Majno and Palade, 1961) and *in vitro* (Ehringer et al., 1996). Ischemia-reperfusion injury enhances the leukocyte-endothelium interaction in postcapillary venules, macromolecular leakage, and ultimately the reduction of functional capillary perfusion (Tauber et al., 2004). Reduction in total vascular cross-sectional area is also linked to the swelling of the endothelial cells during reperfusion (Ward and McCarthy, 1995). The acute loss of endothelial barrier function during ischemia-

reperfusion leads to the abnormal transportation of solutes and water across the microvasculature, resulting in the formation of tissue edema (Menger et al., 1997). However, the molecular mechanism that leads to an increase in permeability during ischemia-reperfusion is still not fully understood.

1.4 Ischemia-Reperfusion Injury

Reperfusion causes damage when the blood supply returns to the tissue after an ischemic period, which is known as ischemia-reperfusion injury (Eltzschig and Collard, 2004). This restoration of blood supply causes tissue inflammation due to release of cytokines and neutrophil activation. Ischemia-reperfusion injury causes intracellular calcium overload, generation of ROS, reduced endothelial NO production (Ambrosio and Tritto, 1999; Lefer and Lefer, 1996), and endothelial hypermeability, which leads to myocardial edema (Garcia-Dorado and Oliveras, 1993; Carden and Granger, 2000). The release of ROS and proteolytic enzymes from activated leukocytes plays a vital role in the damage of myocytes and vascular cells (Jones and Lefer, 2000). However, postischemic swelling of the myocardium has also been observed in saline-perfused hearts (Di Napoli et al., 2001), indicating that there is a leukocytes-independent mechanism leading to edema formation. In line with that, Schäfer et al. (2003) and Gündüz et al. (2010) showed that endothelial barrier dysfunction may be induced due to an activation of the contractile apparatus and distortion of cell–cell adherens junctions, leading to intercellular gap formation.

However, the mechanism of reperfusion-induced injury is not fully understood. The understanding of the pathophysiology of reperfusion injury is one of the important challenges in designing novel therapeutic approaches in acute coronary disease. Therefore, it is important to analyze the mechanism of activation of the contractile machinery of EC as well as loss of cell-cell adhesion proteins during ischemia-reperfusion.

1.5 Endothelial Adherens Junction Proteins

The intercellular junctions between endothelial cells control the movement of solutes between blood and tissues. The interaction of adherens junction proteins with the actin cytoskeleton is important for stabilization of junctions. The adherens junction

protein VE-cadherin has extracellular and cytoplasmic domains. The cytoplasmic domain binds to three related catenins: β-catenin, p120, and γ-catenin (plakoglobin). β-catenin binds to α-catenin, which in turn binds to the actin cytoskeleton; this binding is important for endothelial cell barrier function (Yamada et al., 2005 Vestweber, 2008). Tyrosine phosphorylation of junctional proteins like cadherins and catenins is an indicator of junctional disassembly (Young et al., 2003). Disruption of homophilic binding of VE-cadherin to catenins increases endothelial permeability to macromolecules (Hordijk et al., 1999).

1.6 Endothelial Actomyosin Cytoskeleton

There are three basic mechanisms involved in inflammatory conditions that lead to endothelial barrier dysfunction: (I) cytoskeletal protein reorganization, i.e. the rearrangement of cortical actin into cytosolic stress fibers; (II) activation of the contractile machinery; (III) disassembly of adhesion junctions induced by phosphorylation of cell adhesion molecules. This leads to a dissociation of intracellular regulatory proteins like β-catenin from VE-cadherin, which results in its internalization or degradation of adherens junctions. The fundamental parts of the endothelial cytoskeleton are actin filaments (microfilaments), intermediate filaments, and microtubules, which are responsible to maintain cell shape (Chang and Goldman, 2004; Revenu et al., 2004; Dudek and Garcia, 2001). The dynamic equilibrium of actin cytoskeleton between polymerization and de-polymerization depends on the cellular demand. Normally, there is an equal balance between the amount of G-actin and F-actin (Stossel et al., 1985). Actin polymerization is necessary for the formation of F-actin, which is a basic structural unit for actin cytoskeletal. Actin bundles organize into three structures, the membrane skeleton, cortical actin rim at the cell periphery and stress fibers. The membrane skeleton and stress fibers are made up of short F-actin filaments (Cramer et al., 1997). Stress fibers are contractile actomyosin structures and play an important role in cell contraction (Hotulainen and Lappalainen, 2006); and interendothelial cell gap formation (Dudek and Garcia, 2001).

1.7 Endothelial Contractile Apparatus

The endothelial cell contractility is regulated by actomyosin interactions that require actin polymerization and phosphorylation of MLC. The MLC phosphorylation is controlled by the activity of two key enzymes, Ca^{2+}/calmodulin (CaM)-dependent myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) (Verin et al., 1995).

1.7.1 Role of MLC Phosphorylation

Myosins are contractile proteins and are one of the most abundant proteins in cells. On the basis of their head domain structure, myosins can be grouped into different categories (Weiss and Leinwand, 1996). The most important class of myosins is myosin II, which is an actin-binding protein, originally identified in muscle, but also found in non-muscle cells. It is made up of two heavy chains, containing the head and tail domain, two essential and two regulatory MLC. In non-muscle cells, myosin II activity is controlled by the phosphorylation level of the regulatory MLC. It is reported that MLCK and Rho-associated protein kinase (ROCK) can directly phosphorylate MLC at Thr18 and Ser19 (Hirata et al., 2009; Garcia et al., 1995). It can also be phosphorylated at Ser1/2 and Thr9 (Yamakita et al., 1994). MLC phosphorylation at Ser19 is assumed to promote the contractility and stability of actomyosin (Sellers, 1991; Trybus, 1991). This concept is supported by Haeberle et al. (1988) and Umemoto et al. (1989) showing that phosphorylation of MLC at Ser19 starts actomyosin interaction and generates maximum force in permeabilised smooth muscle cells. Additional phosphorylation at Thr18 does not further increase the sliding speed of actin filaments produced by interaction with myosin in *in vitro* assays.

Actomyosin interactions play an important role in cell structure, contraction, adhesion, stress fibre formation, and the ensuing EC barrier dysfunction. MLCP dephosphorylates MLC, causing cell relaxation and barrier stabilization (Verin et al., 1995; Totsukawa et al., 2000).

1.7.2 Role of Myosin Light Chain Kinase (MLCK)

It has been shown that EC express a MLCK isoform of approximately 214 kDa which has a much higher molecular weight then the 113 kDa isoform found in smooth muscle cells (Garcia et al., 1997; Lazar and Garcia, 1999). Both isoforms share the MLC binding site, the catalytic and Ca^{2+}/CaM regulatory motifs. However, the high-molecular EC-MLCK additionally possesses a unique 922-amino-acid domain at its NH_2-terminus, which contains multiple regulatory serine and tyrosine phosphorylation sites.

The MLCK-mediated actomyosin contraction is correlated with cell function, morphology, and motility. Activation of MLCK elevates MLC phosphorylation, cytoskeleton contraction, stress fibre formation, and intercellular gap formation (Tinsley et al., 2000). The MLCK inhibitor ML-7 enhances the basal barrier function and considerably reduces agonist-induced hyperpermeability in isolated-perfused coronary venules (Yuan et al., 1997). Inflammatory mediator such as thrombin increases EC permeability, which can be attenuated by the inhibition of MLCK activity (Garcia et al., 1995). ML-7, a cell-permeable inhibitor of MLCK, also reduces gap formation during reoxygenation in a model of endothelial monolayers (Schäfer et al., 2003). Thus, under physiological conditions MLCK is an important regulator for solutes, macromolecules and blood cells which pass the endothelium on a paracellular route. Under pathophysiological conditions, however, it becomes a critical determent for hyperpermeability (Shen et al., 2010).

1.7.3 Role of Myosin Light Chain Phosphatase (MLCP)

In smooth muscle cells, as well as in endothelial cells, MLCP is a holoenzyme complex composed of protein phosphatase 1 (the catalytic subunit PP1), myosin phosphatase targeting subunit (the regulatory subunit MYPT1), and a smaller subunit of 20 kDa whose function is still unknown (Alessi et al., 1992; Shimizu et al., 1994).

The PP1 is a serine-threonine phosphatase that regulates different cellular processes such as protein synthesis, cell cycle progression, proliferation, carbohydrate metabolism, and muscle relaxation. It has a molecular weight of

approximately 38 kDa and consists of three isotypes, PP1α, PP1β/δ, and PP1γ (Shima et al., 1993). Principally, PP1 can dephosphorylate a multitude of substrates. Its specificity results from its binding to a regulatory subunit, which specifically recruits the catalytic subunit to its substrate. Therefore, the regulatory subunits are unrelated, but share the same PP1-binding sequence known as the RVxF motif (Terrak et al., 2004). In endothelial cells, as well as in smooth muscles cells, MYPT1 binds PP1δ to form the active MLCP holoenzyme complex which dephosphorylates MLC (Verin et al., 2000; Härtel et al., 2007). Two binding sites of PP1 on MYPT1 have been reported: a strong binding site in the N-terminal region (the so called N-terminal peptide from amino acid 1 to 38), and a weak site in the C-terminal half of the ankyrin repeats (Hartshorne et al., 1998). In addition to that, MYPT1 is essential for MLCP activity because PP1 alone has low phosphatase activity towards substrates like MLC (Hirano et al., 1997; Johnson et al., 1997).

Different isoforms of MYPT have been identified, such as MYPT1, MYPT2, MBS85, MYPT3, and TIMAP (Ito et al., 2004). MYPT1 is also called M110, myosin binding subunit (MBS), or M130/M133. Originally it was cloned from chicken gizzard and rat aorta (Shimizu et al., 1994; Chen et al., 1994), but later, Hirano et al. (1997) found MYPT1 also expressed in EC from porcine aorta. As other regulatory subunits, MYPT1 contains the PP1c-binding RVxF motif followed by 7-8 ankyrin repeats in the N-terminal region. MYPT1 from smooth muscle cells directly binds to myosin via its C-terminal region (Hirano et al*.,* 1997; Matsumura and Hartshorne, 2008). MYPT1 does not only confer substrate specificity but also a site of MLCP regulation. It is well established that MYPT1 possess two important inhibitory phosphorylation sites, threonine (Thr) 850 and Thr696 (for sequence in smooth muscle cells). RhoA associated Rho kinase (ROCK) was the first kinase shown to phosphorylate MYPT1 at Thr850 site, which is present in myosin-binding domain to induce dissociation of MLCP from myosin (Velasco et al., 2002), while phosphorylation at Thr696 leads to inhibition of MLCP without complex dissociation (Feng et al., 1999; Ito et al., 2004). Since then kinases have been reported phosphorylating MYPT1 at its inhibitory sites, like ROCK (Kimura et al., 1996; Feng et al., 1999) ZIP kinase (ZIPK) (MacDonald et al., 2001). The thiophosphorylation of MYPT1 caused the decrease in the activity of the enzyme and myosin light chain phosphorylation in smooth muscle cells. It indicates that the phosphorylation and dephosphorylation of this subunit is important

for the regulation of smooth muscle contraction (Trinkle-Mulcahy et.al., 1995). Furthermore, PKA or PKG have been shown to phosphorylate MYPT1 at Ser695 causing MLCP activation or preventing its inhibition (Wooldridge et al., 2004; Nakamura et al., 2007).

In addition to MYPT1 phosphorylation, MLCP activity is modulated by a group of small molecular weight regulatory proteins, which directly interact with the PP1 catalytic subunit. The PKC-potentiated inhibitory protein of 17 kDa (CPI-17) is of special interest in the context of this study. There are also CPI-17 homologues, PHI (Eto et al., 1999), KEPI (Liu et al., 2002), and GBPI (Liu et al., 2004) in the human genome. Each CPI-17 family member contains a PP1 inhibitory domain, where the sequences are >41 % identical to CPI-17 (Eto et al., 1999). CPI-17 is targeted by a variety of kinases. In its phosphorylated form CPI-17 binds to PP1 leading to an inhibition and disassembly of the MLCP holoenzyme.

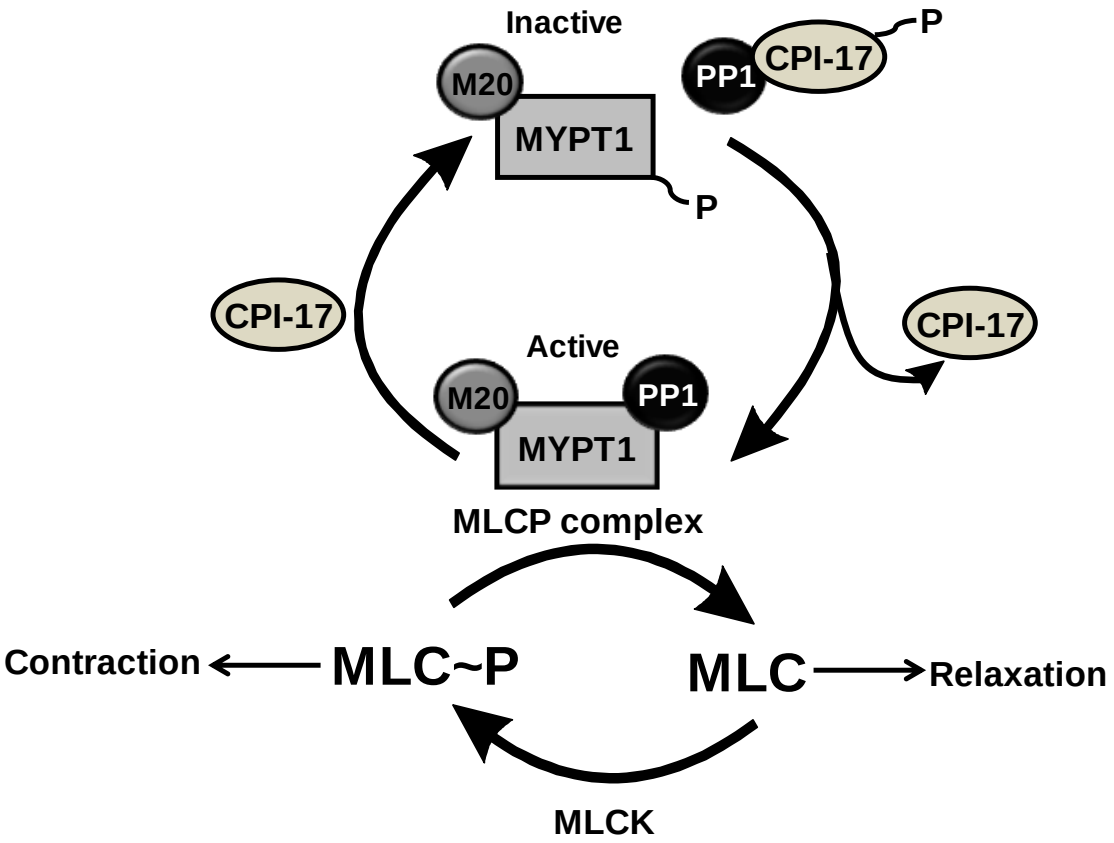


Figure 1.1: The activity of myosin light chain phosphatase (MLCP) is inhibited via phosphorylation of MYPT1 and CPI-17 leading to an increase myosin light chain (MLC) phosphorylation and contractile activation.

1.8 Role of Rho-Kinase

Two isoforms of ROCK have been identified in mammals: ROCKI and ROCKII (Leung et al, 1995; Matsui et al., 1996). In all cells of the cardiovascular system ROCK is controlled by members of Rho-GTPases, particularly RhoA, and is a central intracellular effector of a multitude of mediators, activating cells via G-protein coupled receptors (Somlyo and Somlyo, 2000). Under physiological conditions ROCK is involved in smooth muscle contraction and the ensuing regulation of vascular tone. Furthermore, it is an important determinant for dynamic changes of the cell cytoskeleton, adhesion structures, and triggers cell physiological processes like migration and proliferation (Kaibuchi et al., 1999). Pathophysiological derailment of ROCK activity can cause vasoconstriction up to vasospasms or proliferation of smooth muscle cells of the media. Accordingly, a systemic increase of blood pressure up to hypertension, vascular remodelling and myocardial hypertrophy has been associated with a persistent enhanced ROCK activity (Shimokawa and Takeshita, 2005). This concept is supported by experimental and clinical studies showing that inhibition of ROCK can blunt these diseases (Sawada et al., 2000; Mallat et al., 2003).

In EC activation of RhoA by thrombin leads a ROCK-mediated inhibition of MLCP, an increase in MLC phosphorylation, activation of the contractile apparatus, stress fibre formation, and increased permeability (Essler et al., 1998; van Nieuw Amerongen et al., 1998). Rho-kinase is activated by hypoxia induces MLC phosphorylation that leads to pulmonary vasoconstriction in cultured pulmonary arterial smooth muscle cells and isolated rat pulmonary artery. The Rho-kinase inhibitor Y-27632 abolishes hypoxia-induced pulmonary contraction, indicating that Rho-kinase plays an important role in hypoxia-induced pulmonary vasoconstriction (Wang et al., 2001). Accordingly, Y-27632, which specifically blocks ROCK activity (Ishizaki et al., 2000), lead to MLC phosphorylation, stress fiber formation, and

attenuated thrombin-induced permeability (Carbajal et al., 2000; van Nieuw Amerongen et al., 2000a).

1.9 Role of Protein Kinase C

Protein kinases C (PKCs) are a family of serine-threonine protein kinases and their activities are regulated by Ca^{2+}, diacylglycerol (DAG), and phospholipids. PKC has been reported to regulate different cellular functions such as proliferation and differentiation (Nishizuka, 1984). The various isoforms of PKC have been divided into 3 classes on the basis of their structure, activation, and substrate specificity (Parekh et al., 2000). The conventional or classic PKCs [alpha (α), beta (ß), gamma (γ)] are Ca^{2+}-dependent and are regulated by DAG. The novel PKCs [delta (δ), epsilon (ε), eta (η), theta (θ)] are also regulated by DAG but are Ca^{2+}-independent. The atypical PKCs [zeta (ζ), iota (ι), lambda (λ)] are independent of both Ca^{2+} and DAG. The activity of PKC isoforms depend on their phosphorylation status (Mitchell et al., 1989; Pears et al., 1992; Sonnenburg et al., 2001). The PKC isoforms have four conserved domains (C1–C4) and five variable domains (V1–V5) (Soderling, 1990).

PKC plays a vital role in the endothelial dysfunction in response to different inflammatory mediators, and targeting this signaling molecule with specific inhibitors may constitute an effective therapy for treatment. The regulatory and catalytic domains are important pharmacological targets in designing inhibitors, and many inhibitors have already been reported, e.g. calphostin C and sphingosine, that interact with the regulatory domain (Kobayashi et al., 1989; Hannun et al., 1986). Staurosporine and bisindolylmaleimide (BIM) block PKC activity by interaction with the ATP binding site (Tamaoki et al., 1986; Toullec et al., 1991). The activated PKC by DAG increases both MLC phosphorylation at PKC-specific sites and force development due to inhibition of MLC dephosphorylation rather than stimulation of MLC phosphorylation (Masuo et al., 1994; Ikebe and Brozovich, 1996).

PKC was the first kinase studied in detail during ischemia-reperfusion. The activation and translocation of PKC to the membrane during ischemia was initially analyzed by a protein kinase C activity assay of the cytosol and membrane fractions of hearts exposed to ischemia or ischemia-reperfusion (Prasad and Jones, 1992).

The ischemic translocation was not altered by reperfusion. PKC α, δ, and ε translocation to the membrane and nuclear fractions was reported in rat hearts during ischemia (Yoshida et al., 1996). The translocation of PKC α, ε, and ι was also observed in a rat heart model during myocardial ischemia (Albert and Ford, 1999).

Previously, the crucial role of PKC has been reported in post-ischemic endothelium dysfunction by increased production of superoxides in guinea-pig hearts (Maczewski and Beresewicz, 2000). PKC are activated in response to several inflammatory mediators such as thrombin, bradykinin, and platelet activating factor (Yuan, 2002) and it has been revealed that PKC causes endothelial barrier dysfunction induced by these mediators (Murray et al., 1991; Ramirez et al., 1996; Sandoval et al., 2001) but the molecular mechanism involved in endothelial dysfunction by PKC is not fully understood. The underlying study investigated the role of PKC signalling and the role of VE-cadherin at cell-cell contacts in the regulation of endothelial barrier functions.

Many studies have shown that in smooth muscle cell PKC is involved in the regulation of MLC phosphatase in response to various agonists (Somlyo and Somlyo, 2003). It has been shown that histamine induces MLC phosphorylation, partial inhibition of MLC phosphatase, and disruption of the actin cytoskeleton in corneal endothelium through Ca^{2+} and PKC mediated pathways. This is further influenced by CPI-17 phosphorylation (Srinivas et al., 2006). CPI-17 is phosphorylated by PKCα and PKCδ isoforms and the phosphorylated form inhibits PP1Cδ, the catalytic subunit of MLC phosphatase (Eto et al., 2001). This led to the assumption that the PKC-induced activation of the contractile machinery and increased in permeability may be explained by a PKC/CPI-17-mediated inhibition of MLCP.

1.10 Expression and Regulation of CPI-17

CPI-17 is a soluble globular protein and is described to be an endogenous inhibitor of MLCP (Eto et al., 1995). Two isoforms of CPI-17 have been reported, CPI-17α (147 residues, 16.7 kDa) and CPI-17β (120 residues, 13.5 kDa). The three-dimensional structure of unphosphorylated CPI-17 reveals that it is composed of a four-helix bundle followed by a loop structure (the inhibitory P-loop) at the N-

terminus. The critical phosphorylation site (Thr38) is present in the P-loop structure, in a cavity between helices (Ohki et al., 2001). CPI-17 was initially discovered in smooth muscle tissues (Eto et al., 1997), and the expression level is high in tonic muscles, but lower in phasic muscles. The PKC induced contraction depends on the expression level of CPI-17 in each tissue (Woodsome et al., 2001). The expression level of CPI-17 was also analyzed in embryonic smooth muscle tissues and arterial neointimal lesions. It is highly expressed in mature smooth muscle cells, but the expression level is different under pathological conditions (Kim et al., 2009). Moreover, undetectable level of CPI-17 was observed in arterial smooth muscle from chicken and it produces an insignificant level of the force through G-protein-mediated signals (Kitazawa et al., 2004). CPI-17 expression is also found in platelets (Watanabe et al., 2001) and brain tissues (Dubois et al., 2003). CPI-17 phosphorylation leads to pulmonary hypertension under hypoxia (Dakshinamurti et al., 2005). CPI-17 higher expression and activation has been observed in airway-hyper-responsiveness that is associated with heightened airway resistance and inflammation is a characteristic feature of asthma (Sakai et al., 2005). Thus, the expression level of CPI-17 is an essential determinant for MLC phosphorylation and smooth muscle contraction. Further, it was shown that upregulation of CPI-17 and Rho-kinase mediated Ca^{2+} sensitization leads to high MLC phosphorylation level, slow relaxation in detrusor smooth muscle, and bladder dysfunction in diabetic animals (Chang et al., 2006).

Several phosphatases and kinases control the phosphorylation level of CPI-17. Activation of PKC (Eto et al., 1995), ROCK (Koyama et al., 2000), protein kinase N (Hamaguchi et al., 2000), and ZIP-like kinase (MacDonald et al., 2001), integrin-linked kinase (Huang et al., 2006) induces CPI-17 phosphorylation on Thr38 *in vitro*. PKC α and δ are the dominant kinases that phosphorylate CPI-17 (Eto et al., 2001). CPI-17 is also phosphorylated by PKC α, ε, λ, and ζ, isoforms (Zemlickova et al., 2004). In addition to this, zipper-interacting kinase (MacDonald et al., 2001) and p21-activated kinase (Takizawa et al., 2002) are directly involved to phosphorylate CPI-17 at Thr38. Activation of G-protein-coupled receptors induces a rapid activation of Ca^{2+}-dependent PKC, which in turn phosphorylate CPI-17 leading to an inhibition of MLCP. Maneuver elevating the cellular levels of cAMP (Aslam et al., 2010) or cGMP reduce phosphorylation of CPI-17 (Etter et al., 2001). A similar effect can be

observed in presence of cGMP analogues (Bonnevier and Arner, 2004). In addition, phosphatase 2A and 2C are also attributed to dephosphorylate CPI-17 (Takizawa et al., 2002), indicating the fact that several phosphatases regulate CPI-17 phosphorylation.

CPI-17 phosphorylation at Thr38 results in an inhibition of MLCP activity whereas dephosphorylation causes relaxation of smooth muscle cells. Agonists like histamine, phorbol ester, and phenylepinephrine induce phosphorylation of CPI-17 at Thr38 (Kitazawa et al., 2000). Histamine-induced phosphorylation of CPI-17 is catalysed by PKCα and δ isoforms (Eto et al., 2001). The current concept is that phosphorylated CPI-17 elicits a conformational change and directly binds at the active site of PP1 catalytic subunit and inhibits MLCP (Ohki et al., 2003). Furthermore, there is accumulating evidence that CPI-17 plays an important role in cytoskeletal reorganisation under cell stress. It has been shown that overexpression of CPI-17 in fibroblasts cause abnormal accumulation of cortical F-actin fibres and MLC phosphorylation (Eto et al., 2001). Kolosova et al. (2004) demonstrated that PKC/CPI-17 pathway may be involved in the regulation of endothelial cytoskeleton, focal adhesions, and MLC phosphorylation in response to histamine. Recently, it has been shown that stimulation of the cAMP/PKA-signalling pathway in endothelial cells reduces the phosphorylation level of CPI-17 in EC from human umbilical veins in analogy to the intact arterial smooth muscle (Aslam et al., 2010).

1.11 AIMS OF THE PRESENT PROJECT

In the present study, the role of the contractile machinery in the remodeling of the endothelial cytoskeleton and disruption of the adherens junctions during reperfusion is studied. The integrity of endothelium barrier is maintained by contractile forces and junctional proteins that make a contact between adjacent cells. Under pathological conditions, the loss of this barrier integrity promotes actin-myosin interactions and the opening of intercellular junctions that leads to hyperpermeability. Endothelial hyperpermeability contributes in the progression of different diseases. It was hypothesized that the increased permeability is associated with alteration in the structure and organization of the cytoskeletal junctional complexes in response of reperfusion. The physiological role of actin-myosin interactions in cytoskeletal remodelling during reperfusion is not fully understood. The aims of study were to investigate:

1. the potential mechanism of reperfusion-induced activation of the contractile machinery and increased intercellular gap formation,
2. the role of MLCK and MLCP in the regulation of the MLC phosphorylation status during reperfusion,
3. the role of Rho-kinase and PKC in reperfusion-induced inhibition of MLCP,
4. the functional role of CPI-17, an endogenous inhibitor of MLCP, on reperfusion-induced barrier failure,
5. the effect of reperfusion on localization of adherens junctions proteins VE-cadherin and β-catenin,
6. the role of the actin cytoskeleton in reperfusion-induced barrier failure.

To achieve these aims, underlying experiments were performed with an established cultured model of porcine aortic endothelial cells (PAEC) and human umbilical vein endothelial cells (HUVEC) during reperfusion after simulated ischemia. In pilot sets of experiments, PAEC were exposed to 45 min of simulated ischemia (Po_2 < 1 mmHg, pH 6.4) followed by 45 min of reperfusion (Po_2 = 100 mmHg, pH 7.4). The phosphorylation status of contractile proteins was analyzed in Western blotting. Pharmacalogical inhibitors were used to identify the signaling cascades to be involved in reperfusion-induced barrier failure. The functional assay was performed by measuring macromolecular permeability across endothelial monolayers in CPI-17 downregulated cells. Distortion of adherens junctions proteins and actin rearrangement were visualized by immunocytochemistry.

2 MATERIALS

2.1 Chemicals and Reagents

Acrylamide	Roth, Karlsruhe
Agarose	Invitrogen, Paisley, United Kingdom
6-Aminohexanoic acid	Merck-Schuchardt, Hohenbrunn
APS	Serva, Heideberg
Benzonase	Merck, Darmstadt
Bisacrylamide	Roth, Karlsruhe
Bromphenol blue	Sigma, Deisenhofen
BSA	Sigma, Deisenhofen
Calyculin A	Calbiochem, Bad Soden
Calcium chloride	Merck, Darmstadt
Collagenase	PAA Laboratories, Pasching, Austria
Complete® inhibitor cocktail	Roche, Mannheim
CPI-17 siRNA	Qiagen Hilden
DMSO	Sigma, Deisenhofen
DTT	Sigma, Deisenhofen
EC Oxyrase	Oxyrase, Mansfield, USA
EDTA-Sodium chloride	Sigma, Deisenhofen
Endothelial Growth Medium Kit	PromoCell, Heidelberg
Ethanol	Riedel de Haën, Seelze
FCS	PAA Laboratories, Pasching, Austria
FuGENE 6	Roche, Mannheim
Glucose	Merck, Darmstadt
Glycine	Roth, Karlsruhe
Glycerol (100%)	Roth, Karlsruhe
HBSS	PAA Laboratories, Pasching, Austria
HEPES	Roth, Karlsruhe
High molecular weight standard	Sigma, Deisenhofen
Low molecular weight standard	Sigma, Deisenhofen
Potassium chloride	Merck, Darmstadt
Potassium dihydrogen phosphate	Merck, Darmstadt

Medium 199/Earl`s Salts	Gibco BRL, Eggenstein
Magnesium chloride	Fluka, Neu-Ulm
Magnesium sulfate	Merck, Darmstadt
Methanol	Riedel de Haën, Seelze
Mercaptoethanol	Merck-Suchard, Hohenbrunn
Millipore water	Millipore, Eschborn
Opti-MEM I	Gibco Life Technologies GmbH, Darmstadt
Sodium flouride	Merck, Darmstadt
Sodium chloride	Roth, Karlsruhe
Sodium bicarbonate	Merck, Darmstadt
Di-Sodium hydrogen phosphate	Roth, Karlsruhe
Sodium hydroxide	Roth, Karlsruhe
Neonatal Calf Serum	PAA Laboratories, Pasching, Austria
Paraformaldehyde	Merck, Darmstadt
Penicillin-streptomycine	Gibco BRL, Eggenstein
Ponceau S Solution	Serva, Heidelberg
Sodium dodecyl sulphate	Merck, Darmstadt
N2 gas	Air Liquid, Krefeld
Sodium flouride	Merck, Darmstadt
TEMED	Sigma, Deisenhofen
Tween 20	Amersham, Braunschweig
Tris	Roth, Darmstadt
Triton X-100	Serva, Heidelberg
Trypsin EDTA solution	Biochrom AG, Berlin

2.2 Pharmacalogical Inhibitors

ML-7	Alexis, Lörrach
BIM	Calbiochem, Bad Soden
Y-27632	Alexis, Lörrach

2.3 Antibodies

Primary Antibodies	
Anti-CPI-17; rabbit IgG	Upstate, Charlottesville, USA
Anti-Phospho-CPI-17; rabbit IgG	Upstate, Charlottesville, USA
Anti-MLC; Clone MY-21; mouse IgM	Sigma, Steinheim
Anti-Phospho–MLC; rabbit IgG	Cell Signalling, Danvers, USA
Anti-MYPT1; rabbit gG	Upstate, Charlottesville, USA
Anti-Phospho-MYPT850; rabbit IgG	Upstate, Charlottesville, USA
Anti-RhoA; mouse IgG	Santa Cruz Biotechnology, Heidelberg
Anti-Actin; Clone AC-40; mouse IgG	Sigma, Deisenhofen
Anti-Vinculin; Clone hVIN-1; mouse IgG	Sigma, Steinheim
Anti-α-Tubulin; mouse IgG	Calbiochem, Darmstadt
Anti VE-Cadherin (C-19); goat IgG	Santa Cruz Biotechnology, Heidelberg
Anti-Catenin-beta; TRITC	BD Biosciences, Heidelberg
Anti-phalloidin; TRITC	Sigma, Steinheim

Secondary Antibodies	
Anti-rabbit; IgG; HRP-Conjugate; from donkey	Upstate, Charlottesville, USA
Anti-mouse; IgG; HRP-Conjugate; from sheep	BD Biosciences, Heidelberg
Anti-mouse; IgM; HRP-Conjugate; from goat	Sigma, Steinheim
Anti-goat; IgG; TRITC (Red)	Invitrogen, Karlsruhe
Anti-mouse; IgG; Alexa fluor 488$^{(R)}$ donkey (Green)	Invitrogen, Karlsruhe

2.4 siRNA

Gene	**Sequence**	
CPI-17 siRNA	5'-ACCUGUCGAGGACUUCAUCdTdT-3'	Qiagen, Hilden
	3'-GAUGAAGUCCUCGACAGGUdTdT-5'	
neg siRNA		Qiagen, Hilden

2.5 Laboratory Instruments

Material for cell culture:

CCD camera	Bio Rad Laboratories, Hercules, USA
Culture dishes	Becton-Dickinson, Heidelberg
Demineralisation unit	Millipore, Eschborn
Electroblot chamber	Biotec-Fischer, Reiskirchen
Electrophoresis apparatus	Biometra, Göttingen
Electrophoresis chamber	Biotec-Fischer, Reiskirchen
Filter paper	Biotec-Fischer, Reiskirchen
Glas coverslips	Menzel, Braunschweig
Glass articles	Schott, Mainz
Hamilton syringe	Hamilton, Bonaduz,Switzerland
Incubator	Heraeus, Hanau
LSM-510 confocal microscope	Carl-Zeiss, Heidelberg
Magnet stirrer	Jahnke und Kunkel, Staufen
Magnetic rack	Dynalbiotech ASA Oslo, Norway
Microscope	Olympus, Japan
Neubauer-chamber	Superior, Marienfeld
Nitrocellulose membrane	Schleicher und Schuell, Dassel
pH-Meter	WTW-Weilheim
Pipette tips	Eppendorf Netheler-Hinz, Hamburg
Pipettes	Eppendorf-Netheler-Hinz, Hamburg
Polycarbonate Membrane Transwell Inserts	Corning Life Sciences, Lowell, USA
Power supply	Biometra, Göttingen
Preparation instruments	Aeskulab, Heidelberg
Rubber policeman	Becton-Dickinson, Heidelberg
Shaker	Biometra, Göttingen
Sterile bench	Heraeus, Hanau
Sterile filter (0.2 µm)	Sartorius, Göttingen
Sterile pipettes	Becton-Dickinson, Heidelberg
Table centrifuge	Hereaus, Hanau
Tissue chopper	Harvard Apperatus, March-Hugstetten
Tubes	Eppendorf-Netheler-Hinz, Hamburg

Vortex	Heidolph, Kelheim
Water bath	Julabo, Seelbach

2.6 Softwares

Microsoft Excel 2000	Microsoft Corp, USA
Microsoft Word 2000	Microsoft Corp, USA
Microsoft Windows XP	Microsoft Corp, USA
Quantity One analysis software	Bio-Rad Laboratories, Hercules, USA
LSM 510 confocal microscope	Carl-Zeiss, Heidelberg

3 METHODS

3.1 Isolation and cell culture

Preparation of porcine aortic endothelial cells (PAEC)

Endothelial cells from porcine aorta were isolated as previously described (Spahr and Piper, 1990). Porcine aortas were freshly obtained from the slaughterhouse and kept in 0.9% (wt/vol) NaCl solution until final preparation.

In the first step, aortas were freed from adventitial tissue, opened by a longitudinally cut, mounted onto a board, and rinsed with 0.9% (wt/vol) NaCl solution to flush the intima. Finally, endothelial cells of the upper layer of intima were gently scratched off with a scalpel and transferred into 40 ml of pre-warmed M199 medium. The suspended cells were centrifuged at 260 x g at room temperature for 8 minutes, the supernatant was removed and the pellet was dissolved in fresh cell culture medium. Afterwards, the cells were seeded onto primary cell culture dishes which, contain cell culture medium 199 supplemented with 5% (vol/vol) penicillin/ streptomycin and 10% (vol/vol) NCS. Then cells were cultivated at 37 °C and 5% CO_2 in humidified environment for 3-4 hours. Thereafter, cells were extensively rinsed with pre-warmed HEPES (pH 7.4) to remove cell debris and non-adherent cells. Then adherent endothelial cells were cultured in 15 ml fresh cell culture medium. The next day, cell culture medium was replaced by medium 199 supplemented with 2% (vol/vol) penicillin/streptomycin and 10% (vol/vol) NCS.

Cell culture medium:

M199 medium	x ml
NCS (vol/vol)	10%
Penicillin/streptomycin (100 lU/ml, 100 µg/ml) (vol/vol)	2%

M199 medium: (pH 7.4)

Medium 199 / Earl's salt	9.62 g/l
HEPES	15 mM
NaHCO	24 mM

HEPES/Tyrode's buffer (HBS): (pH 7.4)

NaCl	125 mM
KCl	2.6 mM
KH_2PO_4	1.2 mM
$MgSO_4$	1.2 mM
HEPES	25 mM

Preparation of human umbilical vein endothelial cells (HUVEC)

Endothelial cells from human umbilical veins were isolated as previously described (Jaffe et al., 1973). The umbilical vein was cannulated and perfused with HBSS to remove traces of blood, and was finally filled with collagenase solution (0.2%; wt/vol), using a 30-ml syringe. After 20-30 minutes of incubation at 37 °C, the collagenase solution containing endothelial cells was gently flushed out from the vein with 30 ml of HBSS containing 3% (vol/vol) FCS, to inactivate the collagenase. The cell suspension was collected in a 50 ml falcon tube and centrifuged for 5 minutes at 260 x g. The supernatant was removed and cells were resuspended in culture medium supplemented with 0.1% (vol /vol) gentamycin. The cells were seeded on 3-4 primary 10-cm cell culture dishes and cultured in an incubator (37 °C, 5% CO_2) for 2 hours. Afterwards, cells were washed with pre-warmed HBSS to remove erythrocytes and non-adherent cells. Adherent cells were continued to incubate in fresh cell culture medium in an incubator. The next day, medium was replaced with fresh endothelial cell culture medium.

Collagenase solution

HBSS	xml
Collagenase II (293 Units/mg) (wt/vol)	0.025 %
$MgCl_2$	0.5 mM
$CaCl_2$	1.5 mM

Endothelial cell culture medium

Endothelial cell basal medium (PromoCell) supplemented with following reagents:

FCS (vol/vol)	10%
Endothelial cell growth supplement/Heparin (wt/vol)	0.4%
Hydrocortisone (wt/vol)	0.1%
bFGF (wt/vol)	1 ng/ml
hEGF (wt/vol)	0.1 ng/ml
Penicillin/streptomycin (vol/vol)	2%

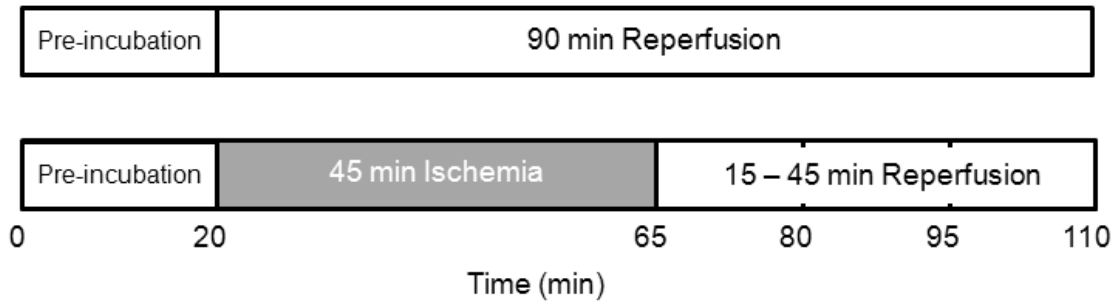
3.2 Subcultivation of endothelial cells

Confluent cultures of primary endothelial cells were washed with pre-warmed HBSS and subsequently trypsinized in 3 ml of trypsin/EDTA solution (composition: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4, 0.05% (wt/vol) trypsin and 0.02% (wt/vol) EDTA). Detached cells were collected into cell culture medium and seeded on cell culture dishes at a density of 2.2 $x10^4$ cells/$cm^{2.}$ For determination of albumin permeability cells were seeded on round polycarbonate membrane transwell inserts. For immunostaining, cells were seeded on 2.5 cm glass cover-slips. Experiments were performed with confluent endothelial monolayers.

3.3 Experimental protocol for ischemia-reperfusion

Monolayers of endothelial cells (80-90% confluence) were exposed to 45 min of simulated ischemia (Po_2 < 1 mmHg, pH 6.4) followed by 15, 30 or 45 min of reoxygenation (Po_2 = 100 mmHg, pH 7.4). Cells were placed on a heating plate at 37

°C and culture medium was replaced with HBSS for 20 minutes to allow the cells to adapt to the incubation conditions (pre-incubation period). Then the cells were exposed to anoxic medium containing 1 U/ml EC-Oxyrase, an oxygen consuming enzyme from bacteria, which reduced the Po_2 in the medium within 1 min below 1 mmHg and maintains it at that low level throughout the ischemic period. Afterwards cells were reoxygenated by change to normoxic medium. In pilot experiments the time course of Po_2 during ischemia and reoxygenation was verified by a polagrographic oxygen electrode. As controls, EC monolayers were incubated at normoxic conditions for 90 min in normoxic medium. In a set of experiments EC were reoxygenated in presence of 10 µM ML-7 (myosin light chain kinase inhibitor), 8 µM bisindolylmaleimide (BIM, a protein kinase C inhibitor), or 20 µM Y-27632 (a ROCK inhibitor), respectively. In pilot experiments the minimum effective concentration of these inhibitors were determined, and stock solutions of Y-27632, BIM, and ML-7 were prepared in DMSO immediately before use. The same final concentration of DMSO was included in all respective control samples, final concentration 0.1 % (vol/vol).



Anoxic Medium (pH 6.4)

NaCl	137.8 mM
$CaCl_2$	1.3 mM
KCl	2.6 mM
KH_2PO_4	1.2 mM
$MgSO_4$ x $7H_2O$	1.2 mM
HEPES	22 mM

Normoxic Medium (pH 7.4)

NaCI	137.8 mM
$CaCl_2$	1.3 mM
KCI	2.6 mM
KH_2PO_4	1.2 mM
$MgSO_4$ x $7H_2O$	1.2 mM
HEPES	22 mM

3.4 Protein detection

3.4.1 Preparation of Samples

After treatment, cells were lysed in pre-heated 2x SDS lysis buffer. Then cell lysate was scraped and collected in a 1.5 ml Eppendorf tube. Samples were denatured for 10 minutes at 65 °C and used immediately or stored at –20 °C.

2x SDS loading sample buffer (100 ml)

Tris/HCI (pH 6.8)	250 mM
Glycerol (vol/vol)	20%
SDS (wt/vol)	4%
β-mercaptoethanol (vol/vol)	1%
bromphenol blue (wt/vol)	0.001%
DTT	10 mM

3.4.2 Preparation of SDS-polyacrylamide gel electrophoresis (SDS PAGE)

The SDS gel apparatus was assembled after cleaning the glass plates with water and ethanol. The resolving gel solution of (12.5%) was prepared according to the volume of solutions as given below. TEMED and ammonium persulfate (APS) were added when the gel was ready to pour. The resolving gel was poured (app. 8.5 cm height), and overlayed a thin layer of water. After polymerization of the resolving gel, the stacking gel solution was poured and a comb was inserted to create the

wells. After polymerization of the stacking gel, the comb was removed and the gel was ready for electrophoresis.

Solutions	**Resolving gel**	**Stacking gel**
	12.5%	**6%**
Distilled water	9.8 ml	17.5 ml
Acrylamide 40% (wt/vol)	12.7 ml	3.8 ml
Bisacrylamide 2% (wt/vol)	7.0 ml	2.0 ml
Separating gel buffer Tris/HCl; 120 mM (pH 8.8)	9.5 ml	------
Stacking gel buffer Tris/HCl; 120 mM (pH 6.8)	------	6.0 ml
SDS 10% (wt/vol)	400 µl	250 µl
TEMED	30 µl	20 µl
APS 10% (wt/vol)	400 µl	250 µl

3.4.3 Electrophoresis

1x running gel buffer was prepared and added to the electrophoresis chamber. The comb was removed and wells were washed. Protein samples were denatured at 65 °C for 10 minutes. The denatured samples were loaded into the wells with a Hamilton syringe. Finally, the gel was run at 45 voltages over night to separate the protein bands.

1x running buffer:

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

3.4.4 Electroblotting (Western blot)

Proteins were transferred onto a nitrocellulose membrane by using semi dry blotting method. Whatman 3M filter papers were cut to size of the gels, wetted in 1x blotting buffer, and three of these were placed onto the anode of the semi dry blotting chamber. The prewetted nitrocellulose membrane was put on the filter papers and the gel placed onto the nitrocellulose membrane. After that three more prewetted filter papers placed onto the gel. The membrane and the gel were gently pressed to remove air bubbles between the layers. The graphite cathode was put on top of the stack. The blot was connected to the power supply and it was run at 0.9 mA/cm^2 of the gel area for 1-2 hours.

1x blotting buffer

Tris/HCl	25 mM
Glycine	150 mM
Methanol (vol/vol)	10%

3.4.5 Ponceau staining

After blotting, the nitrocellulose membrane was washed with water for 4-5 minutes. To estimate the efficiency of protein transfer, the membrane was stained with ponceau S for 5 minutes on a shaker. This stain is reversible and produces pink bands of proteins to show whether the transfer was successful. The membrane was destained by washing with TBST (1x TBS + 0.1% (vol/vol) Tween 20) on a shaker for 10 minutes.

3.4.6 Immunodetection of proteins

After de-staining by washing with TBST, the membrane was incubated in blocking solution (3% (wt/vol) BSA in 1x TBST) for 1 hour at room temperature.

After blocking, the membrane was incubated with primary antibody (1:1000 in 3% (wt/vol) BSA) overnight at 4 °C under constant shaking. Afterwards, the membrane was washed with TBST 3-4 times for 10-15 minutes at room temperature.

Then the membrane was incubated with secondary antibody (1:1000 in 3% (wt/vol) BSA) for 1 hour at room temperature. Subsequently the membrane was washed with TBST 3-4 times for 10-15 minutes.

Then the membrane was incubated with enhanced chemiluminescence (ECL) solution for 1 minute. The luminescence was detected and recorded with Bio-Rad Quantity One gel documentation system and PeqLab Fusion system.

Solutions

10x Tris-buffered saline (TBS)

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

TBS Tween (TBST)

1x TBS
Tween 20 (vol/vol) 0.1%

Primary Antibodies

Antibodies	**Dilution factor**
Anti-CPI-17; rabbit IgG	1:1000
Anti-Phospho-CPI-17; rabbit IgG	1:1000
Anti-MLC; Clone MY-21; mouse IgM	1:1000
Anti-Phospho-MLC; rabbit IgG	1:1000
Anti-MYPT1; rabbit gG	1:1000
Anti-Phospho-MYPT850; rabbit IgG	1:1000
Anti-Actin; Clone AC-40; mouse.IgG	1:1000
Anti-Vinculin; Clone hVIN-1; mouse IgG	1:1000
Anti-α-Tubulin; mouse IgG	1:1000

Secondary antibodies, horseradish peroxidase (HRP)-labeled

Antibodies	Dilution factor
Anti-rabbit; IgG; HRP-Conjugate; from donkey	1:1000
Anti-mouse; IgG; HRP-Conjugate; from sheep	1:1000
Anti-mouse; IgM; HRP-Conjugate; from goat	1:1000

All antibodies were diluted in 3% (wt/vol) BSA in 1x TBST.

3.5 Stripping and reprobing

Stripping solution: (50 ml)
1M Tris/HCl (pH 6.8)
10% SDS (wt/vol)
Millipore H_2O

To reprobe the membranes with antibodies against other proteins of the same or equal size, bound antibodies were removed by incubating the membranes with prewarmed stripping buffer for 2-5 minutes at room temperature under constant shaking. Subsequently membranes were washed extensively with TBST buffer, blocked and reprobed with appropriate antibodies.

3.6 Measurement of endothelial monolayer permeability

The endothelial permeability was measured according to Noll et al. (1999), using a two compartment (luminal and abluminal compartments) system model. The luminal and abluminal compartments were separated by a polycarbonate filter membrane. The medium in the abluminal compartment was continuously stirred and the entire system was placed in a water bath at 37 °C. The luminal compartment contained 2.5 ml while the abluminal compartment contained 6.5 ml of normoxic medium. There was no difference in hydrostatic pressure between the luminal and abluminal compartment. The diffusion of trypan blue-labeled albumin from the luminal to the abluminal compartment was measured continuously with a spectrophotometer (Specord 10, Zeiss Jena, Germany). In a set of experiments, the medium of the

luminal and abluminal compartment contained EC-Oxyrase (1 U/ml) at pH 6.4 for 45 minutes, to expose cell to simulated ischemia. Afterwards, the filters were mounted into the permeability apparatus and albumin flux was determined under reoxygenation conditions.

The albumin flux (F) was measured in mol/ (sec x cm^2) through endothelial monolayer. Area (S) was calculated as the increase in albumin concentration ($d[A]_2$) during the time interval (dt) in the abluminal compartment with the volume (V) as follows:

$$F = \frac{d[A]_2 / dt \times V}{S}$$

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

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

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

3.7 Downregulation of CPI-17

Short interfering RNA (siRNA) was used to silence endogenous CPI-17 in cultured HUVEC. Cells were treated with CPI-17 specific siRNA duplex. Duplex of sense 5'-ACCUGUCGAGGACUUCAUCdTdT-3' and antisense 3'-GAUGAAGUCCUCGACAGGUdTdT-5' siRNA was used for downregulation of CPI-17 as described before (Kolosova et al., 2004). Non-specific siRNA duplex was used as control treatment. Endothelial cells were seeded on 35-mm cell culture dishes (for Western blotting), on Transwell filters (for permeability experiments) and on glass-cover slips (for immunostaining). Confluent cells were transfected with 200 nM CPI-17 specific siRNA or non-specific siRNA using FuGENE 6 transfection reagent according to manufacturer‘s instructions. Experiments were performed after 48 hours

of transfection and downregulation of CPI-17 was determined by Western blot analysis.

Transfection procedure:

Transfection was carried out with 200 nM of siRNA. A calculation for one sample is given below. The total volume was 1200 µl. Two solutions were prepared in two different tubes.
*Solution A. 5*0 µM siRNA (4.8 µl) was mixed with 95.2 µl Opti-MEM I medium.
Solution B. FuGENE (3 µl) was mixed with 97 µl Opti-MEM I medium.

Solution A and solution B were prepared and vortexed for 10 seconds and incubated at room temperature for 10 minutes. Afterwards, the solution A was mixed with solution B, mixed properly by vortexing, and incubated for 40 minutes at room temperature. During this period, cell culture medium was removed, and 1 ml Opti-MEM I medium was added. After 40 minutes, 200 µl transfection medium was added and cells were incubated under standard conditions at 37 ºC, 5% CO_2 for 5 hours. Afterwards the transfection medium was replaced with the cell culture medium and respective experiments were performed after 48 hours.

3.8 Immunostaining

The cell monolayers were exposed to 45 minutes of ischemia followed by 15 min of reperfusion. Then cells were fixed with 4% (wt/vol) paraformaldehyde (PFA) in PBS for 15 minutes at room temperature or with cold methanol for 15-20 minutes at -20 °C. Fixed cells were incubated with 0.2% (vol/vol) Triton X-100/TBS to permeabilize for 10 minutes at room temperature followed by blocking with 5% (wt/vol) BSA/1xTBS/0.1% (vol/vol) Tween 20. After blocking, cells were incubated with primary antibodies at 4 °C for overnight. Primary antibodies (anti-VE-cadherin (C-19); goat IgG and anti-catenin-beta; mouse IgG) were diluted 1:200 in blocking solution. Actin was stained by phalloidin-TRITC. Next day, cells were washed with 1x TBS/0.1% (vol/vol) Tween 20 for 10-20 minutes. Then cells were incubated with desired secondary antibodies diluted in blocking solution at room temperature for 1 hour. Secondary antibodies used: Anti-goat IgG; TRITC 1:500; and Anti-mouse IgG;

Alexa fluor 488 donkey 1:200 respectively. Afterwards, cells were washed with 1x TBS/0.1% (vol/vol) Tween 20 for 10-20 minutes. Cells were mounted with 90% (vol/vol) glycerol/PBS. The prepared slides were visualized under confocal microscope, Zeiss LSM 510.

3.9 Statistical analysis

Data are given as means ± SEM of 3 experiments of independent cell preparations as given in the legends to figures. Data of macromolecule permeability were given as mean $\pm$ SD. Comparison of means of several groups was performed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. Statistical analysis was performed by GraphPAd Prism, version 5 for Windows (GraphPad software Incorporation, San Diego, USA) Probability (P) values less than 0.05 were considered significant ($P< 0.05$).

4 Results

4.1 Effect of ischemia-reperfusion on EC barrier function

To analyze the effect of reperfusion on endothelial barrier function flux of albumin across cultured PAEC monolayers was determined. Under normoxic conditions the permeability did not change during the whole period of incubation (Fig. 4.1). Exposure to simulated ischemia (Po_2 < 1 mmHg, pH 6.4) led to an increase of albumin permeability after 45 min (endischemic value). With the onset of reoxygenation permeability was further increased, reaching a maximum level after 15 min, and then decreased during the ongoing 30 min of incubation.

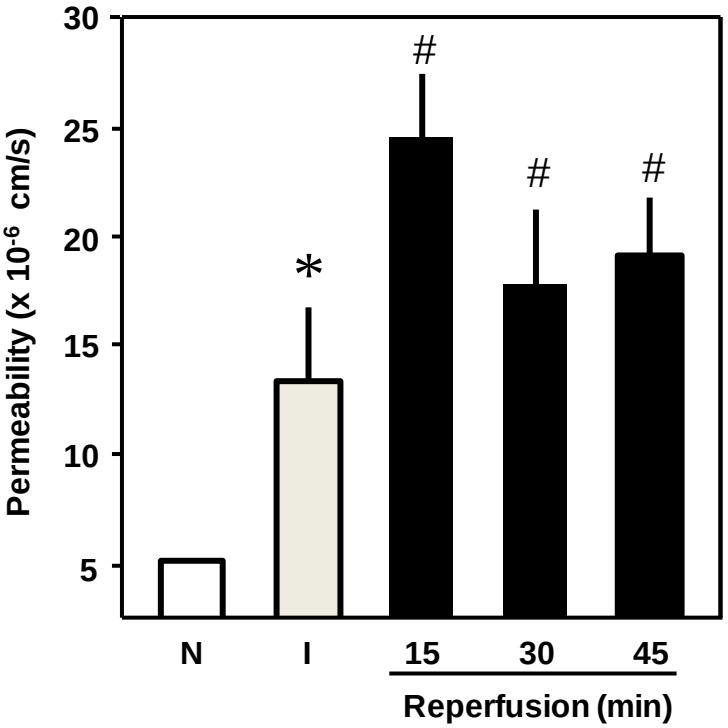


Figure 4.1 Effect of ischemia-reperfusion on albumin permeability of porcine aortic endothelial cells (PAEC). (N) Control cells were exposed to normoxia (Po_2 = 100 mmHg, pH 7.4). (I) Cells were exposed to simulated ischemia (Po_2 < 1 mmHg, pH 6.4) for 45 min. (Reperfusion) Following ischemia cells were reperfused by medium change (Po_2 = 100 mmHg, pH 7.4) and permeability was determined at time 15, 30,

and 45 min, respectively. Data are presented as means ± SEM of 3 separate experiments of independent cell preparations. $P < 0.05$: *I vs. N, #Reperfusion vs. I.

4.2 Effect of ischemia-reperfusion on actin cytoskeleton and adherens junction proteins

A major determinant of endothelial barrier function is the integrity of the actin-based cytoskeleton and cell adhesion structures. It is well established that distortion of both structures lead to cell retraction, opening of intercellular gaps and capillary leakage (Kuhne et al., 1993; Sutton et al., 2003; Glyn and Ward, 2002; Shi et al., 2009).

To analyze the effect of ischemia-reperfusion on actin cytoskeleton and endothelial adhesion structures, PAEC were exposed to simulated ischemia followed by 15 min of reperfusion. As shown in fig. 4.2, actin fibers bundles are arranged as cortical band at the cell periphery in normoxic control cells. After 45 min of ischemia actin cytoskeleton becomes largely rearranged and stress fibers are formed traversing the cells. Disintegration of actin cytoskeleton and stress fiber formation is further enhanced after 15 min of reperfusion. Disintegration of the actin cytoskeleton coincides with VE-cadherin/ß-catenin-based adherense junctions during simulated ischemia and reperfusion, indicated by translocation of both proteins from cell borders, where both nicely decorate cell-cell contacts under normoxic conditions.

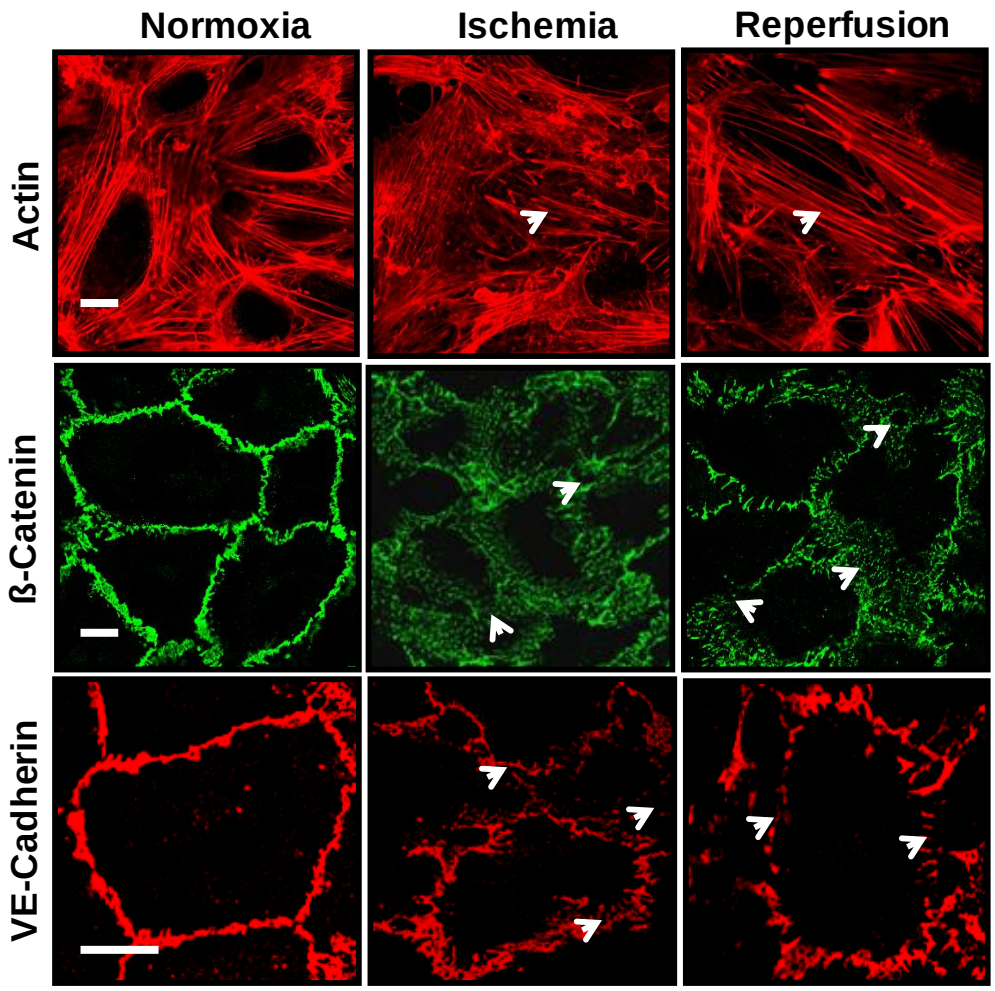


Figure 4.2 Effect of ischemia-reperfusion on localization of actin cytoskeleton and the adherens junction proteins ß-catenin and VE-cadherin in PAEC monolayers. (Normoxia) control cells were exposed to normoxic conditions (Po_2 = 100 mmHg, pH 7.4). (Ischemia) cultured monolayers were exposed to simulated ischemia (Po_2 < 1 mmHg: pH 6.4) for 45 min. (Reperfusion) Following ischemia cells were exposed to reperfusion by medium change (Po_2 = 100 mmHg, pH 7.4) for 15 min. Arrowheads denote F-actin stress fibers, which traverse endothelial cells, or delocalization of ß-catenin and VE-cadherin at endothelial cell borders. One representative image of at least three independent experiments is given. Bar = 20 μm.

4.3 Effect of ischemia-reperfusion on MLC phosphorylation

Activation of the endothelial contractile machinery is the second key determinant of barrier function. Since the endothelial contractile machinery is activated by phosphorylation of the regulatory MLC leading to barrier dysfunction in

macro-vascular and micro-vascular endothelium (Yuan et al., 1997), this parameter was analyzed during ischemia-reperfusion. To that end, PAEC were exposed to simulated ischemia for 45 min followed by reperfusion and MLC phosphorylation was determined at the end of ischemia and during the ongoing reperfusion. As shown in fig. 4.3, MLC phosphorylation was increased during ischemia and persisted at elevated levels during 45 min of reperfusion.

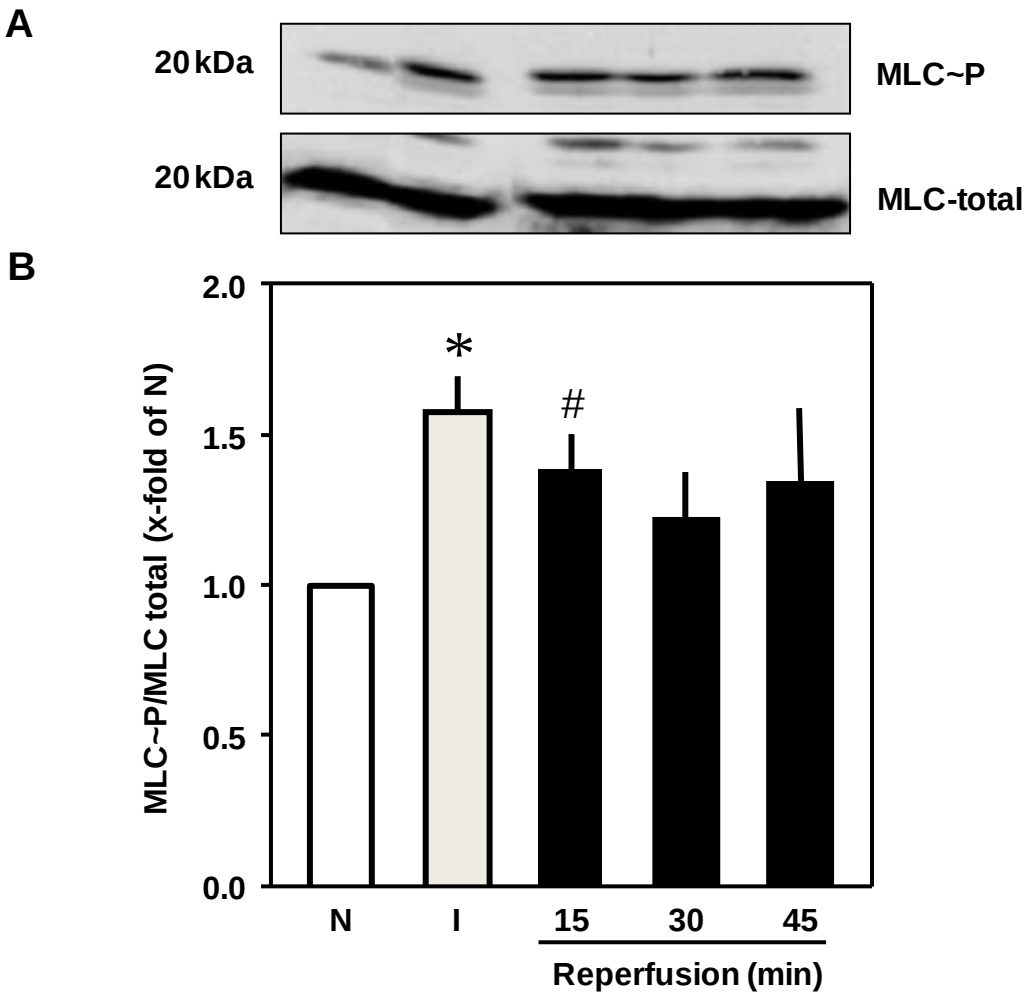


Figure 4.3 Effect of ischemia-reperfusion on MLC phosphorylation of PAEC. (N) Control cells were exposed to normoxia (Po_2 = 100 mmHg). (I) Cells were exposed to simulated ischemia (Po_2 < 1 mmHg, pH 6.4) for 45 min. (Reperfusion) Following ischemia cells were reperfused by medium change (Po_2 = 100 mmHg, pH 7.4) and MLC phosphorylation was determined at time 15, 30, and 45 min, respectively. **(A)** Representative Western blot showing MLC phosphorylation (MLC~P) at Ser19 compared to MLC total. **(B)** Densitometric analysis of the Western

blots. MLC phosphorylation relative to MLC total was given as x-fold increase to normoxia. The ratio of normoxia was set to 1. Data are presented as means ± SEM of 3 separate experiments of independent cell preparations. $P < 0.05$: *I vs. N, #Reperfusion vs. N.

4.4 Effect of MLCK inhibition on ischemia-reperfusion-induced MLC phosphorylation

It is well established that MLCK is one of the most important kinase which directly phosphorylate MLC at Ser19 and causes barrier dysfunction (Garcia et al., 1995; Goeckeler and Wysolmerski, 1995; Yuan et al., 1997). Therefore, the role of this kinase upstream of MLC phosphorylation was assessed during ischemia-reperfusion. PAEC were exposed to simulated ischemia for 45 min followed by 15 min of reperfusion in the presence and absence of 10 µM ML-7, a specific inhibitor added at optimum concentration to completely inhibit MLCK. As shown in fig. 4.4, ML-7 reduces reperfusion-induced MLC phosphorylation, but not below the level of normoxic cells, indicating that MLCK is involved in MLC phosphorylation stimulated by ischemia-reperfusion.

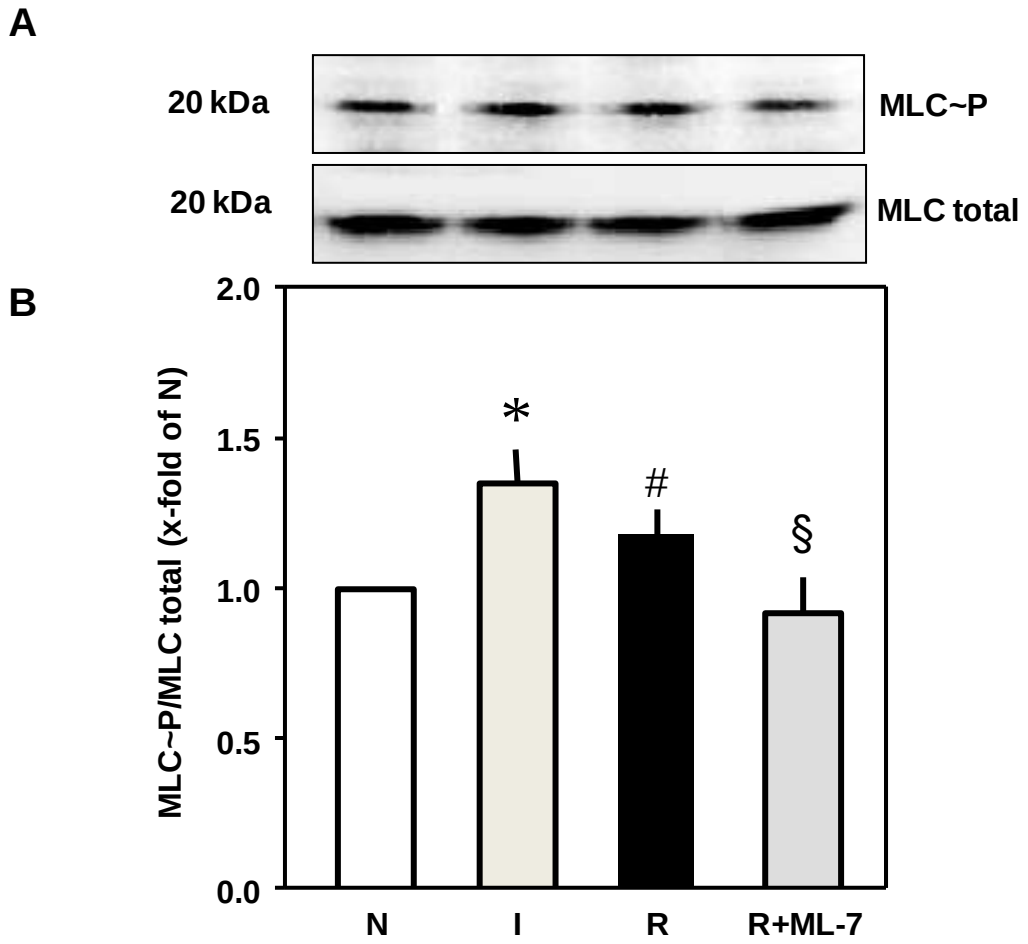


Figure 4.4 Effect of MLCK inhibition on ischemia-reperfusion-induced MLC phosphorylation in PAEC. (N) Control cells were exposed to normoxia (Po_2 = 100 mmHg, pH 7.4). (I) Cells were exposed to simulated ischemia (Po_2 < 1 mmHg, pH 6.4) for 45 min. (Reperfusion) Following ischemia cells were reperfused by medium change (Po_2 = 100 mmHg, pH 7.4) and MLC phosphorylation was determined after 15 min in presence of 10 µM ML-7. **(A)** Representative Western blot showing MLC phosphorylation (MLC~P) at Ser19 compared to MLC total. **(B)** Densitometric analysis of the Western blots. MLC phosphorylation relative to MLC total was given as x-fold increase to normoxia. The ratio of normoxia was set to 1. Data are presented as means ± SEM of 3 separate experiments of independent cell preparations. P < 0.05: *I vs. N, $^{\#}$R vs. N, §R+ML-7 vs. R alone.

4.5 Effect of ischemia-reperfusion on MYPT1 phosphorylation

MLC phosphorylation is balanced by MLCK and MLCP, the latter of which is inhibited by phosphorylation of its regulatory subunit MYPT1 at Thr850. To analyze the activation state of MLCP, the phosphorylation of MYPT1 was determined after 45 min of simulated ischemia and during 45 min of reperfusion in PAEC. As shown in fig. 4.5, ischemia caused an increase in MYPT1 phosphorylation, which is reduced during reperfusion, but is maintained significant elevated compared to the normoxic level, indicating that MLCP is inhibited during ischemia-reperfusion.

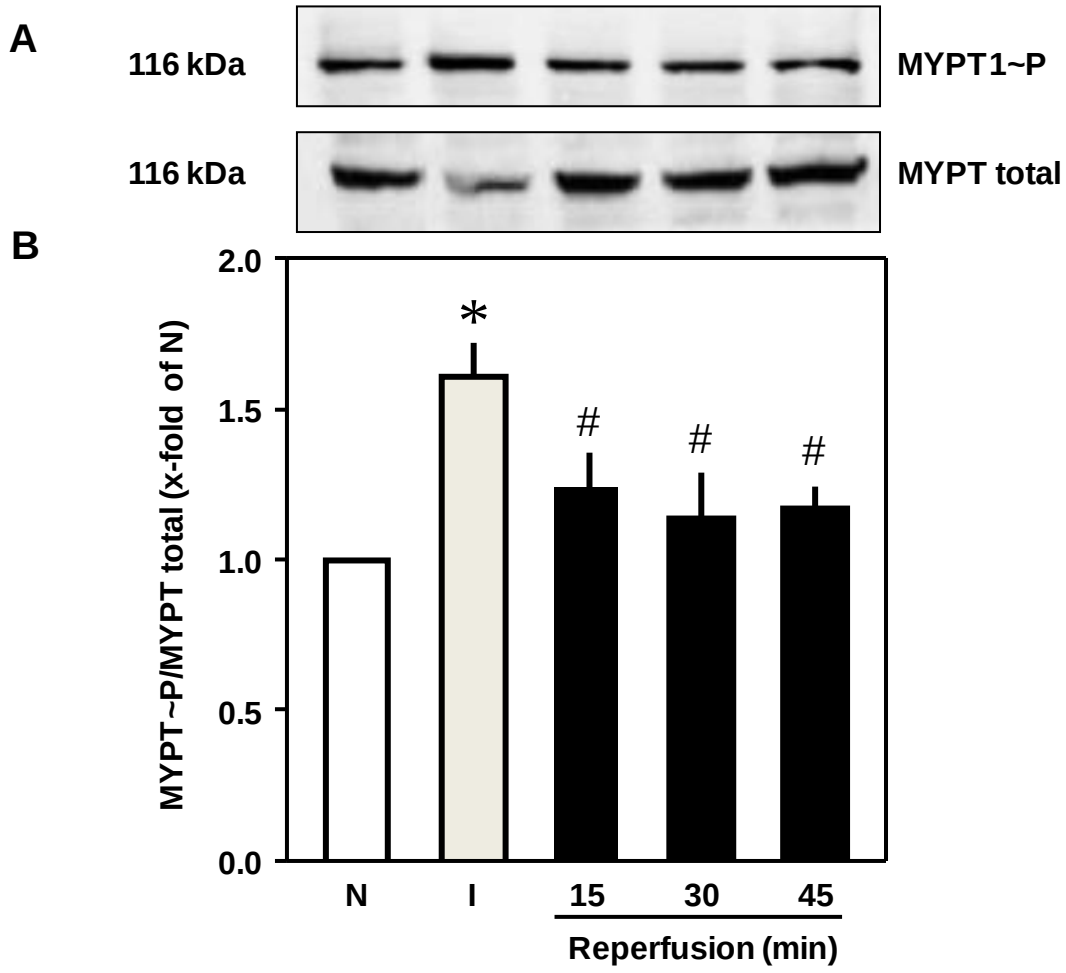


Figure 4.5 Effect of ischemia-reperfusion on MYPT1 phosphorylation in PAEC. (N) Control cells were exposed to normoxia (Po_2 = 100 mmHg, pH 7.4). (I) Cells were exposed to simulated ischemia (Po_2 < 1 mmHg, pH 6.4) for 45 min. (Reperfusion) Following ischemia cells were reperfused by medium change (Po_2 = 100 mmHg, pH 7.4) and MYPT1 phosphorylation was determined at time 15, 30, and 45 min,

respectively. **(A)** Representative Western blot showing MYPT1 phosphorylation at Thr850 relative to MYPT1 total **(B)** Densitometric analysis of Western blot. MYPT1 phosphorylation relative to MYPT1 total was given as x-fold increase to normoxia. The ratio of normoxia was set to 1. Data are presented as means ± SEM of 3 separate experiments of independent cell preparations. $P < 0.05$: * I vs. N, $^{\#}$Reperfusion vs. N.

4.6 Effect of ischemia-reperfusion on CPI-17 phosphorylation

CPI-17 is a small regulatory protein of PP1 catalytic subunit of the MLCP. In its phosphorylated state, CPI-17 can bind to PP1 and inhibit the phosphatase activity. Formerly, CPI-17 has been identified in smooth muscle cells, but is also expressed in endothelial cells and represents another mechanism for inhibition of MLCP activity. Therefore, CPI-17 phosphorylation was analyzed in the present study to support the concept that MLCP is inhibited during ischemia-reperfusion. As shown in fig. 4.6, CPI-17 phosphorylation is below the detection level in normoxic PAEC. However, exposure of the cells to 45 min of simulated ischemia caused a significant increase in CPI-17 phosphorylation, which maintained on the elevated level during reperfusion, indicating that CPI-17 persisted in its inhibitory state during ischemia-reperfusion.

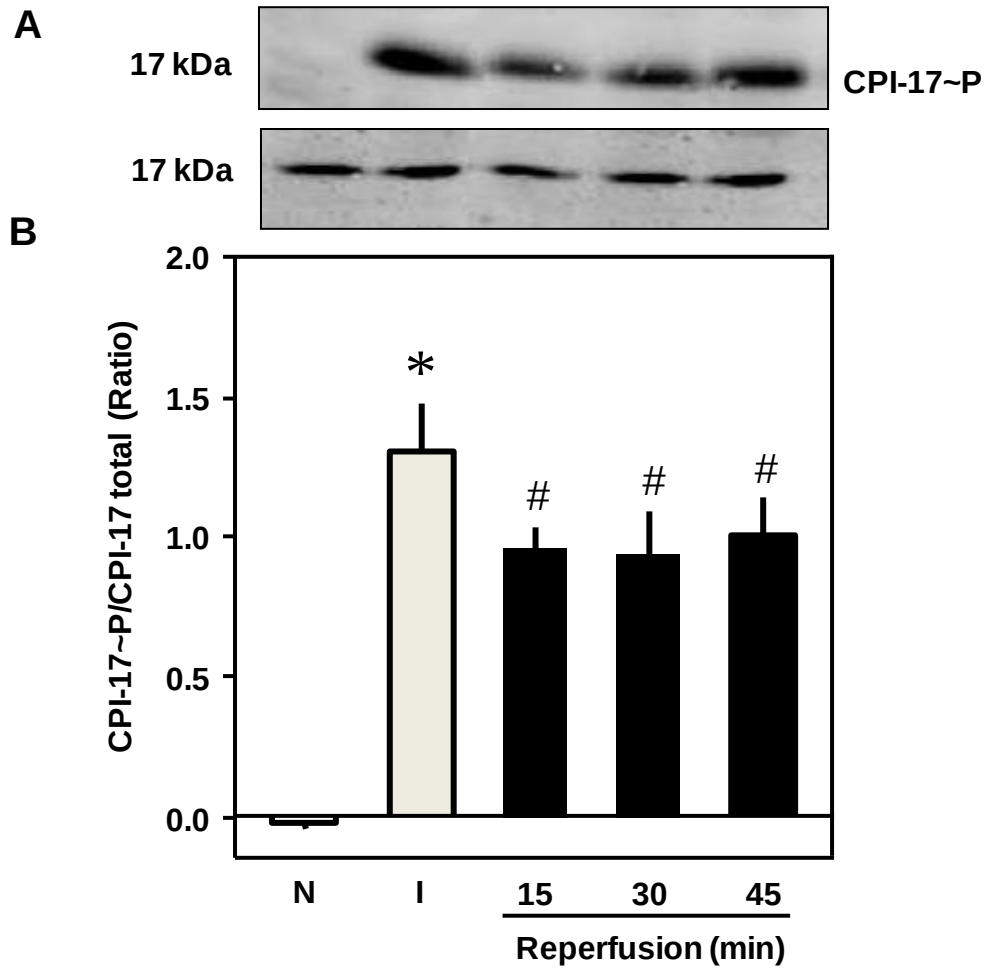


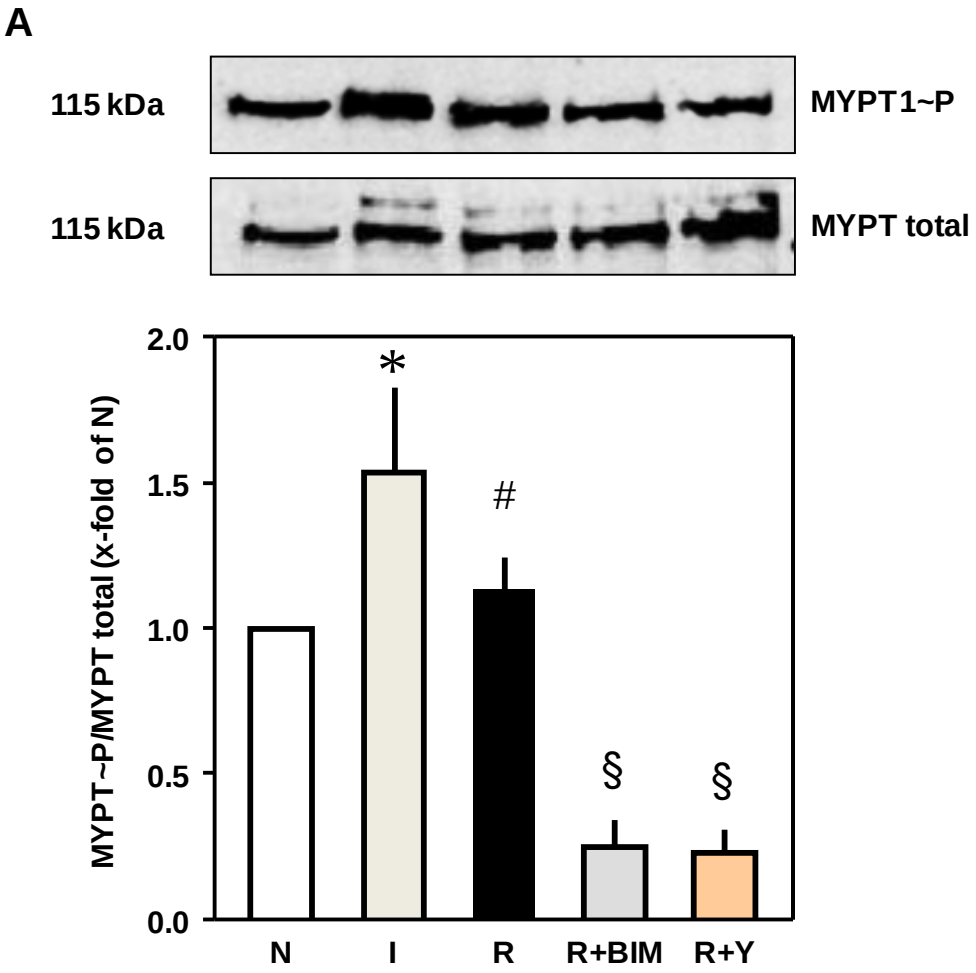
Figure 4.6 Effect of ischemia-reperfusion on CPI-17 phosphorylation at Thr38 in PAEC. (N) Control cells were exposed to normoxia (Po_2 = 100 mmHg, pH 7.4). (I) Cells were exposed to simulated ischemia (Po_2 < 1 mmHg, pH 6.4) for 45 min. (Reperfusion) Following ischemia cells were reperfused by medium change (Po_2 = 100 mmHg, pH 7.4) and CPI-17 phosphorylation was determined at time 15, 30, and 45 min, respectively. **(A)** Representative Western blot showing CPI-17 phosphorylation relative to CPI-17 total. **(B)** Densitometric analysis of the Western blots. CPI-17 phosphorylation relative to CPI-17 total was given. Data are presented as means ± SEM of 3 separate experiments of independent cell preparations. P < 0.05: *I vs. N, #Reperfusion vs. N.

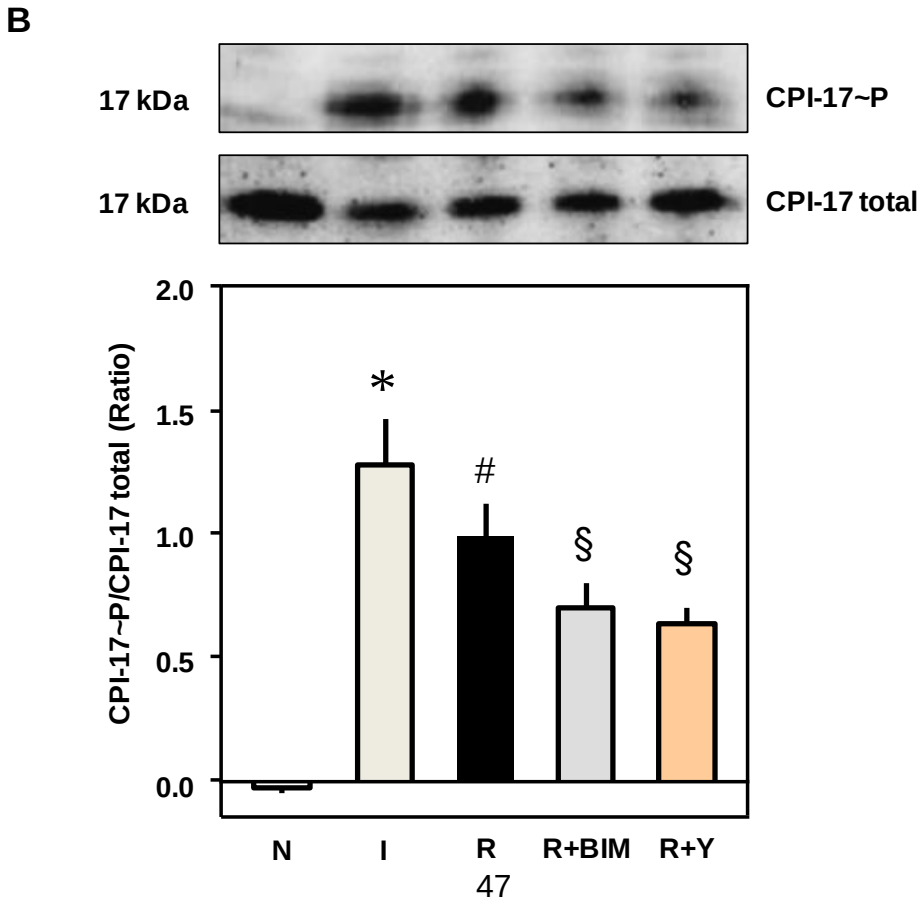
4.7 Effect of RhoA/ROCK and PKC on phosphorylation of MYPT1, CPI-17, and MLC during ischemia-reperfusion

The phosphorylation state of both regulatory subunits of the MLCP holoenzyme complex is controlled by RhoA/ROCK and PKC pathway, leading to inhibition of MLCP activity. Here, the question was tested whether MYPT1 and/or CPI-17 is phosphorylated by one or both kinase pathways during ischemia-reperfusion. To that end, phosphorylation of MYPT1 at Thr850 and CPI-17 at Thr38 was analyzed in PAEC after 45 min of ischemia and 15 min of reperfusion. In sets of experiments 8 µM bisindolylmaleimide (BIM), a pan-specific pharmacological inhibitor of PKC, or 20 µM Y-27632, a specific pharmacological ROCK inhibitor, was added at the onset of reperfusion. As shown in fig. 4.7A, inhibition of the PKC, as well as the RhoA/ROCK pathway, decreased MYPT1 phosphorylation below the normoxic values indicating that both pathways are involved, not only in ischemia-reperfusion-induced MYPT1 phosphorylation, but also in the controls under basal normoxic conditions.

Compared to the effect on MYPT1 phosphorylation, inhibition of ROCK and PKC only blunts ischemia-reperfusion-induced CPI-17 phosphorylation (Fig. 4.7B), indicating that both kinases are only partially involved in the control of the CPI-17 phosphorylation state.

In a final set of experiments it was tested whether the manoeuvre causing dephosphorylation of MYPT1 and CPI-17 affects the phosphorylation state of the regulatory subunit of myosin. As shown in fig. 4.7C, exposure of PAEC to BIM or Y-27632 at the onset of reperfusion in order to block PKC or ROCK, decreased MLC phosphorylation below the normoxic value. The observation that MYPT1 and CPI-17 dephosphorylation in response to inhibition of PKC or ROCK coincide with the reduction in MLC phosphorylation during ischemia-reperfusion supports the concept that inhibition of MLCP is involved in the activation of the contractile machinery under these conditions.


A
115 kDa
MYPT1~P
115 kDa
MYPT total
MYPT~P/MYPT total (x-fold of N)
2.0
1.5
1.0
0.5
0.0
*
#
§
§
N
I
R
R+BIM
R+Y


B
17 kDa
CPI-17~P
17 kDa
CPI-17 total
CPI-17~P/CPI-17 total (Ratio)
2.0
1.5
1.0
0.5
0.0
*
#
§
§
N
I
R
R+BIM
R+Y

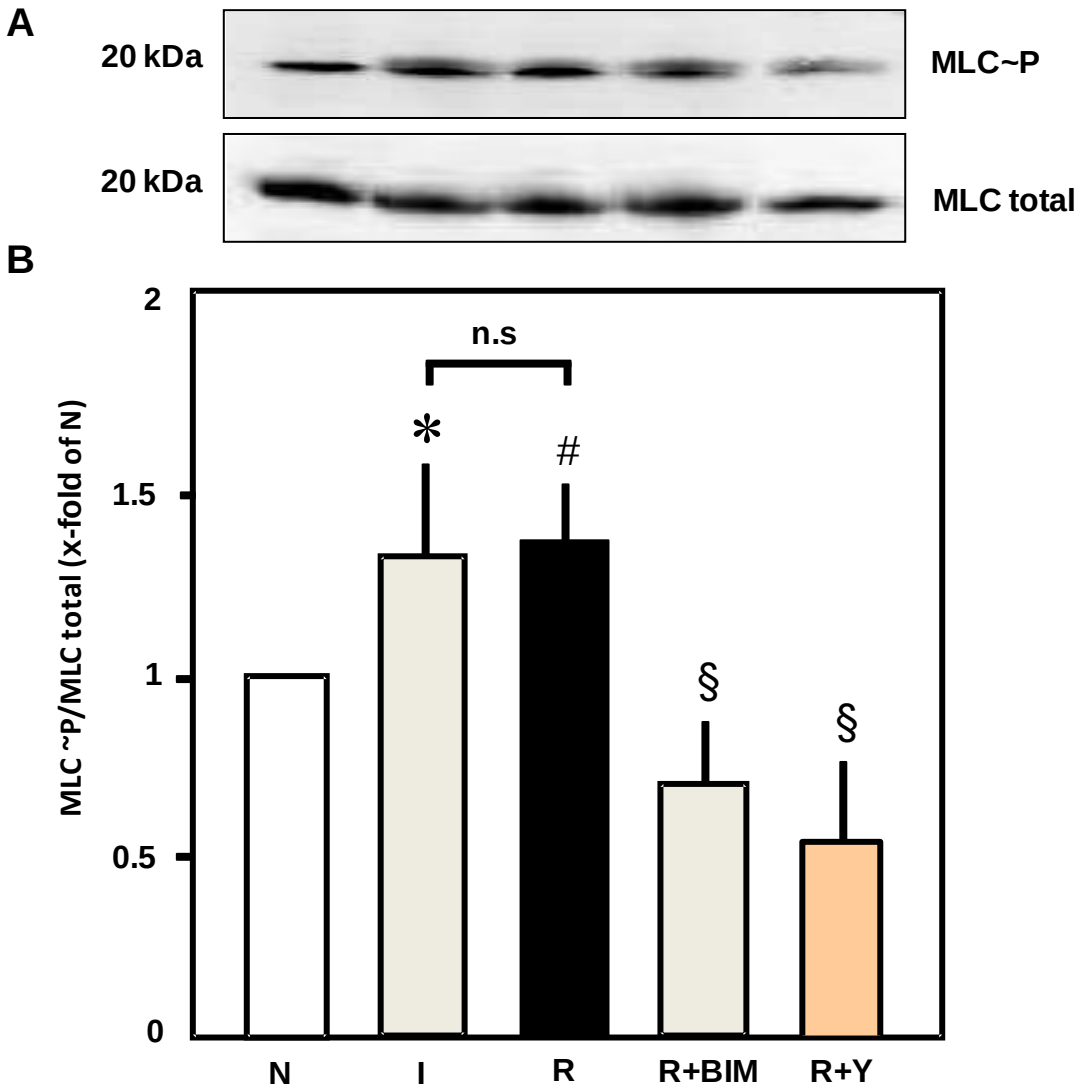


Figure 4.7 Effect of RhoA/ROCK and PKC on phosphorylation of MYPT1, CPI-17, and MLC during ischemia-reperfusion in PAEC. (N) Control cells were exposed to normoxia (Po_2 = 100 mmHg, pH 7.4). (I) Cells were exposed to simulated ischemia (Po_2 < 1 mmHg, pH 6.4) for 45 min. (R) Following ischemia cells were reperfused by medium change (Po_2 = 100 mmHg, pH 7.4) for 15 min in presence of 8 µM BIM (R+BIM) or 20 µM Y-27632 (R+Y). (**A)** Effect of RhoA/ROCK and PKC on phosphorylation of MYPT1. (Top) Representative Western blot showing MYPT1 phosphorylation at Thr850 relative to MYPT1 total. (Bottom) Densitometric analysis of MYPT1 phosphorylation. MYPT1 phosphorylation relative to MYPT1 total was given as x-fold increase to normoxia. The ratio of normoxia was set to one. Data are presented as means ± SEM of 3 separate experiments of independent cell preparations. P < 0.05: * I vs. N, $^{\#}$R vs. N, §R+ BIM or §R+Y vs. R alone. **(B)** Effect of

ROCK and PKC inhibition on CPI-17 phosphorylation. (Top) Representative Western blot showing CPI-17 phosphorylation at Thr38 relative to CPI-17 total. (Bottom) Densitometric analysis of CPI-17 phosphorylation. CPI-17 phosphorylation relative to CPI-17 total was given as. Data are presented as means ± SEM of 3 separate experiments of independent cell preparations. $P < 0.05$: * I vs. N, $^{\#}$R vs. N, §R+BIM or §R+Y vs. R alone. **(C)** Effect of ROCK and PKC inhibition MLC phosphorylation (Top) Representative Western blot showing MLC phosphorylation relative to MLC total. (Bottom) Densitometric analysis of MLC phosphorylation. MLC phosphorylation relative to MLC total was given as x-fold increase to normoxia. The ratio of normoxia was set to one. Data are presented as means ± SEM of 3 separate experiments of independent cell preparations. $P < 0.05$: *I or $^{\#}$R vs. N, §R+BIM or §R+Y vs. R alone, n.s.: not significantly different.

4.8 Effect of PKC inhibition on ischemia-reperfusion-induced macromolecule permeability, F-actin cytoskeleton and adherence junctions

To prove the impact of PKC inhibition on ischemia-reperfusion induced barrier failure, PAEC were exposed to simulated ischemia for 45 min followed by 15 min of reperfusion. In a set of experiments 8 µM BIM was added to the cells. As shown in fig. 4.8A, inhibition of the PKC pathway significantly reduced reperfusion-induced albumin permeability.

In a second step, the role of PKC on reperfusion-induced distortion of actin cytoskeleton and adherens junction was analyzed. To that end, PAEC were exposed to 45 min of ischemia followed by 15 min of reperfusion as described in (4.2). In a set of experiments PAEC were reperfused in presence of 8 µM BIM (8 µM).

As shown in fig. 4.8B, BIM attenuated the ischemia-reperfusion-induced stress fiber formation, and restored the ribbon like distribution of VE-cadherin at cell-cell junctions during reperfusion.

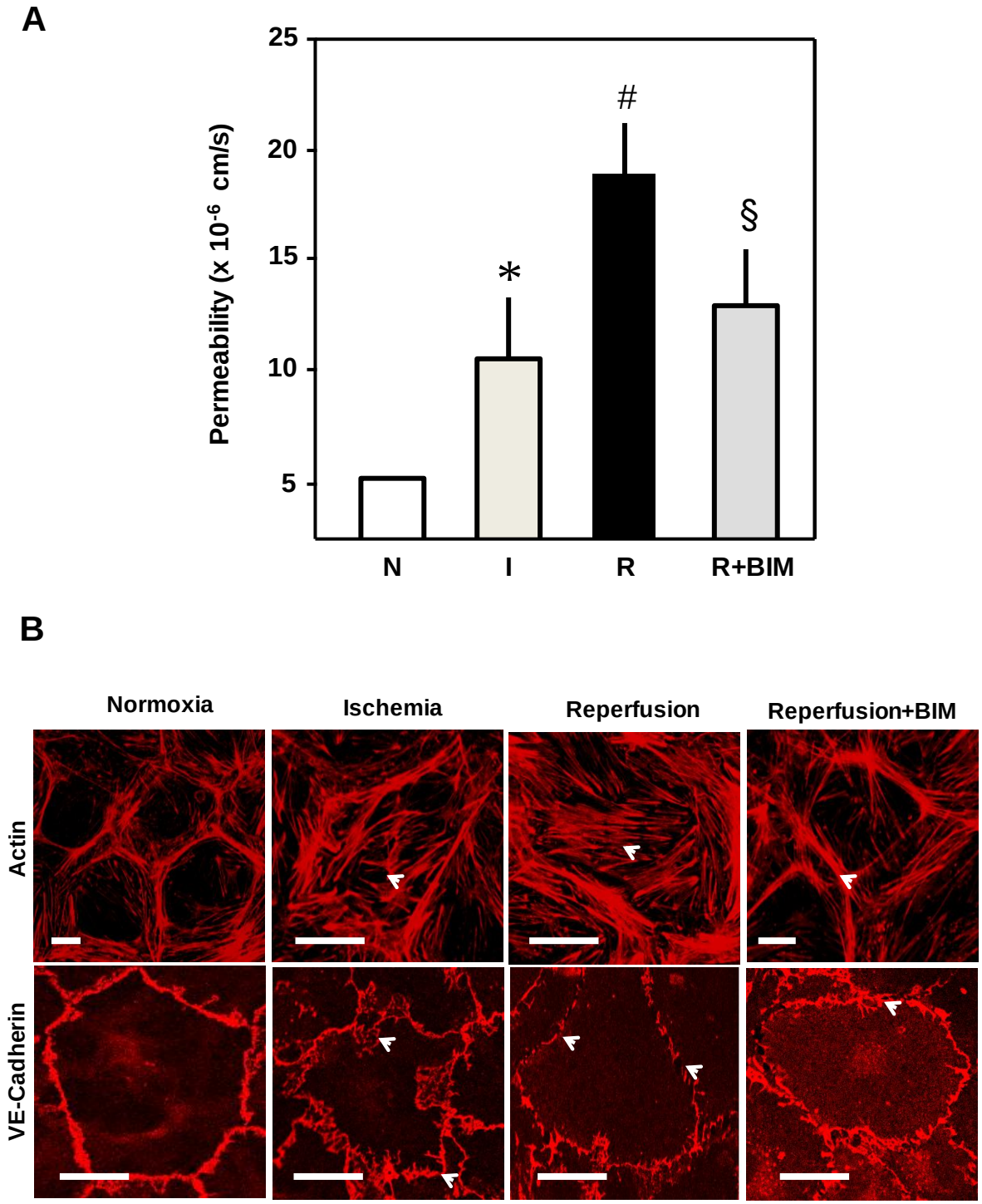


Figure 4.8 (A) Effect of PKC inhibition on ischemia-reperfusion-induced macromolecule permeability in PAEC**.** (N) Control cells were exposed to normoxia (Po_2 = 100 mmHg, pH 7.4). (I) Cells were exposed to simulated ischemia (Po_2 < 1 mmHg, pH 6.4) for 45 min. Following ischemia cells were reperfused by medium change (Po_2 = 100 mmHg, pH 7.4) in absence (R) or presence of 8 µM BIM (R+BIM)

for 45 min. Data are presented as means ± SEM of 3 separate experiments of independent cell preparations. P<0.05: *I vs. N, #R vs. N, §R+BIM vs. R alone. **(B)**. Effect of PKC inhibition on distortion of actin cytoskeleton and VE-cadherin in PAEC monolayers. Control cells were exposed to normoxic conditions (N) and simulated ischemia (I) as in (A). Afterwards cells were reperfused as in (A) by medium change for 15 min with (R+BIM) and without (R) PKC inhibitor 8 µM BIM. Arrowheads denote F-actin stress fibers, which traverse endothelial cells, or delocalization of VE-cadherin at endothelial cell borders. One representative image of at least three independent experiments is given. Bar = 20 µm.

4.9 Effect of CPI-17 silencing on ischemia-reperfusion-induced MLC phosphorylation

As shown above, CPI-17 is strongly phosphorylated during ischemia and persists on a high level during reperfusion (Fig. 4.6 and 4.7B). This high level of CPI-17 phosphorylation coincided with the elevated level of MLC phosphorylation (Fig. 4.3). In contrast, MYPT1 has already declined to the normoxic value during reperfusion. Thus, the CPI-17, the endogenous inhibitor of PP1 catalytic subunit, appears to be an important regulator in MLC phosphorylation and contractile activation in endothelial cells.

To further support this conclusion, HUVEC were treated with CPI-17 specific siRNA (200 nM) or negative siRNA for 48 h to reduce the endothelial content of CPI-17. Afterwards the cells were exposed to 45 min of ischemia followed by 15 min of reperfusion. As shown in fig. 4.9, HUVEC, as PAEC, express CPI-17. Reduction of CPI-17 by gene silencing led to dephosphorylation of MLC under basal conditions and prevented MLC phosphorylation during ischemia as well as reperfusion.

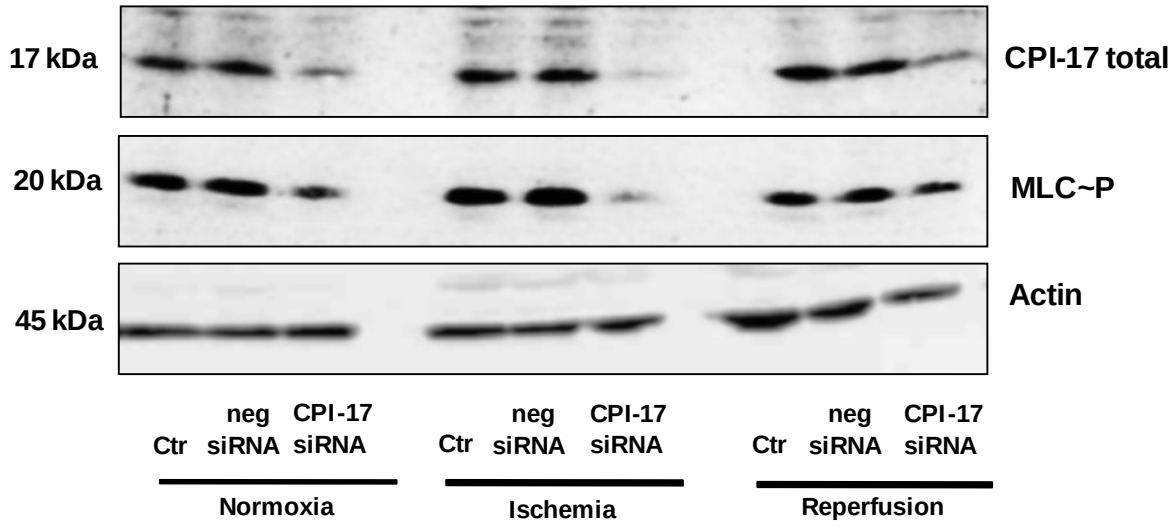


Figure 4.9 Effect of CPI-17 silencing on MLC phosphorylation during ischemia-reperfusion. HUVEC were treated with CPI-17 specific siRNA or negative (neg) siRNA for 48 h. (N) Control cells were exposed to normoxia (Po_2 = 100 mmHg, pH 7.4)). (I) Cells were exposed to simulated ischemia (Po_2 < 1 mmHg, pH 6.4) for 45 min. (R) Following ischemia cells were reperfused by medium change (Po_2 = 100 mmHg, pH 7.4) for 15 min. Representative Western blot shows MLC phosphorylation relative to CPI-17 total. Actin was used as loading control.

4.10 CPI-17 silencing restores ischemia-reperfusion-induced distortion of actin cytoskeleton and adherens junctions

In a second step, the effect of CPI-17 silencing on ischemia-reperfusion-induced disruption of cell adhesion molecules was analyzed. Therefore, HUVEC were treated with CPI-17 specific siRNA (200 nM) or neg siRNA for 48 h, which has been proven to effectively reduce cellular CPI-17 protein content (Fig. 4.9). Afterwards, cells were exposed to 45 min of ischemia followed by 15 min of reperfusion.

As shown in fig. 4.10, silencing of CPI-17 restores disruption of intercellular junction proteins. VE-cadherin reappeared at cell-cell junctions in CPI-17 siRNA transfected cells as compared to control siRNA during reperfusion. The effect of CPI-17 silencing on reperfusion-induced distortion of actin cytoskeleton was also studied.

Formation of actin stress fibers is reduced in CPI-17 siRNA transfected cells, and actin bundles reside at the cell periphery even during ischemia-reperfusion.

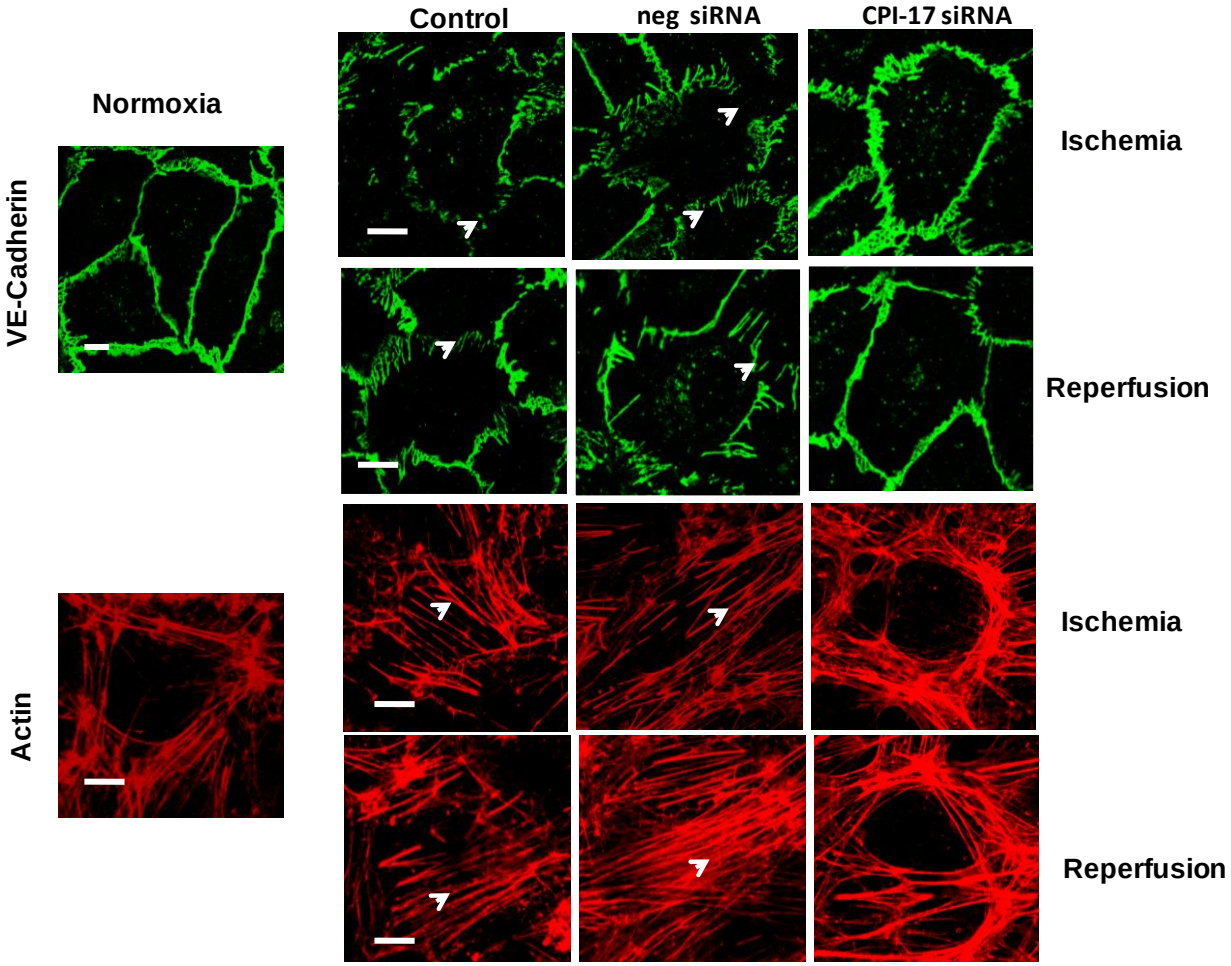


Figure 4.10 Effect of CPI-17 silencing on ischemia-reperfusion-induced distortion of actin cytoskeleton and VE-cadherin. HUVEC were treated with CPI-17 specific siRNA or negative (neg) siRNA for 48 h. (Normoxia) Control cells were exposed to normoxia (Po_2 = 100 mmHg, pH 7.4). (Ischemia) Cells were exposed to simulated ischemia (Po_2 < 1 mmHg, pH 6.4) for 45 min. (Reperfusion) Following ischemia cells were reperfused by medium change (Po_2 = 100 mmHg, pH 7.4) for 15 min. Arrowheads show the dissolution of VE-cadherin and actin stress fiber formation in non-transfected cells. Bar = 20 μm.

4.11 CPI-17 silencing reduces reperfusion-induced albumin permeability

In a final step it was tested whether silencing of CPI-17 by siRNA affects endothelial barrier function during ischemia-reperfusion. To accomplish that, HUVEC were treated as described above to reduce the cellular content of CPI-17. Afterwards cells were exposed to 45 min of ischemia followed by 15 min of reperfusion. As shown in fig. 4.11, reduction of CPI-17 did not affect basal macromolecule permeability under normoxic conditions. However, macromolecule permeability of CPI-17 siRNA treated cells was significantly reduced when exposed to ischemia or reperfusion, indicating that CPI-17 plays an important role in endothelial barrier stabilization.

However, as can be depicted from fig. 4.11, albumin permeability is not completely reduced to the normoxic value in CPI-17 siRNA treated cells, when cells were exposed to reperfusion. Therefore, it was tested whether MLCK may also be involved in this situation. To that end, cells were exposed to ischemia for 45 min followed by reperfusion with 10 µM ML-7, to block MLCK activation during reperfusion. As shown in fig. 4.11, ML-7 reduces reperfusion-induced albumin permeability in siRNA transfected cells, indicating that MLCK is involved in reperfusion-induced hyperpermeability. The Ischemia-reperfusion-induced increase in permeability was completely resored, when MLCK was inhibited in CPI-17 siRNA silenced cells with the onset of reperfusion, supporting the concept that both, MLCK and MLCP, are involved in ischemia-reperfusion-induced hyperpermeability.

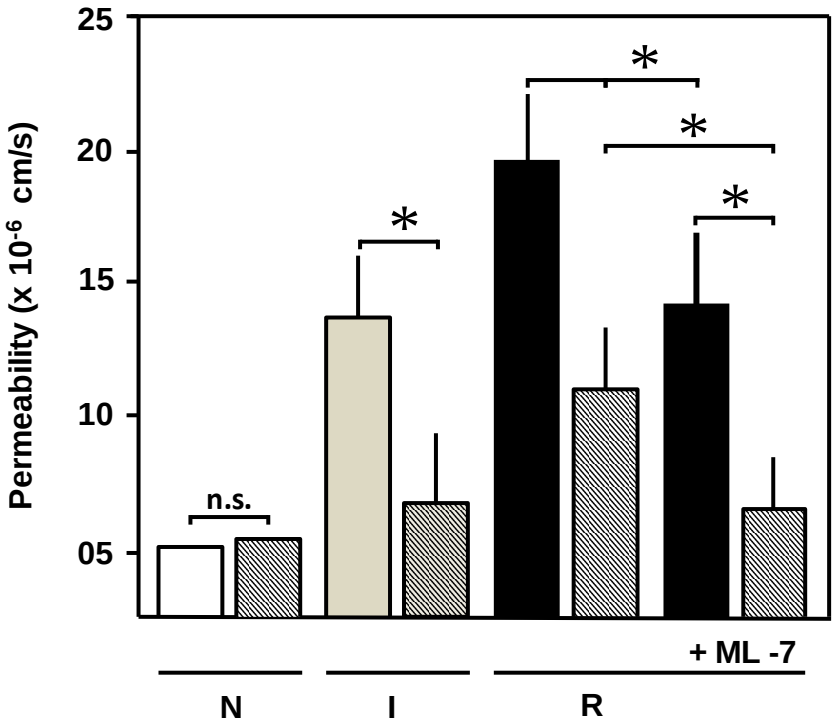


Figure 4.11 Effect of CPI-17 silencing and MLCK inhibition on ischemia-reperfusion-induced hyperpermeability. HUVEC were treated with CPI-17 specific siRNA (dashed bars) or negative siRNA (open bars) for 48 h. (N) Control cells were exposed to normoxia (Po_2 = 100 mmHg, pH 7.4). (I) Cells were exposed to simulated ischemia (Po_2 < 1 mmHg, pH 6.4) for 45 min. (R) Following ischemia cells were reperfused by medium change (Po_2 = 100 mmHg, pH 7.4) for 15 min. In a set of experiments, the cells were exposed to 10 µM ML-7, a specific inhibitor of MLCK, at the onset of reperfusion. *P < 0.05, n.s.: not significantly different.

5 DISCUSSION

Capillary leakage is the first indication of a disturbed endothelial barrier function during reperfusion of ischemic tissue. In the heart, as well as in other organs, failure of endothelial barrier causes extravasation of solutes, macromolecules and blood cells leading to edema formation, which jeopardize functional and structural recovery of the organ during reperfusion (Garcia et al., 2012).

There is a lot of indirect evidence from *in vivo* studies showing that capillary endothelial cells are stimulated to contract in the ischemia-reperfusion myocardium (Glyn and Ward, 2000). Likewise, exposure of endothelial monolayers to simulated ischemia induced an instant increase in cytosolic Ca^{2+} concentration, which coincided with an increase in interendothelial gap formation. During the following reperfusion the cytosolic Ca^{2+} concentration and gap formation was further enhanced, indicating that contractile activation and disturbance of cell adhesion structures are involved in endothelial barrier failure (Ladilov et al., 2000; Gündüz et al., 2006). Maneuver which blocks the Ca^{2+} increase also prevented the increase in gap formation in that model, demonstrating that the hyperpermeability induced by ischemia-reperfusion is triggered by cytosolic Ca^{2+} concentration. In a further study Schäfer et al. (2003) showed that the reperfusion-induced increase in gap formation can be blocked by inhibitors of the MLCK. This led to the concept that endothelial barrier failure induced during ischemia-reperfusion is, at least in part, caused by Ca^{2+}-dependent activation of the contractile machinery. Although one of the most frequent and severe vascular complication, the mechanism of ischemia-reperfusion is not fully understood. Strategies for therapeutic intervention targeting the endothelium are not available today.

The present study analyzed molecular mechanism involved in the endothelial barrier failure. Special focus was laid on the endothelial contractile machinery and cell-cell adhesion structures. Endothelial monolayers from porcine aortas and human umbilical veins, exposed to 45 min of simulated ischemic followed by 45 min of reperfusion, was chosen as a basis for the experimental paradigm.

The major findings of the present study are:

(1) Exposure of PAEC to simulated ischemia induced an increase in macromolecule permeability which was further enhanced when the cells were reoxygenated.
(2) The increase in permeability coincided with disturbance of the F-actin cytoskeleton and cell adhesion structures.
(3) During ischemia-reperfusion phosphorylation of MLC, MYPT1 and CPI-17 was increased. This increase in phosphorylation was reduced, when EC were reperfused in presence of a panspecific inhibitor of PKC or ROCK.
(4) Inhibition of PKC with the onset of reperfusion also reduced the reperfusion-induced increase in macromolecule permeability.
(5) CPI-17 silencing in HUVEC abolished reperfusion-induced MLC phosphorylation and restored reperfusion-induced distortion of the F-actin cytoskeleton and cell adherens junctions. It abolished the ischemia-induced increase in macromolecule permeability and reduced the increase in permeability induced by reperfusion. A complete reduction of the reperfusion-induced increase in permeability was observed when CPI-17 silenced cells were reperfused in the presence of an inhibitor of MLCK.

5.1 Ischemia-Reperfusion induces rearrangement of actin cytoskeleton and distortion of adherens junctions

The actin cytoskeleton provides the mechanical strength to the cell and interacts with myosin filaments to provide the force of contraction. In the present study it was shown that the increase in macromolecule permeability of the endothelial monolayers coincided with disturbance of the F-actin cytoskeleton and cell adhesion structures, when the cells were exposed to ischemia and reperfusion. These findings show that the experimental procedure used in the present study is in accordance with the established concept of endothelial barrier function and integrity of the cytoskeleton (Mehta and Malik, 2006).

In the present study ischemia-reperfusion induced extensive rearrangement of the actin cytoskeleton. Changes in actin cytoskeleton had already started during ischemia and led to stress fiber formation which traversed the cells. During reperfusion distortion of the F-actin cytoskeleton proceeded. Peripheral actin bands were progressively fading while stress fiber formation was enhanced. Collectively, the

data suggests that the increase in permeability during ischemia, and the further rise in response to reperfusion are causally linked with the progressive alterations in actin cytoskeleton and disturbance of cell adhesion structures.

The data of the present study are in accordance to previous work from Partridge (1995). She has reported that hypoxia-reoxygenation induces rearrangement of the actin cytoskeleton, opening of intercellular gaps and an increase in endothelial monolayer permeability. The essential role of F-actin fibers in maintaining cell integrity has also been shown in epithelial cells during ischemia (Molitoris, 1991; Schwartz et al., 1999), indicating that this causal link between cytoskeleton and barrier function is not only confined to endothelial, but also on other epithelial cells. Kuhne et al. (1993) and Watanabe et al. (1991) have shown that even under normoxic conditions, energy depletion alone causes disintegration of actin bundles at the cell periphery and endothelial cell retraction, which coincides withan increase in macromolecule permeability. Disruption of microfilaments and depolymerization of microtubules has also been observed under ATP depletion conditions by other laboratories (Hinshaw et al., 1989), indicating that the disintegration of actin cytoskeleton and cell adhesion structures result, at least in part, from the loss of energy during ischemia, wherease, reorganization of the cell cytoskeleton starts again, when ATP synthesis starts again with the supply of oxygen at the onset of reperfusion.

In addition to the integrity of the F-actin cytoskeleton, maintenance of adherens junctions is important for the regulation of endothelial barrier function (Wallez and Huber, 2008; Bazzoni and Dejana, 2004). Adherens junctions are formed by interactions of VE-cadherin and β-catenin, the latter of which links VE-cadherin to the actin cytoskeleton. Thus, paracellular permeability is increased when VE–cadherin and ß-catenin at cell-cell junctions disrupt (Rabiet et al., 1996; Corada et al., 1999; Weis et al., 2004; Monaghan and Burridge, 2009).

In line with this concept, the data of the present study shows that endothelial monolayers exposed to ischemia-reperfusion undergo distortion of VE-cadherin and β-catenin at the cell periphery. Delocalisation of both proteins from cell borders has already started during ischemia and proceeded during reperfusion.

Taken together, ischemia and reperfusion cause barrier failure due to combined disturbance of cytoskeletal and cell adhesion structures. The process of endothelial malfunction starts during ischemia and is further enhanced when reperfusion sets on. Furthermore, the study has revealed that the experimental model of cultured cells used, shares fundamental characteristics regarding physiological barrier function of the intact coronary system *in vivo*. Therefore, it is an appropriate model to analyze the underlying molecular mechanisms of barrier failure.

5.2 Ischemia-Reperfusion induces MLCK activation and MLCP inhibition

It is well established that the contractile activity of endothelial cells is controlled by phosphorylation of MLC, which in turn is regulated by at least two enzymes: an endothelial specific Ca^{2+}/calmodulin-dependent 210 kDa MLCK (Lazar and Garcia., 1999) and a MLCP (Knapp et al., 1999; Verin et al., 2000; Härtel et al., 2007) which is similar to the one expressed in smooth muscle cells. MLCK is an important kinase which directly phosphorylates MLC at Ser19 and regulates endothelial barrier function (Wysolmerski and Lagunoff, 1991). As already mentioned in the introduction, the endothelial MLCP is a holoenzyme complex formed by the PP1 catalytic subunit and the regulatory subunit MYPT1, which targets PP1 to myosin. When MYPT1 becomes phosphorylated, MLCP is inhibited and the phosphatase complex disassembles (Härtel et al., 2007).

The data of the present study show that ischemia-reperfusion induces phosphorylation of both MLC and MYPT1. Application of the MLCK inhibitor ML-7 at optimum concentration, which completely blocks MLCK activity, reduced MLC phosphorylation only back to the normoxic level. These data are consistent with the assumption that reperfusion-induced activation of the contractile machinery depends on activation of MLCK and inhibition of MLCP. Likewise, addition of ML-7 at the onset of reperfusion caused only a partial reduction of reperfusion-induced hyperpermeability, supporting the concept that other signaling mechanism, in addition to MLCK, are involved in the control of contractile activation and barrier failure.

In accordance with the data of the present study, several reports in literature demonstrate that inhibition of MLCK by ML-7 reduce hypoxia-induced MLC

phosphorylation and barrier failure in cultured endothelial monolayers. Schäfer et al. (2003) showed in monolayers of microvascular coronary endothelial cells from rat that ML-7 or Wortmannin, two chemical different inhibitors of MLCK, partially block reoxygenation-induced hyperpermeability. Kuhlmann et al. (2007) oberved in monolayers of microvascular endothelial cells from bovine brain that hypoxia induces phosphorylation of MLC, which coincided with loss of barrier function. In that model, application of ML-7 reduced MLC phosphorylation below the normoxic level and completely restored barrier integrity, indicating that mechanism counteracting MLC dephosphorylation, like MLCP, may be less active in this cell preparation.

5.3 Reperfusion-induced MLCP inhibition mediated by RhoA activation

As mentioned above, the activity of the MLCP holoenzyme complex is regulated by phosphorylation of MYPT1. One of the most important intracellular trigger of MYPT1 phosphorylation is the RhoA/ROCK pathway (Kimura et al., 1996). In smooth muscle cells, as well as in many non-muscle cells, ROCK-dependent phosphorylation of MYPT1 led to inhibition of MLCP, increase in MLC phosphorylation, and activation of the actomyosin-based contractile apparatus (Feng et al., 1999; Hartshorne, 1987**;** Kimura et al., 1996). Y-27632, a specific pharmacological inhibitor of ROCK, has been shown to reduce phosphorylation of MYPT1 as well as MLC, and causes vasodilation (Shimokawa, 2002). In endothelial cells, two independent groups (Essler et al., 1998; van Nieuw Amerongen et al., 1998) demonstrated that inactivation of the RhoA/ROCK pathway also led to inactivation of MLCP, increase in MLC phosphorylation, and an opening of intercellular gaps by activation of the endothelial contractile apparatus.

In line with those findings, the data of the present study show that inhibition of ROCK by Y-27632 reduced ischemia-reperfusion induce increase in MLC and MYPT1 phosphorylation beyond the normoxic value. It also halved phosphorylation of CPI-17 compared to its endischemic value, indicating that the RhoA/ROCK pathway is activated under these conditions and may inhibit MLCP. This concept is consistent with previous studies showing that hypoxia-induced activation of ROCK contributes to the inhibition of MLCP in endothelial cells (Takemoto et al., 2002) as well as in smooth muscle cells (Wang et al., 2003). The mechanism, however, which

activates RhoA/ROCK during ischemia-reperfusion is only poorly understood. One possible trigger could be the increase in cytosolic Ca^{2+} concentration during ischemia-reperfusion .

5.4 Ischemia-reperfusion-induced MLCP inhibition is mediated by PKC and ROCK activation

PKC isoforms and ROCK are well established modulators of the endothelial barrier function by targeting the contractile apparatus, cytoskeleton and cell adhesion structures at specific sides. Lynch et al. (1990) were among the first who systematically analyzed the effect of direct activation or inhibition of PKC on macromolecule permeability in cultured endothelial monolayers. Hempel et al. (1999) reported that the antagonist nifedipine prevents the ischemia-induced increase in permeability via PKC inhibition in cultured porcine endothelial cells, indicating that endothelial barrier failure can be triggered by Ca^{2+} via activation of PKC activation.

There is accumulating evidence that PKC and RhoA/ROCK signaling pathways are closely linked with each other. Hippenstiel et al. (1998) reported that inhibition of RhoA by Clostridium difficile toxin B prevented translocation and activation of PKC to plasma membrane in lipopolysaccharide as well as phorbol ester-stimulated HUVEC. The author concluded that RhoA is necessary for the activation and translocation of PKC to the endothelial plasma membrane. Patil et al. (2004) as well as Pang and Bitar (2005) could show that the interaction of RhoA and PKC-α is due to direct binding of RhoA to PKC. The resulting complex is translocated to the plasma membrane mediated by HSP27 in acetylcholine stimulated smooth muscle cells. Recently, He et al. (2012) have shown that inhibition of either PKC or Rho/ROCK causes similar reduction in transendothelial resistence of cultured monolayers, indicating that even basal endothelial barrier function is controlled by both pathways. Collectively, these data have led to the assumption that both signaling pathways may also be involved in ischemia-reperfusion-induced barrier failure.

In line with this, inhibition of endothelial PKC by a panspecific inhibitor BIM reduced ischemia-reperfusion induced increase in phosphorylation of MLC and

MYPT1 beyond normoxic values and halved the phosphorylation of CPI-17. The most prominent effect of PKC inhibition is observed on MYPT1 phosphorylation, which is similar to the one induced by ROCK inhibition under the same conditions, indicating that during ischemia-reperfusion PKC and RhoA/ROCK inhibit MLCP by targeting MYPT1. Inhibition of PKC or ROCK had only moderate effects on CPI-17 phosphorylation indicate that there are other kinases involved. In accordance with this assumption, both inhibitors only halved the MLC phosphorylation level induced by ischemia-reperfusion.

In addition to the effect on the contractile machinery, PKC may also target cytoskeletal proteins and cell adhesion structures leading to hyperpermeability. In the present study VE-cadherin and ß-catenin were analyzed because they are central proteins of endothelial junctions which are highly regulated by intracellular signaling mechanisms and important for endothelial barrier function (Rabiet et al., 1996; Gotsch et al., 1997; Hordijk et al., 1999; Corada et al., 2001; Waschke et al., 2004). The stability of the VE-cadherin-ß-catenin adhesion structures may by indirectly or directly influenced by PKC. Activation of the contractile machinery by PKC may affect VE-cadherin-mediated cell adhesion via ß-catenin, which links the actin cytoskeleton to cell adhesion protein complexes (Lampugnani et al., 1995; Dejana, 2004). On the other hand, PKC phosphorylation of strategic proteins of adhesion structures may weaken the VE-cadherin binding to the structures, leading to disassembly of adherens junctions and loss of barrier function (Vandenbroucke St Amant et al., 2012).

In the present study it was shown that ischemia-reperfusion induced actin stress fiber formation, disruption of VE-cadherin and β-catenin at cell-cell junctions. Inhibition of PKC prevented the distortion of actin cytoskeleton and cell-cell junctions. Accordingly, inhibition of PKC reduced the reperfusion-induced permeability increase toward endischemic levels. This indicates that activation of PKC is responsible, at least in part, for the rearrangement of the actin cytoskeleton, disruption of adherens proteins and finally barrier malfunction. However, it does not fully account for ischemia-reperfusion hyperpermeability. In so far both, PKC and RhoA/ROCK are only partially involved in the deleterious mechanism.

To test new options for a therapeutic intervention against barrier failure, it was intriguing to identify a signaling element on which PKC and RhoA/ROCK converge. This led to the idea to test signaling mechanisms down-stream of both kinases.

5.5 CPI-17 silencing reduces reperfusion-induced hyperpermeability

CPI-17 is phosphorylated by several kinases including PKC, Rho kinase, zipper-interacting protein kinase, and integrin-linked kinase in smooth muscle cells (Hirano, 2007; Koyama et al., 2000). Phosphorylation of CPI-17 at Thr38 by PKC increases its inhibitory potency and activity towards MLCP (Eto et al., 1997; Kitazawa et al., 2000), which in turn controls the MLC phosphorylation level. Kitazawa et al. (2000) also reported that pharmacological inhibitors of PKC and ROCK-reduced CPI-17 phosphorylation and smooth muscle contraction.

CPI-17 is also found in endothelial cells. Kolosova et al. (2004) described for the first time the expression of CPI-17 on mRNA level in lung microvascular and microvascular endothelial cells as well as in HUVEC. Because of its low protein expression, the authors overexpressed CPI-17 in pulmonary artery endothelial cells to analyze the possible role in endothelial cells. They found that expression of CPI-17 enhances formation of stress fibers, focal adhesions and MLC phosphorylation if cells are stimulated by direct activators of PKC or by inflammatory mediators like histamin. Srinivas et al. (2006) showed expression of CPI-17 on protein level in corneal endothelial cells. Stimulation of histamine and thrombin led to an increase in CPI-17 phosphorylation in these cells. Most recently, Aslam et al. (2010) demonstrated that CPI-17 is also expressed in HUVEC. In this model thrombin enhanced CPI-17 phosphorylation and recruitment of the activated inhibitor to PP1. Phosphorylation of CPI-17 and its recruitment to MLCP coincided with an inhibition of PP1 activity. In addition, in CPI-17-silenced HUVEC the thrombin-induced increase in permeability was reduced.

In the present study, using the same experimental model, downregulation of CPI-17 caused a decrease in MLC phosphorylation under normoxic conditions, indicating that MLC dephosphorylation is enhanced. On the other side, the fact that CPI-17 is phosphorylated suggested that there is a kinase activity already active

under basal conditions in HUVEC. Reduction of the MCLP inhibitor in siRNA-downregulated cells obviously leads to an enhanced dephosphorylation. Under ischemia this enhanced phosphatase activity prevented MLC phosphorylation nearly completely and blunted it during reoxygenation. Downregulation of CPI-17 also reduced formation of actin stress fiber, disturbance of cell adhesion structures induced by ischemia, and enhanced the reorganization of VE-cadherin at the cell periphery during reperfusion. CPI-17 downregulation also abolished the ischemia-induced increase in macromolecule permeability and reduced the increase in permeability induced by reperfusion. A complete reduction of the reperfusion-induced increase in permeability was observed when CPI-17-silenced cells were reperfused in the presence of an inhibitor of MLCK.

Collectively, these data demonstrate that CPI-17 stabilizes actin cytoskeleton, cell-cell adhesion and reduces the contractile activation of endothelial cells. Furthermore, in combination with an inhibitor of MLCK it protects endothelial barrier against imminent failure. This makes CPI-17 an interesting target of therapeutic intervention to stabilize endothelial barrier and to prevent edema formation induced by ischemia-reperfusion and beyond.

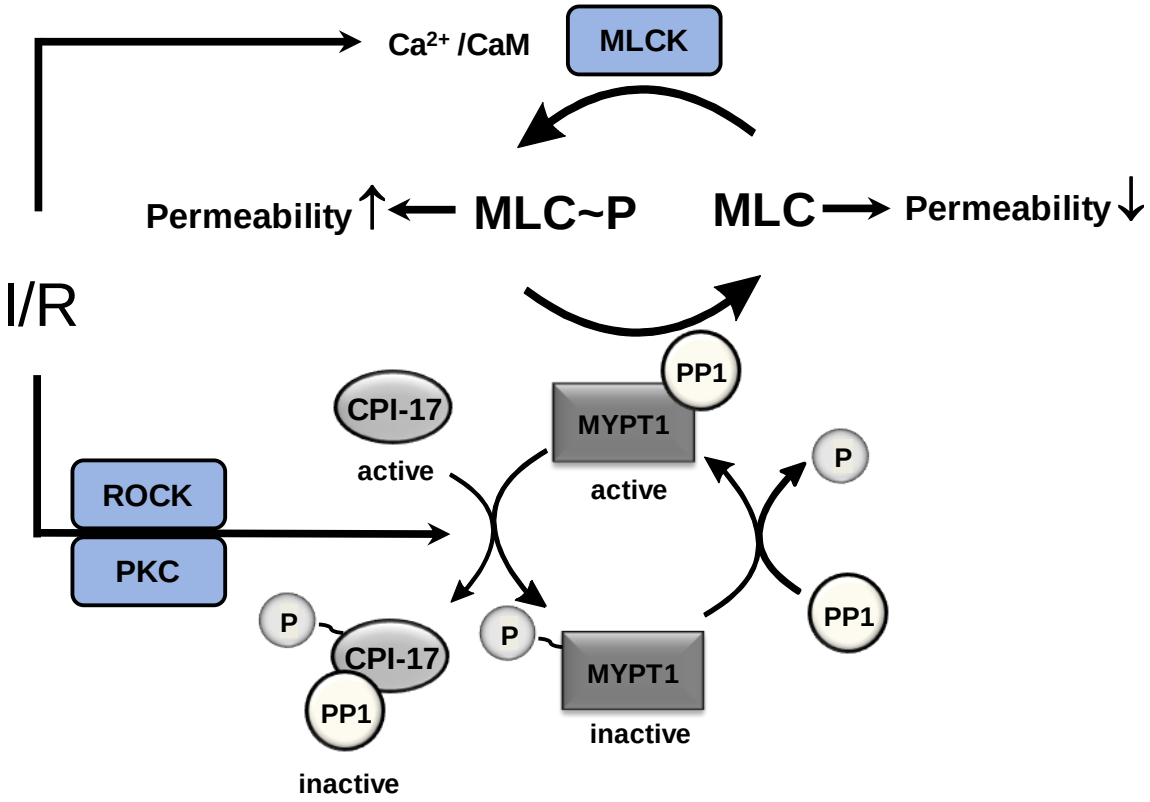


Figure 5.1: In endothelial cells, ischemia-reperfusion (I/R) leads to an activation of the Ca^{2+}-dependent MLCK and inhibition of the MLCP. The activation of the MLCK is triggerd by the increase in cytosolic Ca^{2+} concentration (Gündüz et al., 2006). Inhibition of MLCP may be mediated by PKC and RhoA/ROCK signaling pathways targeting the endogenous CPI-17 and MYPT1.

6 REFERENCES

Albert C, Ford D *(*1999*)* Protein kinase C translocation and PKC dependent protein phosphorylation during myocardial ischemia. Am J Physiol; 276: 642–650

Alessi D, MacDougall LK, Sola MM, Ikebe M, Cohen P (1992) The control of protein phosphatase-1 by targetting subunits. The major myosin phosphatase in avian smooth muscle is a novel form of protein phosphatase-1. Eur J. Biochem; 210: 1023-1035

Allingham MJ, van Buul JD, Burridge K (2007) ICAM-1-mediated, Src- and Pyk2-dependent vascular endothelial cadherin tyrosine phosphorylation is required for leukocyte transendothelial migration. J Immunol; 179: 4053-4064.

Ambrosio G, Tritto I (1999) Reperfusion injury: experimental evidence and clinical implications. Am Heart J; 138: 69–75

Anderson JM, Balda MS, Fanning AS (1993) The structure and regulation of tight junctions. Curr Opin Cell Biol; 5: 772-778

Andriopoulou P, Navarro P, Zanetti A, Lampugnani MG , Dejana E (1999) Histamine induces tyrosine phosphorylation of endothelial cell-to-cell adherens junctions. Arterioscler Thromb Vasc Biol; 19: 2286-2297

Angelini DJ, Hyun SW, Grigoryev DN, Garg P, Gong P, Singh IS, Passaniti A, Hasday JD, Goldblum SE (2006) TNF-alpha increases tyrosine phosphorylation of vascular endothelial cadherin and opens the paracellular pathway through fyn activation in human lung endothelia. Am J Physiol Lung Cell Mol Physiol; 291: 1232-1245

Aslam M, Härtel FV, Arshad M, Gündüz D, Abdallah Y, Sauer H, Piper HM, Noll T (2010) cAMP/PKA antagonizes thrombin-induced inactivation of endothelial myosin light chain phosphatase: Role of CPI-17. Cardiovascular Research; 87:375-384

Bazzoni G, Dejana E (2004) Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. Physiol. Rev; 84: 869–901

Beckman JA, Goldfine AB, Gordon MB, Garrett LA, Keaney JF Jr, Creager MA (2003) Oral antioxidant therapy improves endothelial function in type 1 but not type 2 diabetes mellitus. Am J Physiol Heart Circ Physiol; 285: 2392–2398

Beyer EC (1993) Gap junctions. Int Rev Cytol; 137: 1-37

Bolton CH, Downs LG, Victory JG, Dwight JF, Tomson CR, Mackness MI, Pinkney JH (2001) Endothelial dysfunction in chronic renal failure: roles of lipoprotein oxidation and pro-inflammatory cytokines. Nephrol Dial Transplant; 16: 1189–1197

Bonnevier J, Arner A (2004) Actions downstream of cyclic GMP/protein kinase G can reverse protein kinase C-mediated phosphorylation of CPI-17 and Ca^{2+} sensitization in smooth muscle. J Biol Chem; 279: 28998-29003

Carbajal JM, Gratrix ML, Yu CH, Schaeffer RC, Jr (2000) ROCK mediates thrombin's endothelial barrier dysfunction. Am J Physiol Cell Physiol; 279: 195–204

Carden DL, Granger DN (2000) Pathophysiology of ischaemia–reperfusion injury. J Pathol; 190**:** 255–266

Chang L, Goldman RD (2004) Intermediate filaments mediate cytoskeletal crosstalk. Nat Rev Mol Cell Biol; 5: 601–613

Chang S, Hypolite JA, DiSanto ME, Changolkar A, Wein AJ, Chacko S (2006) Increased basal phosphorylation of detrusor smooth muscle myosin in alloxan-induced diabetic rabbit is mediated by upregulation of Rho-kinase β and CPI-17. Am J Physiol Renal Physiol*;* 290: 650-656

Chen YH, Chen MX, Alessi DR, Campbell DG, Shanahan C, Cohen P, Cohen PT (1994) Molecular cloning of cDNA encoding the 110 kDa and 21 kDa regulatory subunits of smooth muscle protein phosphatase 1M. FEBS Lett; 356: 51–55

Corada M, Liao F, Lindgren M, Lampugnani MG, Breviario F, Frank R, Muller WA, Hicklin DJ, Bohlen P, Dejana E (2001) Monoclonal antibodies directed to different regions of vascular endothelial cadherin extracellular domain affect adhesion, and clustering of the protein, and modulate endothelial permeability. Blood; 97: 1679–1684

Corada M, Mariott M, Thurston G, Smith K, Kunkel R, Brockhaus M, Lampugnani MG, Martin-padura I, Stoppacciaro A, Ruco I, Mcdonald DM, Ward PA, Dejana E (1999) Vascular endothelial-cadherin is an important determinant of microvascular integrity *in vivo*. Proc Nate Acad Sci USA*;* 96: 9815–9820

Cramer LP, Siebert M, Mitchison TJ (1997) Identification of novel graded polarity actin filament bundles in locomoting heart fibroblasts: implications for the generation of motile force. J Cell Biol; 136: 1287–1305

Dakshinamurti S, Mellow L, Stephens NL (2005) Regulation of pulmonary arterial myosin phosphatase activity in neonatal circulatory transition and in hypoxic pulmonary hypertension: a role for CPI-17. Pediatr Pulmonol*;* 40: 398-407

Dejana E (2004) Endothelial cell-cell junctions: happy together. Nat Rev Mol Cell Biol; 5: 261–270

Dejana E, Corada M, Lampugnani MG (1995) Endothelial cell-to-cell junctions. FASEB J; 9: 910–918

Di Napoli P, Antonio Taccardi A, Grilli A, Spina R, Felaco M, Barsotti A, De Caterina R (2001) Simvastatin reduces reperfusion injury by modulating nitric oxide synthase expression: an ex vivo study in isolated working rat hearts. Cardiovasc Res; 51*:* 283–293

Dubois T, Howell S, Zemlickova E, Learmonth M, Cronshaw A, Aitken A (2003) Novel in vitro and in vivo phosphorylation sites on protein phosphatase 1 inhibitor CPI-17. Biochem Biophys Res Commun; 302: 186-192

Dudek SM, Garcia JG (2001) Cytoskeletal regulation of pulmonary vascular permeability. J Appl Physiol; 91: 1487–1500

Ehringer WD, Edwards MJ, Miller FN (1996) Mechanisms of alpha-thrombin, histamine, and bradykinin induced endothelial permeability. J Cell Physiol*;* 167: 562-569

Eltzschig HK, Collard CD (2004) Vascular ischaemia and reperfusion injury. Br Med Bull; 70: 71-86

Endemann D, Pu Q, De Ciuceis C, Savoia C, Virdis A, Neves MF, Touyz RM, Schiffrin EL (2004) Persistent remodeling of resistance arteries in type 2 diabetic patients on antihypertensive treatment. Hypertension; 43: 399-404

Esser S, Lampugnani MG, Corada M, Dejana E, Risau W (1998) Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. J. Cell Sci; 111: 1853-1865

Essler M, Amano M, Kruse HJ, Kaibuchi K, Weber PC, Aepfelbacher M *(*1998*)* Thrombin inactivates myosin light chain phosphatase via Rho and its target Rho kinase in human endothelial cells*.* J Biol Chem*;* 273*:* 21867–21874

Eto M, Karginov A, Brautigan DL (1999) A novel phosphoprotein inhibitor of protein type-1 phosphatase holoenzymes. Biochemistry; 38: 16952-16957

Eto M, Kitazawa T, Yazawa M, Mukai H, Ono Y, Brautigan DL (2001) Histamine-induced vasoconstriction involves phosphorylation of a specific inhibitor protein for myosin phosphatase by protein kinase C alpha and delta isoforms. J Biol Chem; 276: 29072-29078

Eto M, Ohmori T, Suzuki M, Furuya K, Morita F (1995) A novel protein phosphatase-1 inhibitory protein potentiated by protein kinase C. Isolation from porcine aorta media and characterization. J Biochem; 118: 1104–1107

Eto M, Senba S, Morita F, and Yazawa M (1997) Molecular cloning of a novel phosphorylation-dependent inhibitory protein of protein phosphatase-1 (CPI-17) in smooth muscle: its specific localization in smooth muscle. FEBS Lett; 410: 356–360

Eto M, Wong L, Yazawa M, Brautigan DL (2000) Inhibition of myosin/ moesin phosphatase by expression of the phospho inhibitor protein CPI-17 alters microfilament organization and retards cell spreading. Cell Motil Cytoskeleton; 46: 222–234

Etter EF, Eto M, Wardle RL, Brautigan DL, Murphy RA (2001) Activation of myosin light chain phosphatase in intact arterial smooth muscle during nitric oxide-induced relaxation. J Biol Chem; 276: 34681-34685

Fanning AS, Jameson BJ, Jesaitis LA, Anderson JM (1998) The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. J Biol Chem; 273: 29745–29753

Feng D, Nagy JA, Hipp J, Dvorak HF, Dvorak AM (1996) Vesiculo-vacuolar organelles and the regulation of venule permeability to macromolecules by vascular permeability factor, histamine, and serotonin. J Exp Med; 183: 1981–1986.

Feng J, Ito M, Ichikawa K, Nishikawa M, Hartshorne DJ, Nakano T (1999) Inhibitory phosphorylation site for Rho-associated kinase on smooth muscle myosin phosphatase. J Biol Chem; 274: 37385–37390

Garcia JG, Davis HW, Patterson CE (1995) Regulation of endothelial cell gap formation and barrier dysfunction: role of myosin light chain phosphorylation. J Cell Physiol; 163: 510-522

Garcia JG, Lazar V, Gilbert-McClain LI, Gallagher PJ, Verin AD (1997) Myosin light chain kinase in endothelium: molecular cloning and regulation. Am J Respir Cell Mol Biol; 16: 489–494

Garcia-Dorado D, Andres-Villarreal M, Ruiz-Meana M, Inserte J and Barba I (2012) Myocardial edema: A translational view. Journal of Molecular and Cellular Cardiology; 52: 931-939

Garcia-Dorado D, Oliveras J (1993) Myocardial oedema: a preventable cause of reperfusion injury? Cardiovasc Res; 27*:* 1555-1563

Glyn CPM, Ward BJ (2000) Contraction in cardiac endothelial cells contributes to changes in capillary dimensions following ischaemia and reperfusion. Cardiovasc Res; 48: 346-356

Glyn MC, Ward BJ (2002) Changes in the actin cytoskeleton of cardiac capillary endothelial cells during ischaemia and reperfusion: the effect of phalloidin on cell shape. J Vasc Res; 39: 72-82

Goeckeler ZM, Wysolmerski RB (1995) Myosin light chain kinase-regulated endothelial cell contraction: the relationship between isometric tension, actin polymerization, and myosin phosphorylation. J Cell Biol; 130: 613–627

Gotsch U, Borges E, Bosse R, Boggemeyer E, Simon M, Mossmann H, Vestweber D (1997) VE-cadherin antibody accelerates neutrophil recruitment in vivo. J Cell Sci; 110: 583–588

Gumbiner BM (1993) Breaking through the tight junction barrier. J Cell Biol; 123: 1631-1633

Gündüz D, Kasseckert SA, Härtel FV, Aslam M, Abdallah Y, Schäfer M, Piper HM, Noll T, Schäfer C (2006) Accumulation of extracellular ATP protects against acute reperfusion injury in rat heart endothelial cells. Cardiovasc Res; 71: 764-773

Gündüz D, Thom J, Hussain I, Lopez D, Härtel FV, Erdogan A, Grebe M, Sedding D, Piper HM, Tillmanns H, Noll T, Aslam M (2010) Insulin stabilizes microvascular endothelial barrier function via phosphatidylinositol 3-kinase/Akt-mediated Rac1 activation. Arterioscler Thromb Vasc Biol; 30:1237-1245.

Haeberle JR, Sutton T A, Trockman BA (1988) Phosphorylation of two sites on smooth myosin effects on contraction of glycerinated vascular smooth muscle. J Biol Chem; 263:4424–4429

Hamaguchi T, Ito M, Feng J, Seko T, Koyama M, Machida H, Takase K, Amano M, Kaibuchi K, Hartshorne DJ, Nakano T (2000) Phosphorylation of CPI-17, an inhibitor of myosin phosphatase, by protein kinase N. Biochem Biophys Res Commun; 274: 825-30

Hannun YA, Loomis CR, Merrill AH, Bell RM (1986) Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding in vitro and in human platelets. J Biol Chem; 261: 12604–12609

Härtel FV, Rodewald CW, Aslam M, Gündüz D, Hafer L, Neumann J, Piper HM, Noll T (2007) Extracellular ATP induces assembly and activation of the myosin light chain phosphatase complex in endothelial cells. Cardiovasc Res; 74:487-496.

Hartshorne DJ (1987) Biochemistry of the contractile process in smooth muscle. In: Physiology of the Gastrointestinal Tract (2nd ed.), edited by Johnson LR. New York: Raven: 423–482

Hartshorne DJ, Ito M, Erdodi F *(*1998*)* Myosin light chain phosphatase: subunit composition, interactions and regulation. J Muscle Res Cell Motil*;* 19*:* 325–341

He F, Yin F, Omran A, Yang LF, Xiang QL, Peng J (2012) PKC and RhoA signals cross-talk in Escherichia coli endotoxin induced alterations in brain endothelial permeability. Biochem Biophys Res Commun; 425: 182-188

Hempel A, Lindschau C, Maasch C, Mahn M, Bychkov R, Noll T, Luft FC, Haller H (1999) Calcium antagonists ameliorate ischemia-induced endothelial cell permeability by inhibiting protein kinase C. Circulation; 99: 2523-2529

Hinshaw DB, Burger JM, Armstrong BC, Hyslop PA (1989) Mechanism of endothelial cell shape change in oxidant injury. J Surg Res; 46: 339-349

Hippenstiel S, Kratz T, Krull M, Seybold J, von Eichel-Streiber C, Suttorp N (1998) Rho protein inhibition blocks protein kinase C translocation and activation. Biochem Biophys Res Commun; 245: 830–834

Hirano K (2007) Current topics in the regulatory mechanism underlying the Ca^{2+} sensitization of the contractile apparatus in vascular smooth muscle. J Pharmacol Sci; 104: 109–115

Hirano K, Phan BC, Hartshorne DJ (1997) Interactions of the subunits of smooth muscle myosin phosphatase. J Biol Chem; 272: 3683-3688

Hirata N, Takahashi M, Yazawa M (2009) Diphosphorylation of regulatory light chain of myosin IIA is responsible for proper cell spreading. Biochem. Biophys. Res. Commun; 381: 682–687

Hordijk PL, Anthony E, Mul FP, Rientsma R, Oomen LC, Roos D (1999) Vascular-endothelial-cadherin modulates endothelial monolayer permeability. J Cell Sci; 112: 1915–1923

Hotulainen P, Lappalainen P (2006) Stress fibers are generated by two distinct actin assembly mechanisms in motile cells. J Cell Biol; 173: 383–394

Huang J, Mahavadi S, Sriwai W, Hu W, Murthy KS (2006) Gi-coupled receptors mediate phosphorylation of CPI-17 and MLC20 via preferential activation of the PI3K/ILK pathway. Biochem J*;* 396: 193-200

Hudry-Clergeon H, Stengel D, Ninio E, Vilgrain I (2005) Platelet-activating factor increases VE-cadherin tyrosine phosphorylation in mouse endothelial cells and and its association with the PtdIns3'-kinase. FASEB J; 19: 512-520

Ikebe M, Brozovich FV (1996) Protein kinase C increases force and slows relaxation in smooth muscle: evidence for regulation of the myosin light chain phosphatase. Biochem Biophys Res Commun; 225: 370-376

Ishizaki T, Uehata M, Tamechika I, Keel J, Nonomura K, Maekawa M, Narumiya S. *(*2000*)* Pharmacological properties of Y-27632, a specific inhibitor of Rho-associated kinases*.* Mol Pharmacol*;* 57: 976–983

Ito M, Nakano T, Erdodi F, Hartshorne DJ (2004) Myosin phosphatase: structure, regulation and function. Mol Cell Biochem; 259: 197-209

Ivanov D, Philippova M, Antropova J, Gubaeva F, Iljinskaya O, Tararak E, Bochkov V, Erne P, Resink T, Tkachuk V (2001) Expression of cell adhesion molecule T-cadherin in the human vasculature. Histochem Cell Biol; 115: 231–242

Jaffe EA, Nachman RL, Becker CG, Minick CR (1973) Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest; 52: 2745-56

Johnson D, Cohen P, Chen MX, Chen YH, Cohen PT (1997) Identification of the regions on the M110 subunit of protein phosphatase 1M that interact with the M21 subunit and with myosin. Eur J Biochem; 244: 931-939

Jones SP, Lefer DJ (2000) Myocardial reperfusion injury: insights gained from gene-targeted mice. News Physiol Sci*;* 15:303-308

Kaibuchi K, Kuroda S, Amano M (1999) Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. Annu Rev Biochem; 68: 459-486

Kim JI, Young GD, Jin L, Somlyo AV, Eto M (2009) Expression of CPI-17 in smooth muscle during embryonic development and in neointimal lesion formation. Histochem Cell Biol; 132: 191-198

Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Feng J, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K (1996) Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science; 273: 245–248

Kitazawa T, Eto M, Woodsome TP, Brautigan DL (2000) Agonists trigger G protein-mediated activation of the CPI-17 inhibitor phosphoprotein of myosin light chain phosphatase to enhance vascular smooth muscle contractility. J Biol Chem; 275: 9897–9900

Kitazawa T, Polzin AN, Eto M (2004) CPI-17-deficient smooth muscle of chicken J Physiol; 557: 515-528

Knapp J, Boknik P, Lüss I, Huke S, Linck B, Lüss H, Müller FU, Müller T, Nacke P, Noll T, Piper HM, Schmitz W, Vahlensieck U,Neumann J (1999) The protein phosphatase inhibitor cantharidin alters vascular endothelial cell permeability. J Pharmacol Exp Ther; 289: 1480-1486

Kobayashi E, Nakano H, Morimoto M, Tamaoki T (1989) Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. Biochem Biophys Res Commun*;* 159: 548-553

Kolosova IA, Ma SF, Adyshev DM, Wang P, Ohba M, Natarajan V, Garcia JG, Verin AD (2004) Role of CPI-17 in the regulation of endothelial cytoskeleton. Am J Physiol Lung Cell Mol Physiol; 287: 970-80

Koyama M, Ito M, Feng J, Seko T, Shiraki K, Takase K, Hartshorne DJ, Nakano T (2000) Phosphorylation of CPI-17, an inhibitory phospho- protein of smooth muscle myosin phosphatase, by Rho-kinase. FEBS Lett*;* 475: 197–200

Kuhlmann CR, Tamaki R, Gamerdinger M, Lessmann V, Behl C, Kempski OS, Luhmann HJ (2007) Inhibition of the myosin light chain kinase prevents hypoxia-induced blood-brain barrier disruption. J Neurochem; 102: 501-507

Kuhne W, Besselmann M, Noll T, Muhs A, Watanabe H, Piper HM (1993) Disintegration of cytoskeletal structure of actin filaments in energy-depleted endothelial cells. Am J Physiol; 264: 1599-608

Ladilov Y, Schäfer C, Held A, Schäfer M, Noll T, Piper HM (2000) Mechanism of Ca^{2+} overload in endothelial cells exposed to simulated ischemia. Cardiovasc Res; 47:394–403

Lampugnani MG, Corada M, Caveda L, Breviario F, Ayalon O, Geiger B, Dejana E (1995) The molecular organization of endothelial cell to cell junctions: differential association of plakoglobin, beta-catenin, and alpha-catenin with vascular endothelial cadherin (VE-cadherin). J Cell Biol; 129: 203-217

Landmesser U, Spiekermann S, Dikalov S, Tatge H, Wilke R, Kohler C, Harrison DG, Hornig B, Drexler H (2002) Vascular oxidative stress and endothelial dysfunction in patients with chronic heart failure: role of xanthine-oxidase and extracellular superoxide dismutase. Circulation; 106: 3073–3078

Laude K, Thuillez C, Richard V (2001) Coronary endothelial dysfunction after ischemia and reperfusion: a new therapeutic target? Braz J Med Biol Res; 34: 1-7

Lazar V, Garcia JG (1999) A single human myosin light chain kinase gene (MLCK; MYLK). Genomics; 57: 256–267

Lefer AM, Lefer DJ (1996) The role of nitric oxide and cell adhesion molecules on the microcirculation in ischaemia-reperfusion. Cardiovasc Res; 32: 743–751

Leung T, Manser E, Tan L, Lim L (1995) A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. J Biol Chem; 270: 29051

Lewalle JM, Bajou K, Desreux J, Mareel M, Dejana E, Noël A, Foidart JM (1997) Alteration of interendothelial adherens junctions following tumor cell-endothelial cell interaction in vitro. Exp Cell Res; 237: 347–356

Liu QR, Zhang PW, Lin Z, Li QF, Woods AS, Troncoso J, Uhl GR (2004) GBPI, a novel gastrointestinal- and brain-specific PP1-inhibitory protein, is activated by PKC and inactivated by PKA. Biochem J; 377: 171-181

Liu QR, Zhang PW, Zhen Q, Walther D, Wang XB, Uhl GR (2002) KEPI, a PKC dependent protein phosphatase 1 inhibitor regulated by morphine. J Biol Chem; 277: 13312-20

Lynch JJ, Ferro TJ, Blumenstock FA, Brockenauer AM, Malik AB (1990) Increased endothelial albumin permeability mediated by protein kinase C activation. J Clin Invest; 85: 1991–1998

MacDonald JA, Eto M, Borman MA, Brautigan DL, Haystead TA (2001) Dual Ser and Thr phosphorylation of CPI-17, an inhibitor of myosin phosphatase, by MYPT-associated kinase. FEBS Lett*;* 493: 91-94

Maczewski M, Beresewicz A (2000) The role of endothelin, protein kinase C and free radicals in the mechanism of the post-ischemic endothelial dysfunction in guinea-pig hearts. J Mol Cell Cardiol; 32: 297–310

Majno G, Palade GE (1961) Studies of inflammation. 1. Effect of histamine and serotonin on vascular permeability: an electron microscopic study. J Biophys Biochem Cytol; 11: 571-605

Mallat Z, Gojova A, Sauzeau V, Brun V, Silvestre JS, Esposito B, Merval R, Groux H, Loirand G, Tedgui A (2003) Rho-associated protein kinase contributes to early atherosclerotic lesion formation in mice. Circ Res; 93: 884-488

Masuo M, Reardon S, Ikebe M, Kitazawa T (1994) A novel mechanism for the Ca^{2+}-sensitizing effect of protein kinase C on vascular smooth muscle: inhibition of myosin light chain phosphatase. J Gen Physiol; 104: 265-286

Matsui T, Amano M, Yamamoto T, Chihara K, Nakafuku M, Ito M, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K (1996) Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. EMBO J; 15: 2208

Matsumura F, Hartshorne DJ. (2008) Myosin phosphatase target subunit: Many roles in cell function. Biochem Biophys Res Commun; 369: 149-156

Mehta D, Malik AB (2006) Signaling mechanisms regulating endothelial permeability. Physiol Rev; 86: 279-367

Menger MD, Rücker M, Vollmar B (1997) Capillary dysfunction in striated muscle ischemia/reperfusion: on the mechanism of capillary "no-flow." Shock; 8: 2-7

Mitchell FE, Marais RM, Parker PJ (1989) The phosphorylation of protein kinase C as a potential measure of activation. Biochem J; 261: 131-136

Mitic LL, Anderson JM (1998) Molecular architecture of tight junctions. Annu Rev Physiol; 60: 121–142

Molitoris BA (1991) Ischemia-induced loss of epithelial polarity: potential role of the actin cytoskeleton. Am J Physiol*;* 260: 769–778

Monaghan-Benson E, Burridge K (2009) The regulation of vascular endothelial growth factor induced microvascular permeability requires Rac and reactive oxygen species. J Biol Chem; 284: 25602-25611

Monnink SH, van Haelst PL, van Boven AJ, Stroes ES, Tio RA, Plokker TW, Smit AJ, Veeger NJ, Crijns HJ, van Gilst WH (2002) Endothelial dysfunction in patients with coronary artery disease: A comparison of three frequently reported tests. J Investig Med; 50: 19–24

Murray MA, Heistad DD, Mayhan WG (1991) Role of protein kinase C in bradykinin-induced increases in microvascular permeability. Circ Res; 68: 1340–1348

Nakamura K, Koga Y, Sakai H, Homma K, Ikebe M (2007) cGMP-dependent relaxation of smooth muscle is coupled with the change in the phosphorylation of myosin phosphatase. Circ Res;101:712-722.

Navarro P, Caveda L, Breviario F, Mandoteanu I, Lampugnani MG, Dejana E (1995) Catenin-dependent and -independent functions of vascular endothelial cadherin. J. Biol. Chem; 270: 30965-30972.

Nishizuka Y, 1984 The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature: 308: 693-698

Noll T, Wozniak G, McCarson K, Hajimohammad A, Metzner HJ, Inserte J, Kummer W, Hehrlein FW, Piper HM (1999) Effect of factor XIII on endothelial barrier function. J Exp Med; 189:1373-82

Ohki S, Eto M, Kariya E, Hayano T, Hayashi Y, Yazawa M, Brautigan D, Kainosho M (2001) Solution NMR structure of the myosin phosphatase inhibitor protein CPI-17 shows phosphorylation-induced conformational changes responsible for activation. J Mol Biol; 314: 839–849

Ohki S, Eto M, Shimizu M, Takada R, Brautigan DL, Kainosho M (2003) Distinctive solution conformation of phosphatase inhibitor CPI-17 substituted with aspartate at the phosphorylation-site threonine residue. J Mol Biol; 326: 1539–1547

Pang H, Guo Z, Su W, Xie Z, Eto M, Gong MC (2005) RhoA/Rho kinase mediates thrombin- and U-46619- induced phosphorylation of a myosin phosphatase inhibitor, CPI-17, in vascular smooth muscle cells. Am J Physiol Cell Physiol; 289: 352-60

Panza JA, Quyyumi AA, Brush JE Jr, Epstein SE (1990) Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. N Engl J Med : 323: 22–27

Parekh DB, Ziegler W, Parker PJ (2000) Multiple pathways control protein kinase C phosphorylation. Embo J; 19: 496-503

Park JB, Charbonneau F, Schiffrin EL (2001) Correlation of endothelial function in large and small arteries in human essential hypertension. J Hypertens; 19: 415–420

Partridge CA (1995) Hypoxia and reoxygenation stimulate biphasic changes in endothelial monolayer permeability. Am J Physiol; 269: 52-58

Patil SB, Pawar MD, Bitar KN (2004) Phosphorylated HSP27 essential for acetylcholine-induced association of RhoA with PKCα. Am J Physiol Gastrointest Liver Physiol ; 286: 635–644

Pears C, Stabel S, Cazaubon S, Parker PJ (1992) Studies on the phosphorylation of protein kinase C-alpha. Biochem J; 283: 515-518

Prasad MR, Jones RM *(*1992*)* Enhanced membrane protein kinase C activity in myocardial ischemia*.* Basic Res Cardiol; 87:19–26

Rabiet MJ, Plantier JL, Rival Y, Genoux Y, Lampugnani MG, Dejana E (1996) Thrombin-induced increase in endothelial permeability is associated with changes in cell-to-cell junction organization. Arterioscler Thromb Vasc Biol; 16: 488–496

Ramirez MM, Kim DD, Duran WN (1996) Protein kinase C modulates microvascular permeability through nitric oxide synthase. Am J Physiol; 271: 1702–1705

Revenu C, Athman R, Robine S, Louvard D (2004) The co-workers of actin filaments: from cell structures to signals. Nat Rev Mol Cell Biol; 5: 635–646

Rizzoni D, Porteri E, Guelfi D, Muiesan ML, Valentini U, Cimino A, Girelli A, Rodella L, Bianchi R, Sleiman I, Rosei EA (2001) Structural alterations in subcutaneous small arteries of normotensive and hypertensive patients with non-insulin-dependent diabetes mellitus. Circulation; 103: 1238–1244

Roberts JM, Taylor RN, Musci TJ, Rodgers GM, Hubel CA, McLaughin MK (1989) Preeclampsia: an endothelial cell disorder. American Journal of Obstetrics and Gynecology; 161: 1200–1204

Rubin, LL (1992) Endothetial cells: adhesion and tight junctions. Curr Opin Cell Biol; 4: 830-833

Sakai H, Chiba Y, Hirano T, Misawa M (2005) Possible involvement of CPI-17 in augmented bronchial smooth muscle contraction in antigen-induced airway hyper-responsive rats. Mol Pharmacol; 68: 145-151

Salomon D, Ayalon O, Patel-King R, Hynes RO, Geiger B (1992) Extrajunctional distribution of N-cadherin in cultured human endothelial cells. Cell Sci; 102**:** 7-17

Sandoval R, Malik AB, Minshall RD, Kouklis P, Ellis CA, Tiruppathi C (2001) Ca2+ signalling and PKC-alpha activate increased endothelial permeability by disassembly of VE-cadherin junctions. J Physiol; 533: 433–445

Sawada N, Tani E, Fujikawa H, Kaibuchi K (2000) Inhibition of Rho-associated kinase results in suppression of neointimal formation of balloon-injured arteries. Circulation; 101: 2030

Schachinger V, Britten MB, Zeiher AM (2000) Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. Circulation; 101: 1899-1906

Schäfer C, Walther S, Schäfer M, Dieterich L, Kasseckert S, Abdallah Y, Piper H.M (2003) Inhibition of contractile activation reduces reoxygenation-induced endothelial gap formation. Cardiovasc Res*;* 58: 149-155

Schiffrin EL (2004) Persistent remodeling of resistance arteries in type 2 diabetic patients on antihypertensive treatment. Hypertension; 43: 399–404

Schiffrin EL, Park JB, Intengan HD, Touyz RM (2000) Correction of arterial structure and endothelial dysfunction in human essential hypertension by the angiotensin receptor antagonist losartan. Circulation; 101: 1653–1659

Schofield I, Malik R, Izzard A, Austin C, Heagerty A (2002) Vascular structural and functional changes in type 2 diabetes mellitus: evidence for the roles of abnormal myogenic responsiveness and dyslipidemia. Circulation; 106: 3037–3043

Schwartz N, Hosford M, Sandoval RM, Wagner MC, Atkinson SJ, Bamburg J, Molitoris BA (1999) Ischemia activates actin depolymerizing factor: role in proximal tubule microvillar actin alterations. Am J Physiol; 276: 544–551

Sellers JR *(*1991*)* Regulation of cytoplasmic and smooth muscle myosin*.* Curr Opin Cell Biol*;* 3*:* 98–104

Shasby DM, Ries DR, Shasby SS, Winter MC (2002) Histamine stimulates phosphorylation of adherens junction proteins and alters their link to vimentin. Am J Physiol Lung Cell Mol Physiol; 282: 1330-1338

Shen Q, Rigor RR, Pivetti CD, Wu MH, Yuan SY (2010) Myosin light chain kinase in microvascular endothelial barrier function. Cardiovasc Res; 87: 272-280

Shi T, Moulton VR, Lapchak PH, Deng GM, Dalle Lucca JJ, Tsokos GC (2009) Ischemia-mediated aggregation of the actin cytoskeleton is one of the major initial events resulting in ischemia-reperfusion injury. Am J Physiol Gastrointest Liver Physiol; 296: 339-347

Shima H, Hatano Y, Chun YS, Sugimura T, Zhang Z, Lee EY, Nagao M (1993) Identification of PP1 catalytic subunit isotypes PP1 gamma 1, PP1 delta and PP1 alpha in various rat tissues. Biochem Biophys Res Commun; 192: 1289-1296

Shimizu, H, Ito, M, Miyahara, M, Ichikawa, K, Okubo, S, Konishi, T, Naka,M, Tanaka, T, Hirano, K, Hartshorne, DJ, et al. (1994) Characterization of the myosin-binding subunit of smooth muscle myosin phosphatase. J Biol Chem; 269: 30407–30411

Shimokawa H (2002) Rho-kinase as a novel therapeutic target in treatment of cardiovascular diseases. J Cardiovasc Pharmacol; 39:319–327

Shimokawa H, Takeshita A (2005) Rho-kinase is an important therapeutic target in cardiovascular medicine. Arterioscler Thromb Vasc Biol; 25: 1767-1775

Simon AM, Goodenough DA (1998) Diverse functions of vertebrate gap junctions. Trends Cell Biol; 8: 477–483

Soderling TR (1990) Protein kinases. Regulation by autoinhibitory domains. J Biol Chem; 265: 1823–1826

Somlyo AP, Somlyo AV (2000) Signal transduction by G-proteins, Rho-kinase and protein phosphatase to smooth muscle and nonmuscle myosin II. J Physiol; 522; 177

Somlyo AP, Somlyo AV (2003) Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. Physiol Rev; 83: 1325-1358

Sonnenburg ED, Gao T, Newton AC (2001) The phosphoinositide-dependent kinase, PDK-1, phosphorylates conventional protein kinase C isozymes by a mechanism that is independent of phosphoinositide 3-kinase. J Biol Chem; 276: 45289-45297

Spahr R, Piper HM (1990) Microcarrier cultures of endotheha1 cells. In: Cell Culture Techniques in Cardiovascular Research, edited by H. M. Piper. Heidelberg, Germany: Springer-Verlag: 220-229

Srinivas SP, Satpathy M, Guo Y, Anandan V (2006) Histamine-induced phosphorylation of the regulatory light chain of myosin II disrupts the barrier integrity of corneal endothelial cells. Invest Ophthalmol Vis Sci; 47:4011-4018

Stossel TP, Chaponnier C, Ezzell RM, Hartwig JH, Janmey PA, Kwiatkowski DJ, Lind SE, Smith DB, Southwick FS, Yin HL, et al. (1985) Nonmuscle actin-binding proteins. Annu Rev Cell Biol; 1: 353–402

Sutton TA, Mang HE, Campos SB, Sandoval RM, Yoder MC, Molitoris BA (2003) Injury of the renal microvascular endothelium alters barrier function after ischemia. Am J Physiol Renal Physiol; 285: 191-198

Taddei S, Salvetti A (2002) Endothelial dysfunction in essential hypertension: clinical implications. J Hypertens; 20: 1671-1674

Takemoto M, Sun J, Hiroki J, Shimokawa H, Liao JK (2002) Rho-kinase mediates hypoxia-induced downregulation of endothelial nitric oxide synthase. Circulation; 106: 57-62.

Takizawa N, Koga Y, Ikebe M (2002) Phosphorylation of CPI17 and myosin binding subunit of type 1 protein phosphatase by p21-activated kinase. Biochem Biophys Res Commun; 297: 773-778

Tamaoki T, Nomoto H, Takahashi I, Kato Y, Morimoto M, Tomita F (1986) Staurosporine, a potent inhibitor of phospholipid/Ca^{2+} dependent protein kinase. Biochem Biophys Res Commun; 135: 397–402

Tauber S, Menger MD, Lehr HA (2004) Microvascular *in vivo* assessment of reperfusion injury: significance of prostaglandin E(1) and I(2) in postischemic “no-reflow” and “reflow-paradox”. J Surg Res; 120: 1-11

Terrak M, Kerff F, Langsetmo K, Tao T, Dominguez R (2004) Structural basis of protein phosphatase 1 regulation. Nature; 429: 780-784

Thambyrajah J, Landray MJ, McGlynn FJ, Jones HJ, Wheeler DC, Townend JN (2000) Abnormalities of endothelial function in patients with predialysis renal failure. Heart; 83: 205–209

Thiagarajan RR, Winn RK Harlan JM (1997) The role of leukocyte and endothelial adhesion molecules in ischemia reperfusion injury. Thromb Haemost; 78: 310-314

Tinsley JH, De Lanerolle P, Wilson E (2000) Myosin light chain kinase transference induces myosin light chain activation and endothelial hyperpermeability. Am J Physiol Cell Physiol; 279: 1285–1289

Totsukawa G, Yamakita Y, Yamashiro S, Hartshorne DJ, Sasaki Y,Matsumura F (2000) Distinct roles of ROCK (Rho-kinase) and MLCK in spatial regulation of MLC phosphorylation for assembly of stress fibers and focal adhesions in 3T3 fibroblasts. J Cell Biol; 150: 797–806

Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F, Duhamel L, Charon D, Kirilovsky J (1991) The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. J Biol Chem; 266: 15771-15781

Trinkle-Mulcahy L, Ichikawa K, Hartshorne DJ, Siegman MJ, Butler TM (1995) Thiophosphorylation of the 130-kDa subunit is associated with a decreased activity of myosin light chain phosphatase in alpha-toxin-permeabilized smooth muscle. J Biol Chem; 270: 18191–18194

Trybus KM *(*1991*)* Regulation of smooth muscle myosin*.* Cell Motil Cytoskelet*;* 18: 81–85

Turowski P, Martinelli R, Crawford R, Wateridge D, Papageorgiou AP, Lampugnani MG, Gamp AC, Vestweber D, Adamson P, Dejana E, Greenwood J (2008) Phosphorylation of vascular endothelial cadherin controls lymphocyte emigration. J Cell Sci; 121: 29-37

Umemoto S, Bengur AR, Sellers JR (1989) Effect of multiple phosphorylations of smooth muscle and cytoplasmic myosins on movement in an in vitro motility assay. J Biol Chem; 264: 1431–1436

Van Nieuw Amerongen GP, Draijer R, Vermeer MA, van Hinsbergh VW (1998) Transient and prolonged increase in endothelial permeability induced by histamine and thrombin. Circ Res 83: 1115-1123

Van Nieuw Amerongen GP, Van Delft S, Vermeer MA, Collard JG, Van Hinsbergh VW (2000a) Activation of RhoA by thrombin in endothelial hyperpermeability: role of Rho kinase and protein tyrosine kinases. Circ Res; 87: 335–340

Velasco G, Armstrong C, Morrice N, Frame S, Cohen P (2002). Phosphorylation of the regulatory subunit of smooth muscle protein phosphatase 1M at Thr850 induces its dissociation from myosin. FEBS Lett; 527: 101–104

Verin AD, Csortos C, Durbin SD, Aydanyan A, Wang P, Patterson CE, Garcia JG (2000) Characterization of the protein phosphatase 1 catalytic subunit in endothelium: involvement in contractile responses. J Cell Biochem; 79: 113-25

Verin AD, Patterson CE, Day MA (1995) Regulation of endothelial cell gap formation and barrier function by myosin-associated phosphatase activities. Am J Physiol; 269: 99–108

Verma S, Anderson TJ (2002) Fundamentals of endothelial function for the clinical cardiologist. Circulation; 105: 546–549

Vestweber D (2008) VE-cadherin: the major endothelial adhesion molecule controlling cellular junctions and blood vessel formation. Arterioscler Thromb Vasc Biol; 28: 223–232

Wallez Y, Huber P (2008) Endothelial adherens and tight junctions in vascular homeostasis, inflammation and angiogenesis. Biochim Biophys Acta; 1778: 794-809

Wang Z, Jin N, Ganguli S, Swartz DR, Li L, Rhoades RA (2001) Rho-kinase activation is involved in hypoxia-induced pulmonary vasoconstriction. Am J Respir Cell Mol Biol; 25: 628–635

Wang Z, Lanner MC, Jin N, Swartz D, Li L, Rhoades RA (2003) Hypoxia inhibits myosin phosphatase in pulmonary arterial smooth muscle cells: role of Rho-kinase. Am J Respir Cell Mol Biol; 29: 465-471

Ward B, McCarthy A (1995) Endothelial cell "swelling“ in ischaemia and reperfusion. J Mol Cell Cardiol; 27: 1293–1300

Waschke J, Drenckhahn D, Adamson RH, Curry FE (2004) Role of adhesion and contraction in Rac 1-regulated endothelial barrier function in vivo and in vitro. Am J Physiol Heart Circ Physiol; 287: 704-11

Watanabe H, Kuhne W, Spahr R, Schwartz P, Piper HM (1991) Macromolecule permeability of coronary and aortic endothelial monolayers under energy depletion. Am J Physiol; 260: 1344-52

Watanabe Y, Ito M, Kataoka Y, Wada H, Koyama M, Feng J, Shiku H, Nishikawa M (2001) Protein kinase C-catalyzed phosphorylation of an inhibitory phosphoprotein of myosin phosphatase is involved in human platelet secretion. Blood; 97: 3798-805

Weis S, Shintani S, Weber A, Kirchmair R, Wood M, Cravens A, McSharry H, Iwakura A, Yoon YS, Himes N, Burstein D, Doukas J, Soll R, Losordo D, Cheresh D (2004) Src blockade stabilizes a Flk/cadherin complex, reducing edema and tissue injury following myocardial infarction. J Clin Invest; 113: 885-94

Weis WI, Nelson WJ (2006) Re-solving the cadherin-catenin-actin conundrum. J. Biol. Chem; 281: 35593-35597

Weiss A, Leinwand LA (1996) The mammalian myosin heavy chain gene family. Annu Rev Cell Dev Biol; 12: 417-39

Woodsome TP, Eto M, Everett A, Brautigan DL, Kitazawa T (2001) Expression of CPI-17 and myosin phosphatase correlates with Ca2+ sensitivity of protein kinase C-induced contraction in rabbit smooth muscle. J Physiol; 535: 553-64

Wooldridge AA, MacDonald JA, Erdodi F, Ma C, Borman MA, Hartshorne DJ, Haystead TA (2004) Smooth muscle phosphatase is regulated in vivo by exclusion of phosphorylation of threonine 696 of MYPT1 by phosphorylation of Serine 695 in response to cyclic nucleotides. J Biol Chem; 279: 34496-34504

Wysolmerski RB, Lagunoff D (1991) Regulation of permeabilized endothelial cell retraction by myosin phosphorylation. Am J Physiol; 261: 32-40

Yamada S, Pokutta S, Drees F, Weis WI, Nelson WJ (2005) Deconstructing the cadherin-catenin-actin complex. Cell; 123: 889 –901

Yamakita Y, Yamashiro S, Matsumura F (1994) In vivo phosphorylation of regulatory light chain of myosin II during mitosis of cultured cells. J Cell Biol; 124: 129–137

Yoshida K, Hirata T, Akita Y, Mizukami Y, Yamaguchi K, Sorimachi Y, Ishihara T, Kawashiama S *(*1996*)* Translocation of protein kinase C-alpha, delta, and epsilon isoforms in ischemic rat heart. Biochim Biophys Acta; 1317: 36–44

Young BA, Sui X, Kiser TD, Hyun SW, Wang P, Sakarya S, Angelini DJ, Schaphorst KL, Hasday JD, Cross AS, Romer LH, Passaniti A, Goldblum SE (2003) Protein tyrosine phosphatase activity regulates endothelial cell-cell interactions, the paracellular pathway, and capillary tube stability. Am J Physiol Lung Cell Mol Physiol; 285: 63–75

Yuan SY (2002) Protein kinase signaling in the modulation of microvascular permeability. Vascul Pharmacol; 39: 213–223

Yuan Y, Huang Q, Wu HM (1997) Myosin light chain phosphorylation: modulation of basal and agonist-stimulated venular permeability. Am J Physiol; 272: 1437–1443

Zemlickova E, Johannes FJ, Aitken A, Dubois T (2004) Association of CPI-17 with protein kinase C and casein kinase I. Biochem Biophys Res Commun; 316: 39-47

7 SUMMARY

Failure of endothelial barrier leading to capillary leakage and edema formation during reperfusion represents major impediment for the recovery of an organ. The endothelial barrier function is maintained by equilibrium of competing contractile and adhesive forces, generated by the actomyosin cytoskeleton and adherens junction proteins, respectively. The activity of the endothelial contractile machinery is regulated by the phosphorylation state of the myosin light chain (MLC), the activity of which is regulated by activities of MLC-kinase (MLCK) and MLC-phosphatase (MLCP). In the present study, the molecular mechanism of ischemia-reperfusion-induced activation of the contractile machinery was analyzed, focusing on the involvement of protein kinase C (PKC) and Rho-associated kinase (ROCK)-mediated inhibition of MLCP. Furthermore, rearrangement of the actin cytoskeleton, loss of VE-cadherin, and β-catenin from cell-cell adhesions were analyzed during ischemia-reperfusion. Exposure of endothelial cells to severe ischemia (Po_2 < 1 mmHg, pH 6.4) for 45 min, followed by 45 min of reperfusion, caused an increase in macromolecule permeability and phosphorylation of MLC, myosin-targeting subunit of MLCP (MYPT1), and C-kinase potentiated protein phosphatase-1 inhibitor (CPI-17). ML-7 (10 µM), a pharmacological inhibitor of MLCK, partially reduced ischemia-reperfusion-induced MLC phosphorylation and hyperpermeability. But also PKC inhibitor bisindolylmaleimide (8 µM) and Rho kinase inhibitor Y-27632 (20 µM) reduced ischemia-reperfusion-induced barrier dysfunction and phosphorylation of MLC, MYPT1, and CPI-17. Downregulation of CPI-17 by siRNA significantly abolished the ischemia-reperfusion-induced increase in permeability and led to actin cytoskeleton rearrangement and restoration of VE-cadherin at cell-cell adhesions.

The data of the present study show for the first time that under ischemia-reperfusion CPI-17 is activated, it stimulates the contractile machinery and induces distortion of adherens junction proteins. These effects are prevented by downregulation of CPI-17. The development of therapeutic strategies targeting CPI-17 provides a new option to protect against ischemia-reperfusion-induced barrier failure.

8 ZUSAMMENFASSUNG

Das Versagen der endothelialen Schranke führt zur kapillären Leckage und Ödembildung und ist ein Haupthindernis bei der Wiedererholung der Organfunktion im Verlauf der Reperfusion. Die endotheliale Barrierenfunktion wird durch das Gleichgewicht zweier konkurrierender Kräfte aufrechterhalten: den kontraktilen Kräften, vermittelt über das Actomyosin-Zytoskelett und den Haltekräften, vermittelt über *Adherens Junctions*. Die Aktivität des endothelialen kontraktilen Apparates wird über Phosphorylierung der Myosinleichtketten (MLC) reguliert, welche wiederum durch die Aktivitäten von MLC-Kinase (MLCK) und MLC-Phosphatase kontrolliert wird. In der vorliegenden Arbeit wurde der molekulare Mechanismus der Ischämie-Reperfusion-induzierten Aktivierung des kontraktilen Apparates analysiert. Dabei lag der Schwerpunkt der Arbeit auf der Proteinkinase C (PKC)- und Rho-abhängigen Kinase (ROCK)-vermittelten Hemmung der MLKP. Darüber hinaus wurden Änderungen des Aktin-Zytoskeletts, die Translokation von VE-Cadherin und β-Catenin aus den Zell-Zell-Adhäsionsstrukturen während Ischämie-Reperfusion untersucht. Eine Ischämie (Po2 < 1 mmHg, pH 6,4) für 45 min, gefolgt von einer 45-minütigen Reperfusion, verursachte einen Anstieg der Makromolekülpermeabilität und Phosphorylierung der MLC, Myosin-bindender Untereinheit der MLCP (MYPT1) und dem *C-kinase potentiated protein phosphatase-1 inhibitor* (CPI-17). ML-7 (10 µM), ein pharmakologischer Inhibitor der MLCK, reduzierte die Ischämie-Reperfusion-induzierte MLC-Phosphorylierung und Hyperpermeabilität teilweise. Der PKC-Inhibitor Bisindolylmaleimid (8 uM) und Rho-Kinase-Inhibitor Y-27632 (20 µM) reduzierte die Ischämie-Reperfusion-induzierte Schrankenstörung und Phosphorylierung von MLC, MYPT1 und CPI-17. Herunterregulation von CPI-17 durch siRNA verminderte die Ischämie-Reperfusion-induzierte Steigerung der Permeabilität und bewirkte die Reorganisation des Aktin-Zytoskeletts und von VE-Cadherin an den Zell-Zell Kontakten.

Die Daten der vorliegenden Studie zeigen zum ersten Mal, dass unter Bedingungen einer Ischämie-Reperfusion CPI-17 aktiviert wird, welches den kontraktilen Apparat stimuliert und zur Störung der Adherens junctions beiträgt. Diese Wirkung kann durch Herunterregulieren von CPI-17 verhindert werden. Die Entwicklung von therapeutischen Strategien, die auf CPI-17 abzielen, stellt eine neuartige Option dar, um vor dem Ischämie-Reperfusion-induzierten Schrankenversagen zu schützen.

9 DECLARATION

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Sabiha Nazli
Giessen, October 2012

10 ACKNOWLEDGEMENTS

I express my deep sincere gratitude to the Almighty of **Allah**, the Most Merciful, the Most Gracious, and the Compassionate, whose overflowing blessings bestowed upon me to complete thesis work.

My special praise for the Holy Prophet **Hazrat Muhammad (Peace be upon him),** the greatest educator, the everlasting source of guidance and knowledge for humanity. He taught the principles of morality and eternal values.

I deem it a great honour to express my deep sense of obligation and gratitude to my honourable and admirable supervisor **Prof. Dr.Thomas Noll**, for his kind and great guidance, valuable comments, inspiring and encouraging attitude throughout the course of my studies. Without his help and kindness, this achievement would have been far away.

I feel great pleasure to express my special thank to the former director of the Institute of Physiology, **Prof. Dr. Dr. H. M. Piper**, for providing me a place and for essential facilities to carry on my studies.

I would like to express my special thanks to my senior **Dr. Muhammad Aslam**, who told me how to plan and perform the research work.

I do not find appropriate words to express my thanks to **Dr. Frauke V. Härtel**, for her valuable guidance, sympathetic behaviour, and corrected my thesis.

I am also thankful for the help and support of **Hermann Holzträger**, **Anna Reis** and **Annika Krautwurst** for their technical assistance and their interest in my success.

I offer my special thanks to all my lab fellows especially **Kiran, Krishnaveni, Tatyana, Daniel, Arshad** and **Assad** for their sincerely cooperation and cheerful company for the progress of my research work.

This acknowledgement would be incomplete unless I offer my humble admiration to my **loving parents** and other family members whose love and endless prayers boost up my success. May Allah grant of the above cited personalities with success and honor.

Specially and most sincerely, I would like to pay my warmest tribute to my beloved **husband** whose great patient, love, and encouragement made my life pleasant. A shower of thanks to my little daughters **Sumaya Shahid** and **Mahveen Shahid** whose smile fill my days with joy.

Thanks

11 PUBLICATIONS

1 **Nazli S**, Aslam M, Härtel FV, Piper HM, Schulz R , Noll T (2013) Role of CPI-17 in ischemia-reperfusion induced barrier failure (Manuscript in preparation)

2 Aslam M, Schlüter KD, Rohrbach S, Rafiq A, **Nazli S**, Piper HM, Noll T, Schulz R, Guenduez D (2012). Hypoxia/reoxygenation-induced endothelial barrier failure: Role of RhoA, Rac1, and MLCK. J. Physiol. 591(2): 461-473

3 Riazuddin S, **Nazli S**, Ahmed ZM, Yang Y, Zulfiqar F, Shaikh RS, Zafar AU, Khan SN, Sabar F, Javid FT, Wilcox ER, Tsilou E, Boger ET, Sellers JR, Belyantseva IA, Riazuddin S, Friedman TB. 2008. Mutation spectrum of MYO7A and evaluation of novel nonsyndromic deafness DFNB2 allele with residual function. Hum Mutat. 29(4): 502-511

4 Ain Q, **Nazli S**, Riazuddin S, Jaleel AU, Riazuddin SA, Zafar AU, Khan SN, Husnain T, Griffith AJ, Ahmed ZM, Friedman TB, Riazuddin S. 2007. The autosomal recessive nonsyndromic deafness locus DFNB72 is located on chromosome 19p13.3. Hum Genet. 122(5): 445-50

5 Riazuddin S, Khan SN, Ahmed ZM, Ghosh M, Caution K, **Nazli S**, Kabra M, Zafar AU, Chen K, Naz S, Antonellis A, Pavan WJ, Green ED, Wilcox ER, Friedman PL, Morell RJ, Riazuddin S, Friedman TB. 2006. Mutations in TRIOBP, which encodes a putative cytoskeletal-organizing protein, are associated with nonsyndromic recessive deafness. Am. J. Hum. Genet. 78(1): 137-43

6 Shaikh RS., Ramzan K., **Nazli S**., Sattar S., Khan SN., Riazuddin S., Ahmed ZM., Friedman TB., Wilcox ER., Riazuddin S. 2005. A new locus for nonsyndromic deafness DFNB51 maps to chromosome 11p13-p12**.** Am J Med Genet A. 138A (4): 392-395

7 Tasawar Z and **Nazli S**. 1999. Prevalence of copepod parasites of Hybrid fish. J.Sc and Tech. Univ. Peshawar., Pakistan: 23

12 PUBLISHED ABSTRACTS

1 **Nazli. S**., Aslam. M., Khawaja. K., Härtel F.V., Piper H.M., Noll. T. Activation of endothelial myosin phosphatase via inhibition of CPI-17 ameliorates reperfusion induced endothelial barrier failure. 76-jahrestagung der Deutschen Gesellschaft für Kardiologie. 08-10 April 2010. Mannheim, Germany.

2 **Nazli. S**., Aslam. M., Khawaja.K., Härtel F.V., Piper H.M., Noll. T. Ischemia reperfusion induces endothelial barrier failure via CPI-17 mediated activation of endothelial contractile machinery. Joint Meeting of the Scandinavian and German Physiological Societies. 27-30th March 2010, Copenhagen Denmark.

3 **Nazli. S**., Aslam. M., Härtel F.V., Riaz A.R., Noll. T. Activation of endothelial myosin phosphatase via inhibition of CPI-17 ameliorates reperfusion induced endothelial barrier failure. Circulation. 2009; 120:S1162**.** (American Heart Association-Scientific Session. Nov. 2009, Orlando, FL USA.

4 **Nazli, S**., Aslam, M., Härtel F.V., Noll, T. Activation of endothelial myosin phosphatase via inhibition of Cpi-17 ameliorates reperfusion induced endothelial barrier failure. 2nd 1st conference organized by Giessen Graduate School for the Life Sciences. 2009. Giessen, Germany.

5 Aslam, M., **Nazli, S**., Härtel F.V., Guenduez.D., Piper H.M., Noll, T. Epac/Rap1 activation, presents a novel therapeutic target against reperfusion-induced endothelial barrier failure organized by European society of cardiology. 2009. Barcelona, Spain.

6 **Nazli, S**., Aslam, M., Härtel F.V., Noll, T. Ischemia-reperfusion induces endothelial barrier failure: Role of CPI-17. 1st conference organized by Giessen Graduate School for the Life Sciences. 2008. Giessen, Germany.

7 Aslam, M., **Nazli, S**., Härtel F.V., Piper H.M., Noll, T. Ischemia reperfusion induces endothelial barrier failure via CPI-17 mediated activation of endothelial contractile machinery organized by European society of cardiology. 2008. Munich, Germany.

8 Khan, S.N., Ramzan, K., Sadiq, R., Ahmed, J., **Nazli, S** and Riazuddin, S. Genetic of hereditary hearing impairment in Pakistan at 17th International Biennial Conference of Pakistan Association & Scientific Conference of Paediatric Association of SAARC Countries organized by Pakistan Paediatric Association Punjab. Feb.19-22, 2004. Pakistan.

9 Ahmad J., Ahmed ZM., Riazuddin S., **Nazli S**., Shaikh RS., Ramzan K., Awais M., Khan SN., Riazuddin S. Genotype-phenotype correlation in DFNB23 and USH1F at 2nd International Symposium on Biotechnology organized by Institute of Biotechnology & Genetic Engineering University of Sindh, Jamshoro and Nuclear Institute of Agriculture (NIA) Tando Jam. Jan. 19-21, 2004. Pakistan.

10 Khan SN, **Nazli S**, Shaikh RS, Ramzan K, Ahmad J, Riazuddin S, Ahmed ZM, and Riazuddin S. Molecular studies of hereditary hearing impairment at the First National Conference on Health Biotechnology organized by National Commission on Biotechnology at University of Health Sciences. Jan. 27-28, 2005. Lahore, Pakistan.

11 Shaikh RS**, Nazli S**, Riazuddin S, Ramzan K, Ahmad J, Ahmed ZM, Khan SN and Riazuddin S. Genetic Heterogeneity of Usher Syndrome Type 1 in Pakistan at 1st National Conference on Health Biotechnology, organized by National Commission on Biotechnology at University of Health Sciences. Jan 27-28, 2005. Lahore, Pakistan.

12 Khan SN, **Nazli S,** Ramzan K, Shaikh RS, Tasneem S, Kalsoom S, and Riazuddin S. Genetic studies of hereditary hearing impairment. 18th FAOBMB Symposium organized by school of biological Sciences University of Punjab. Nov 20-23, 2005. Lahore, Pakistan.

édition scientifique
VVB LAUFERSWEILER VERLAG

VVB LAUFERSWEILER VERLAG
STAUFENBERGRING 15
D-35396 GIESSEN

Tel: 0641-5599888 Fax: -5599890
redaktion@doktorverlag.de
www.doktorverlag.de

ISBN: 978-3-8359-6053-4

Cover photo: © freshidea - Fotolia.com