Research Center Borstel
Leibniz-Center for Medicine and Bioscienes
Department of Lungpharmacology

The role of caveolae in the regulation of endothelial cells barrier function

Dissertation

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Eike Reppien
aus Rostock

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ICH KANN, WEIL ICH WILL, WAS ICH MUSS.

Immanuel Kant

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Publications

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Reppien E., Yin J., Kasper M. Kübler WM., Uhlig S., Platelet-activating factor decreases NO levels in pulmonary endothelial cells by sphingomyelinase dependent recruitment of caveolin-1. *Manuscript in preparation*.

Lindner K., Winoto-Morbach S., Reppien E., Drab M., Martin Ch., Dombrowsky H., Ochs M., Schütz S., Uhlig S.,
Thrombin-induced transcellular albumin transport in human pulmonary microvascular endothelial cells is mediated by sphingomyelinase-induced caveolin recruitment into lipid rafts. *Manuscript in preparation*

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Sphingomyelinase-dependent recruitment of caveolin-1 in platelet-activating factor (PAF) induced edema.

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Abbreviations

ARDS	acute respiratory distress syndrom
ASM	acid sphingomyelinase
BPAE	bovine pulmonary artery endothelial cells
Ca^{2+}	calcium
CaSR	calcium sensing receptor
Cav-1	caveolin-1
Cer	ceramide
COP	colloid oncotic pressure
CPAE	calf pulmonary artery endothelial cells
CHX	cycloheximinde
EC	endothelial cells
fdr	false discovery rate (multiple-comparisons) procedure
eNOS	endothelial Nitric-oxide synthase
ERK	extracellular-regulated protein kinases
GPCR	G protein-goupled receptor
HES	hydroxyethylstarch
HMVEC-L	human microvascular endothelial cells from lung
Imi	imipramine
IP_3	inositol 1,4,5-trisphosphate
IPL	Isolated perfused rat lung
MAPK	mitogen activated protein kinases
MLCK	myosin light chain kinases
min	minutes
NO	nitric oxide
PAF	platelet-activating factor
PAR	proteinase-activated receptor
PLC	phospholipase C
PI3K	phosphatidylinositol-3-OH kinase
SEM	standard error
SM	sphingomyelinase
SOC	store-operated channel
TRPC	transient receptor potential channel

1 Introduction

1.1 The lungs

The lungs are the essential organ of respiration in air-breathing vertebrates. Its principal function is to transport oxygen from the atmosphere into the bloodstream, and to excrete carbon dioxide (CO_2) from the bloodstream into the atmosphere. The lungs are a target for airborne infectious, microbial particles and toxic gases. On the vascular side, the lungs receive the whole cardiac output and therefore the lungs are also a target for blood-borne pathogens and toxins.

1.1.1 Morphology of the lungs

The lungs are located inside the thoracic cavity, protected by the bony structure of the rib cage and enclosed by a double-walled sac called pleura. In humans, the left lung is smaller than the right one to give way for the heart. The lungs are divided into lobes. The right lung has three lobes and the left lung has two. Figure 1.1 shows a picture from the lungs and its anatomy, which is described below.

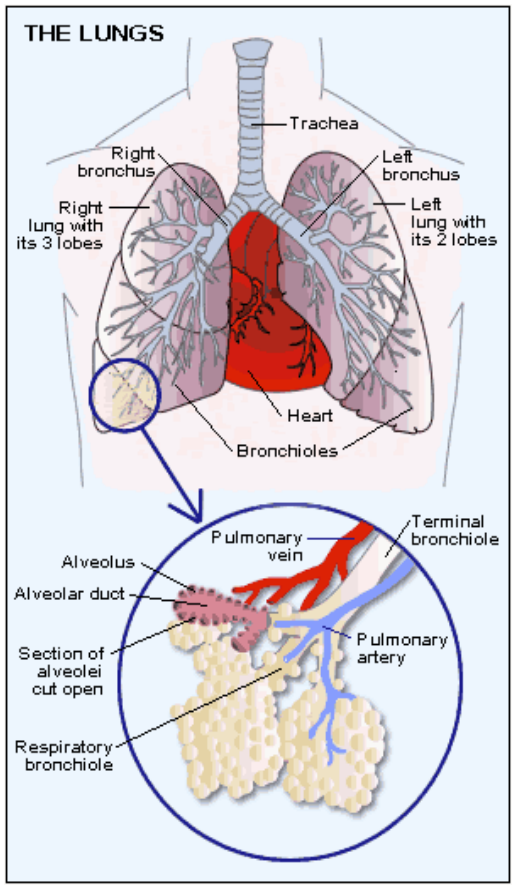


Figure 1.1: ***Anatomy of the lungs.*** *For more details see text. Adapted from: www.mydr.com.au*

The lungs are connected to the upper airways by the trachea and bronchi. The trachea runs down the neck and divides into left and right bronchi. The bronchi enter the lungs and branch out to form the bronchial tree. The bronchi divide into smaller bronchioles, which terminate into alveoli. An alveolus is composed of respiratory tissue and is the site of gas exchange in the lung. The bronchi split up, depending upon species through up to 25 generations into the bronchioles of decreasing diameter. With each generation, the number of

airways increases expotential, while the diameter of each single airways decreases. The last seven generations end blindly in the alveoli. The blind ending alveoli are 0.2 to 0.3 mm in diameter and surrounded by a net of capillaries (5µm in diameter) with a surface area of up to 300 m^2 in humans.

Two different blood systems supply the lungs: the pulmonary and the bronchial vessels. The pulmonary circulation, a low pressure system, starts at the right ventricle leaving the heart through the pulmonary artery where it gets enriched with oxygen and ends in the left heart ventricle. The bronchial circulation supplies the central airways and vessels as well as the lungs lymph nodes with oxygen.

The lungs are a complex cellular system, consisting of more than 40 highly organised cells types. The alveolar wall is built up mainly of flat alveolar type I pneumocytes and thicker type II pneumocytes. The functions of these cells in the lungs are different. The main function of type I cells, which have a low number of organelles, is to facilitate gas exchange. The type II cells synthesise, store and releaese the pulmonary surfactant, a complex mixture of 90% phospholipids and 10% proteins. In addition, these cells are rich in organelles, which indicates a high metabolic activity.

To protect the body from particles, toxins and organisms transported by the incoming air, the lungs possess a variety of defense mechanisms. The ciliary epithelium and mucus secreting glands in the upper airways clear most of the air, while alveolar macrophages clean the alveolar surface. Inflammatory defense mechanims can be triggered by type II, endothelial and epithelial cells and lead to neutrophil migration and subsequent activation of specific immune responses. Specific immune cells like lymphocytes are found mainly in the upper airways.

1.1.2 Lung endothelium

The endothelium is the layer of thin, flat cells that lines the interior surface of blood vessels, forming an interface between the intravascular space and the vessel wall. Its permeability is selective and can be regulated by the organism. The lung endothelium is composed of the macrovascular and microvascular endothelium. The macrovascular endothelial cells are involved in vasoconstriction and vasodilation. And therefore in regulating the vascular tone. The microvascular endothelium controls the exchange of metabo-

lic substances and cells from the blood with the surrounding tissue. The endothelial cells are connected through thight junctions.

The large surface area of the lungs makes them a major target for microorganisms, toxins and other pathogenic patricles out of the enviroment penetrating the lungs capillaries. Therefore the pulmonary endothelium plays a central role in the induction of inflammation processes. Its functions can be altered by a variety of circulating vasoactive proteins, hormones, cytokines and inflammatory mediators. The activation of endothelial cells promotes the adhesion of leukocytes and migration into the lungs. The same processes that allow defensive reactions in order to protect the organism from the environment can also result in barrier dysfunction and lung edema development.

1.1.3 The alveolar-capillary unit

The major function of lungs are to transport O_2 from the atmosphere into the bloodstream and excrete CO_2 from the bloodstream into the atmosphere. This exchange of gases is accomplished in the alveolar-capillary unit. This blood-air barrier, with a diameter of 0.2-0.6 µm, is built up by several thin cell membrane layers: the endothelium, facing the blood compartment, the interstitial space and the alveolar epithelium, lining the air spaces. The total alveolar surface area sums up to 50-100 m² and is about 50 times bigger than the whole outer body surface area [238].

Each aveolus is covered with a fine capillary network. One capillary segment has a diameter of 10-14 µm, each alveolus holds up to 100 segments. The surface area of the lung capillaries has been estimated at 70 m², whereas the combined surface area of arteries, arterioles, veins and venules is only about 5 m².

An electron microscopic images of the alveolar-capillary unit is shown in figure 1.2. It shows an erythrocyte in the position to exchange blood gases (O_2 and CO_2) with the air in the alveolar space.The alveolar-capillary membrane has to be thin enough to allow gas diffusion, but still has to provide protection against the enviroment. All cells involved have a flat shape and a low number of organelles. The inner alveolar surface is covered by the surface active surfactant, which among other functions seals the lung almost completely against salty solutions.

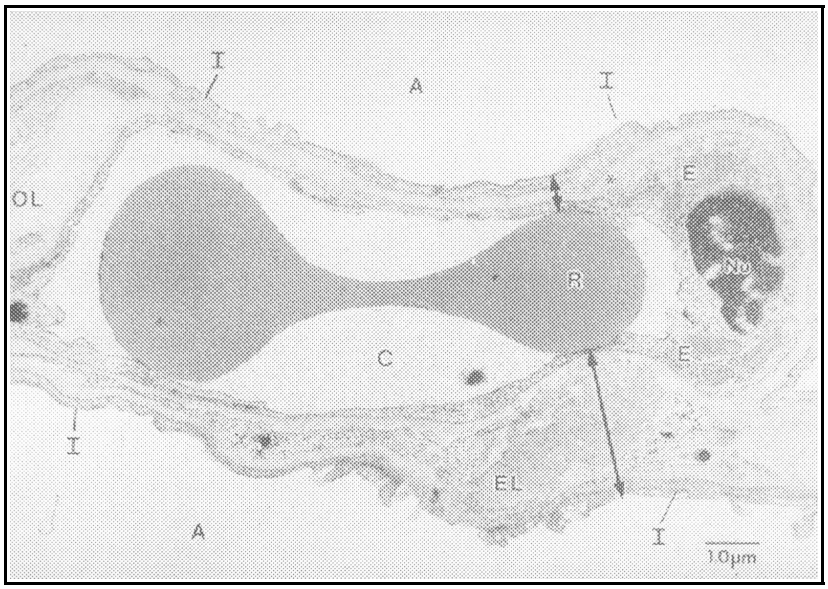


Figure 1.2: ***The alveolar-capillary unit****: electron microscopic image. A (alveolus), C (capillary), E (endothelial cell), I (interstitium, R (erythrocyte), COL (collagen fibers). Adapted from K. Lindner*

The diffusion of oxygen into the body is driven by the difference of the partial gas pressure in the air and the blood. The amount of oxygen that is taken up in a certain time can be determined from artial and venous oxygen concentrations and the passing blood volumina (Fick's principle):

$$V_{O2} / t = V_{blood} \times (C_a - C_v)$$

V_{O2} / t is the volume of oxygen taken up per time

V_{blood} is the volume of blood passing per time

$C_a - C_v$ is the difference of arterial and venous oxygen concerntration [2].

1.2 Vascular permeability in lungs

1.2.1 Physiology of endothelial permeability

The vascular endothelium lining the intima of the blood vessels regulates a variety of functions. The maintencance by the endothelium of a semi-permeable barrier is important in controlling the passage of macromolecules and fluid between blood and interstitial space.

The characteristic permeability of transported macromolecules is dependent on molecular radii as well as the barrier properties of particular endothelium.

The barrier properties of the different endothelial membranes in the pulmonary microvasculatur are not uniform: macromolecular diffusion in postcapillary venules is greater than in arterioles, when measured in whole lung model [221]. Cultured microvascular endothelial cells exhibit tenfold higher barrier properties than macrovascular cells when measured by electrical resistance across the monolayer [26]. Differences in vascular permeability can be explained, when two main physiological mechanims for permeability of endothelium are considered. First, vascular leakage due to the endothelial gap formation leads to increased diffusion rates across the endothelial barrier (Fig. 1.3) [14, 178].

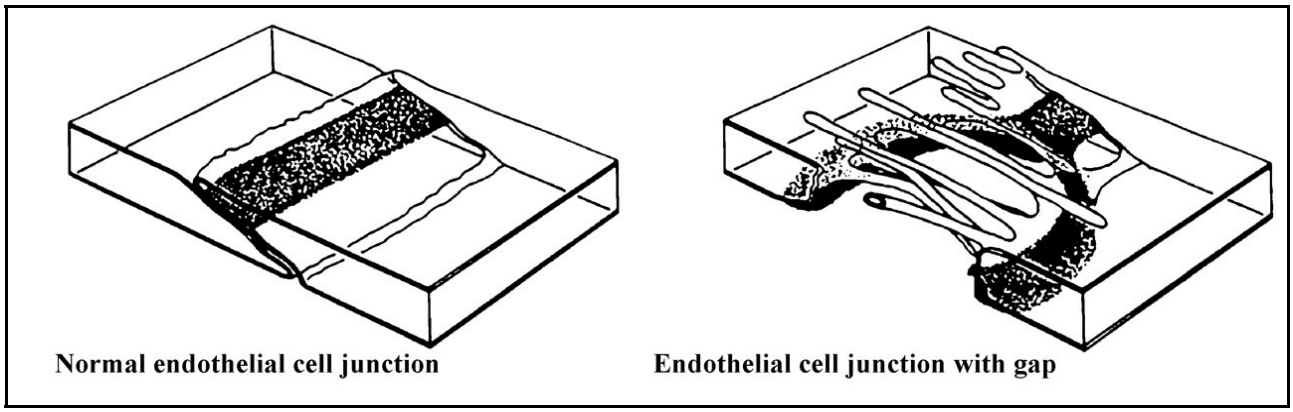


Figure 1.3: ***Endothelial gap formation:*** *Comparison of a normal endothelial cell junction and one with an endothelial gap formation from McDonald* et al. *1999 [178]*

One driving force for liquid flow is the transmicrovascular osmotic gradient. This gradient can be actively established by protein transport across vascular wall through gap formation between the endothelial cells. Theoretical analysis together with experimental findings indicate that protein convection together with basal diffusion must be the primary mechanism, and not transcellular transport [184].

A pathway for proteins with effective diameter of 3 to 4 nm is provided by pores in the endothelium [182]. Also, size dependent passing of macromolecules through endothelial junctions has been reported, but electron microscopic data show no evidence for albumin located within the junctions [106]. A second route for liquid and proteins across the endothelial barrier is transcellular transport via caveolae. Small vesicles can be formed out of membrane invaginations called caveolae. One of the inital observations of caveolae mediated transcellular transport, also called transcytosis, was performed with filipin. This ste-

rol-binding agent is an inhibitor of caveolae formation. Therefore, filipin can reduce the transcytosis of albumin and insulin without affecting chlathrin-dependent pathways, the classic mechanism by which macromolecules are transported via vesicles across the cells [247]. The mechanisms of the transcellular transport via caveolar vesicles is poorly understood.

1.2.2 Regulation of endothelial permeability

The integrity of endothelial barrier is usually maintained by a healthy organism. This is a critical requirement for preservation of pulmonary function. During inflammation, the endothelium can be activated to allow fluid, proteins and polymorphonuclear neutrophils to migrate from the vascular space into the interstitium and subsequently into the surrounding tissue. This is regulated by a variety of inflammatory mediators and intracellular signalling cascades.

Regulation of endothelial cell gap formation and therefore permeability is a function of a dynamic balance between competing adhesive, barrier-promoting tethering forces and contractile, tension-producing forces. Both events have been linked to changes of the actin-based cytoskeleton.

The most intensively studied model of endothelial barrier dysfunction is the thrombin-induced increase of cell monolayer permeability in cell culture. Thrombin evokes numerous endothelial cell responses which contributes to hemostasis, thrombosis, vessel wall pathophysiology and also the development of intercellular gap formation. Thrombin induces rapid cytoskeletal rearrangement and contractile force in the cell, dependent on actomyosin interaction. This mechanism leads to stress fiber formation and cell contraction. It is mediated by phosphorylation of myosin light chains (MLC) by Ca^{2+}/calmodulin (Ca^{2+}/CaM) dependent myosin light chain kinase (MLCK) activity [278, 313]. The MLCK activity was shown to depend on tyrosine phosphorylation of the protein [92]. In addition, the small GTPase Rho and its downstream effector Rho kinase regulates stress fiber formation and subsequent endothelial barrier dysfunction [2, 141]. Rho kinase phosphorylates the myosin binding subunit of MLC phosphotase, which leads to inhibition of its phosphatase activity. In addition, direct phosphorylation of MLC by Rho kinase is possible.

Adhesive forces between cells exist in a dynamic equilibrium with the described contractile

forces in order to maintain and reestablish barrier integrity. The primary adhesive protein present in human endothelium is vascular endothelial (VE) cadherin [55]. Blocking of VE-cadherin increases endothelial permeability as shown in cultured endothelial cells (human umbilical vein endothelial cells, HUVEC) [139] and in vivo (mouse) [50].

The transcellular pathway is generally considered to be of less significance for fluid transport. It was shown that this pathway is tyrosine kinase dependent and gp60 mediated, but its regulation and function is still unclear [248, 281].

1.2.3 Pulmonary edema formation

Pulmonary edema is defined by the accumulation of serous fluid by transsudation from the pulmonary vascular space into the interstitium and the alveolar space. This condition may causes hypoxemia and hypercapnia [29]. Pulmonary edema is either due to direct damage to the tissue (non-cardiogenic edema) or a result of inadequate functioning of the heart or the circulatory system (cardiogenic edema). The cause of cardiogenic or hydrostatic edema is a high lung capillary pressure. The edema which is independent of enhanced hydrostatic pressure is called high permeability or non-cardiogenic edema. The cause of this edema formation is altered permeability of the endothelial cells for water and proteins. Non-cardiogenic edema and hypoxemia represent the hallmarks of ARDS. Table 1.1 shows some causes of cardiogenic and non-cardiogenic edema formation.

Table 1.1: ***A range of several causes of pulmonary edema***

Cardiogenic causes:	***Non-cardiogenic causes***
• *Heat failure by left heart insufficiency* • *Severe cardiac arrhythmia (Tachycardia/fast heartbeat or bradycardia/slow heartbeat)* • *Severe heart attack* • *Hypertensive crisis* • *Excess body fluids, e.g. from kidney failure* • *pericardial effusion with tamponade* • *Fluid overload, e.g. from kidney failure*	• *Inhalation of toxic gases* • *Blood transfusions* • *Severe infection* • *Pulmonary contusion i.e. high-energy trauma* • *Multitrauma, e.g. severe car accident* • *Aspriation of gastrine contents* • *Certain types of medication* • *Upper airway obstruction* • *Reexpansion, i.e. postpneumonectomy or large volume thoracentesis*

The liquid flux between the vessels and the interstitial space is affected by several parameters. The total liquid flux can be described by the modified Starling equation of Pappenheimer and Soto-Rivera [210]:

$$\mathbf{Jv = Kf,c\ [(Pc - Pi) - \sigma\ (\pi p - \pi i)]}$$

Where J_v represents the filtrations rate, $K_{f,c}$ the capillary filtration coefficent, Pc-Pi the difference between capillary blood and hydrostatic tissue pressure, σ the reflection coefficent of the plasma proteins and (πp-πi) the difference between oncotic and interstitial pressure. Under physiological conditions the forces in this system are in balance and small amounts of excess water are drained by the lymphatic system. Imbalances in the „Starling system" lead to an accumulation of fluid in the interstitial space causing problems in gas exchange. Depending on the extent of interstitial fluid accumulation and the integrity of the epithelial barrier interstitial edema may develop into alveolar edema. The cause of edema formation in non-cardiogenic or high permeability edema is altered permeability for water ($K_{f,c}$ enhanced) and/or proteins (σ enhanced).

1.3 Acute respiratory distress syndrome (ARDS)

1.3.1 ARDS

ARDS is a clinical manifestation of severe, acute lung injury. It is characterised by the acute onset of diffuse, bilateral pulmonary infiltrates and later by development of non-cardiogenic pulmonary edema, refractory hypoxia (defined as the ratio of partial oxygen tension (PaO_2) as fraction of inspired oxygen (FiO_2); PaO_2:FiO_2 < 200 mmHg) and decreased lung compliance. Acute respiratory distress syndrome occurs most frequently in the setting of direct or indirect lung injury. Table 1.2 shows an overview of conditions associated with ARDS. Its complex pathophysiology involves an inciting local or systemic event that initiates pulmonary endothelial and epithelial damage and subsequent increased permeability. Tachypnea, hypoxia, and respiratory alkalosis are typical early clinical manifestations, and they are usually followed by the appearance of diffuse pulmonary infiltrates and respiratory failure within 48 hours.

This condition is life-threatening and often lethal. The mortality of ARDS ranges between

40-60%. A less severe form is called acute lung injury (ALI, see below).

Table 1.2: ***Conditions associated with ARDS.*** *Ware* et.al. 2000 [306]

Direct lung injury	***Indirect lung injury***
• *Pneumonia*	• *Sepsis*
• *Aspiration of gastric contents*	• *Multiple trauma*
• *Pulmonary contusion*	• *Multiple blood transfusion*
• *Fat emboli*	• *Cardiopulmonary bybass*
• *Inhalation injury*	• *Burns*
• *Near-drowning*	• *Acute pancreatitis*
• *Reperfusion pulmonary edema*	• *Drug overdose*

The first description of ARDS appeared in 1967 by Ashbaugh and colleagues [11]. Initially, there was no general definition, resulting in controversy over incidence and mortality. In 1988 an expanded definition was proposed that quantified the physiologic respiratory impairment through the use of a four point lung-injury scoring system that was based on the level of positive end-expiratory pressure (PEEP), the ratio of the partial pressure of arterial oxygen to the fraction of inspired oxygen, the static lung complaince, and the degree of infiltration evident on chest radiographs [11, 192]. Other factors included in the assement were the inciting clinical disorder and the presence or absence of no-pulmonary organ dysfunction [11, 192]. In 1994 a new definition was recommended by the American-European Consensus Conference Committee [18]. The consensus definition has two advantages. First, it recognizes that severity of clinical lung injury varies: patient with less severe hypoxemia (is defined by a ratio of partial pressure of arterial oxygen to the fraction of inspired oxygen of 300 mmHg or less) are considered to have acute lung injury (ALI), and those with more severe hypoxemia (as defined by a ratio of 200 mmHg or less) are considered to have the acute respiratory distress syndrome (ARDS). Second, the definition is simple to apply in the clinical setting [18].

1.3.2 Pathophysiology

The development of ARDS is characterised by biological and clinical complexity. A major characteristic is the initially occuring high permeability edema [267]. The lungs histology shows denudation of epithelial lining cells, endothelial cell swelling and marked neu-

trophilic inflammation.

The clinical and physiological manifestations of ARDS appear to arise from lung cell injury like structural damage of cells that may lead to necrosis or apoptosis, cell denudation, ischemic cell death, or metabolic dysfunction. The injury to the endothelium results in increased capillary permeability and the influx of protein-rich fluid into the alveolar space. Injury to the alveolar lining cells also promotes pulmonary edema formation. Damage to type I cells increases entry of fluid into the alveoli and decreased clearance of fluid from the alveolar space. Damage to type II cells results in decreased production of surfactant. Thogether this results in decreased compliance and alveolar collapse.

The neutophils (also some T-lymphocytes) quickly migrate into the inflamed lung parenchyma and can release oxidants, proteases, LTC_4, and other proinflammatory molecules, such as platelet-activating factor (PAF) [18, 192]. Neutrophils are also present in the bronchoalveolar lavage (BAL) [215].

Cytokines secreted by local epithelial and endothelial cells, such as tumor necrosis factor alpha (TNFα), interleukin-1, 6, 8 and 10 (IL-1, IL-6, IL-8, IL-10), leukotrienes (LTC), macrophage inhibitory factor (MIF), and numerous others, along with platelet sequestration and activation, also are important in the development of ARDS [176, 215].

The mechanisms of ARDS and edema formation have been intensively investigated, but many questions are still open. It is known that the pathological changes seen in ARDS are mediated by a variety of inflammatory mediators participating in complex networks of interactions. In this respect, products of cyclo- (COX) and lipooxygenase (LOX) [31, 73], as well as cytokines and reactive oxygen species (ROS) [276] have been discussed. One particularly important mediator is the platelet-activating factor (PAF) [175, 188, 193].

1.4 Platelet-activating factor (PAF)

PAF (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a biologically active lipid mediator that is thought to contribute to a variety of inflammatory lung diseases. After discovery of PAF in rabbit platelets in 1972 by Benveniste *et al* [17] it has been shown that is produced by a variety of different cells. The three dimensional model and chemical structure of PAF is show in figure 1.4. PAF is one of the most potent proinflammatory mediators and in

some instances PAF is effective at concentrations as low as 10^{-12}M [287]. In addition, PAF has been implicated in asthma, sepsis, ALI and ischemia/reperfusion injury [4, 111, 112, 263].

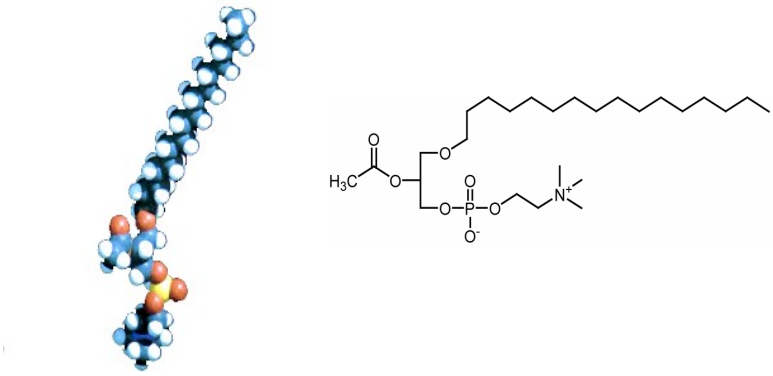


Figure 1.4: A typical molecule of PAF (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine)

1.4.1 PAF synthesis

PAF is synthesised by two different pathways (Fig. 1.5), the remodelling pathway and the *de novo* pathway [190]. The remodelling pathway for PAF synthesis is show in figure 1.5.a. and described below. The PAF synthesis is initiated by the activation of phospholipase A_2 (PLA_2), which may act on 1-O-alkyl-2-arachidonoyl-glycerolphosphocholine to yield lyso-PAF and free arachidonic acid (AA). The lyso-PAF is acetylated by a specific lyso-PAF acetyltransferase using acetyl coenzyme A (Co~A) as a donor. Alternatively PLA_2 may act on arachidonate-containing plasmalogen phosphatidylethanolamine to release free arachidonate.

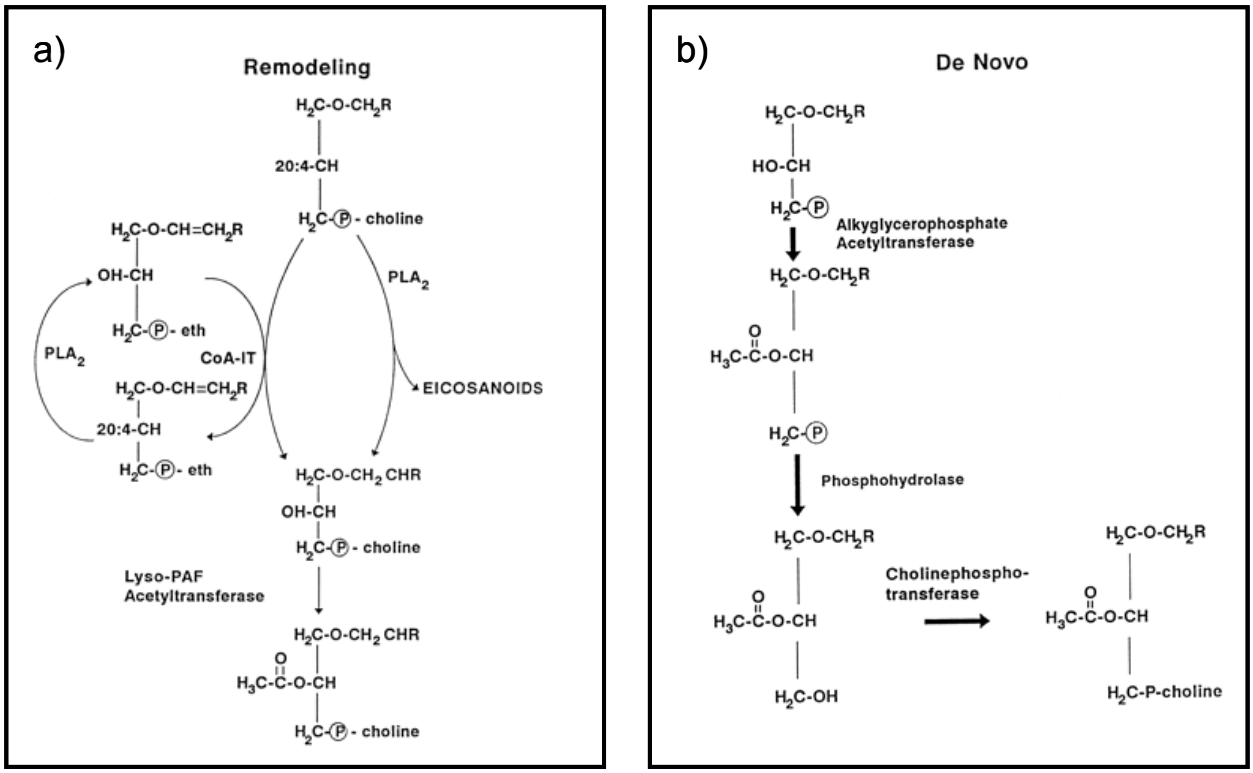


Figure 1.5: ***The remodelling pathway for PAF synthesi (a),*** *PLA2 cleaves AA from 1-O-alkyl-2-arachidonoyl glycerophosphocholine to yield lyso-PAF. Lyso-PAF is acetylated using acetyl Co~A as a donor. Alternatively PLA2 acts on plasmalogen phosphatidyl-ethanolamine to release arachidonate. The generated lysophosphatidylethanolamine acts as an acceptor for arachidonate from the PAF-precursor with formation of lyso-PAF.* ***The de novo pathway for PAF synthesis (b):*** *1-O-alkyl-sn-glycero-3-phosphate is acetylated by 1-O-alkyl-sn-glycero-3-phosphate:acetyltransferase and then transformed into 1-O-alkyl-2-acetyl-sn-glycerols. The latter gets converted to PAF by CDP-cholinephosphotransferase*

The lyso-phosphatidylethanolamine may act as an acceptor for arachidonate in a transacylation reaction from the PAF precursor with formation of lyso-PAF. Therefore, the activation of PLA_2 is essential for generation of lyso-PAF and triggering the remodelling pathway. The activation of lyso-PAF acetyltransferase, which is modulated by a phosphorylation/dephosphorylation cycle, is a limiting factor for generation of biologically active PAF. Figure 5.1.b shows the *de novo* pathway of PAF synthesis and is described below. In brief, the direct precursors of PAF (1-O-alkyl-2-acetyl-sn-glycerols, formed via an acetylation-dephosphorylation sequence catalysed by acetyl Co~A: 1-alkyl-2-lyso-sn-glycero-3-phosphate acetyltransferase and 1-alkyl-2-acetyl-sn-glycero-3-phosphate phosphohydrolase) are converted to PAF by a specific CDP-choline: 1-alkyl-2-sn-acetyl-sn glycerol cholinephospho-transferase. This pathway is mainly involved in the constitutive PAF synthesis and is regulated only by the availability of substrates [35, 226, 257]. In addition, PAF

catabolism is mainly due to PAF acetylhydrolase (PAF-AH) action by cleavage of the short acetyl chain at the 2-sn position to form the biologically inactive lyso-PAF.

1.4.2 PAF receptor and signal transduction

In various cells and tissues a wide variety of physiological and pathophysiological effects are triggered by PAF. PAF signals through specific receptors and a variety of signal transduction systems to elicit its diverse biochemical responses. In many different tissues including lung, heart and liver the PAF receptor (PTAFR, encoded by the human *PTAFR* gene) is expressed on the cell surface [197, 287]. The PTAFR consists of seven transmembrane domains, a characteristic of the G protein-coupled receptor superfamily (GPCRs). Although it is generally accepted that the PTAFR is coupled to various cellular effector systems such as PLA_2 and PLC through G-proteins, the particular of proteins involved in signal transduction is sometimes unclear and probably differs depending on the cell type. For example, it has been shown that PAF stimulates PLC via G_q and G_p, whereas adenylate cyclase is inhibited via G_i [41]. The identity and properties of the G-protein regulating the activity of PLA_2 remains uncertain [41]. One key factor in PAF-induced signal transduction is the polyphosphoinositide turnover [41, 257]

1.4.3 PAF-induced biochemical effects and mediator release

PAF causes phosphorylation of PTAFR, tyrosine phosphorylation of various proteins, activation of PLA_2 and PLC and of phosphatidylinositol-3-kinase (PI3K) and Akt, increased phosphatidylinositol turnover, activation of mitogen activated protein kinase (MAPK) and increased cytosolic calcium levels [71, 115]. The $G_{i/q}$-protein dependent phosphatidylcholine specific phospholipase C (PI-PLC) mediates the hydrolyses of phosphatidylcholine and leads to generation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 mobilises intracellular calcium via IP_3 receptors (IP_3-R) whereas DAG mediates protein kinase C (PKC) activation [41, 257]. In addition to the formation of DAG and IP_3, PTAFR activation also induces the generation of arachidonic acid [287] a precursor of eicosanoids. These lipid mediators play an important role in many physiological and pathophysiological processes such as bronchoconstriction, vasoconstriction and lethal shock [214, 242, 269, 318].

1.4.4 PAF actions in lungs

The platelet-activating factor plays a central role in many pulmonary disorders [286, 287]. In the past years, our group investigated and characterised the mechanisms by which PAF causes airway hyperresponsiveness, bronchoconstriction, pulmonary vasoconstriction and edema formation. All these actions of PAF start within minutes and are mediated by lipid mediators. On the next page figure 1.6 summarises the established PAF actions in lungs.

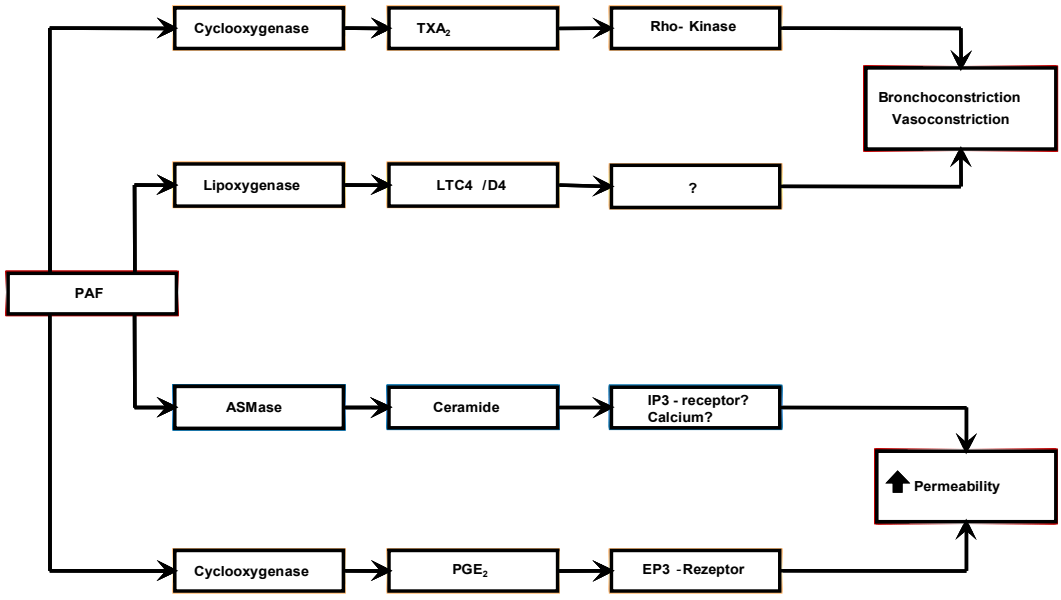


Figure 1.6: ***Mechanisms of PAF-induced bronchoconstriction, vasoconstriction and edema.*** *TXA = thromboxane, LT = Leukotriene, ASM = acid sphingomyelinase, PGE = prostaglandine E, PTGER3 = prostaglandine E recptor 3. For futher details see text. Adapted from Uhlig S and Reppien E. 2005 [287]*

The airway hyperresponsiveness is mediated by thromboxane [108], the vaso- and bronchoconstiction by both thromboxane and leukotrienes [64, 286, 289, 292] that contract bronchial and vascular smooth cells largely by activation of Rho-kinase [173].

Rho-kinase appears to play a dual role, because it regulates both thromboxane release and thromboxane action. Leukotrienes, particularly LTC_4/ LTD_4, are synthesised from arachidonic acid (AA) by 5-lipooxygenase, and do also mediate the effects in PAF-induced vaso- and bronchoconstriction [64, 292]. Inhibition of leukotriene (with AA861) alone or in combination with thromboxane receptor antagonism (SQ29548) prevents the vaso- and bronchoconstriction, but does not affect edema formation in isolated perfused rat lung [64].

Thus edema formation induced by PAF occurs independent from changes in hydrostatic pressure. Also, in our model of the isolated perfused rat lung hydrostatic pressure as a cause of pulmonary edema is excluded by constant pressure perfusion [287]. The fact that PAF causes edema by increasing vascular permeability is supported by measurements of the capillary filtrations coefficient [64, 291]. Over the past years, our group observed that the PAF-induced edema formation is blocked independently and additively by cyclooxygenase inhibition and by quinolines [94, 95]. Therefore our group began to examine these pathways.

One pathway is cyclooxygenase-dependent and mediated by PGE_2 [94]. PGE_2 is a mediator that has long been suspected of playing a critical role in many models of edema formation [9, 170] The first direct evidence for PGE_2 as a mediator of edema formation was described in *in vivo* model (rat) that used a neutralizing anti-PGE_2 antibody to prevent carrageenan-induced paw edema [216]. In isolated perfused rat lung it was shown that PAF administration stimulates release of PGE_2 and that the perfusion with a neutralizing anti-PGE_2 antibody attenuated the PAF-induced edema formation [94]. Further investigations showed that PGE_2 triggers pulmonary edema by activation of EP3 receptors [94, 268]. The oedematogenic properties of EP3 receptors may be explained by their ability to decrease intracellular cAMP levels and increase cellular calcium concentration, two phenomena that are frequently linked to increased vascular permeability [191, 252].

The other pathway was subsequently shown to involve activation of acid sphingomyelinase and ceramide formation [96]. The conclusion, that ASM and ceramide contribute to PAF-induced edema, was based on the following findings in mice *in vivo* and in isolated perfused rat lung experiments [96]: in isolated perfused rat lung perfusion with C_2-ceramide (long chain ceramide such as C_{16} or C_{18} (natural forms) are insoluble) caused severe edema and application of ceramide-specific antibodies in mice *in vivo* and the IPL reduced the extent of PAF-induced edema. Inhibition of ASM by pharmacological (D609, inhibitor of ceramide biosynthesis [252] or imipramine that induces proteolytic degradation of ASM [1, 118]) or genetic means ($Smpd1^{-/-}$ mice) prevented the increase in ceramide and attenuated pulmonary edema formation in response to PAF. A complete inhibition was only achieved by combining ASM deficiency or D609 with the cyclooxygenase inhibitor acetyl salicylic acid (ASA). This simultaneous blockade of ASM and cyclooxygenase

demonstrated that PAF-induced edema through ASM/ceramide independent of cyclooxygenase mechanisms [96]. How PAF induces edema by ASM dependent production of ceramide is largely unknown. There are reasons to believe that ceramide may be generated in the outer leaflet of the cell membrane by extracellularly active ASM:(i) an extracellular form of ASM is now well established [178], (ii) vascular endothelial cells are rich and regulated source of secretory ASM [178], (iii) 95% of sphingomyelin, the substrate of ASM, is present in the outer leaflet of the membrane [147, 273] and (iv) PAF increases the activity and the amount of extracellular ASM [96]. How ASM-derived ceramide causes pulmonary edema was partly investigated by Lindner *et al* [156]. They observed that ceramide increases transendothelial permeability by a non-apoptotic mechanism, which is independent of cytoskeletal rearrangement [156]. The mechanism by which ASM-metabolites regulate vascular permeability are unknown and subject of the present thesis. One hypothesis is that ceramide may be simply necessary to form lipid rafts (caveolae) [99, 100] which are required as a platform for signalling molecules of several cascades.

1.5 Thrombin

Thrombin is a serine protease that is generated in circulating plasma by the cleavage of prothrombin, via the prothrombinase. It converts fibrinogen to fibrin in the blood coagulation complex and activates factor X, V, Ca^{2+} and membrane phospholipids [98], other components of this complex, which are essential for the proteolytic reaction. The proteolytic active form is called α-thrombin.

The thrombin receptors (protease-activated receptor, PAR-1, 2, 3, 4) are present on many different cell types, including endothelial cells, platelets, fibroblasts, mast cells, macrophages and leukocytes [199]. In addition to this, thrombin activates G protein-coupled protease activated receptors (PAR-1, PAR-3, and PAR-4) and promotes numerous cellular effects. Also, thrombin induces the release of specific inflammatory mediators, which are involved in tissue repair and inflammatory and proliferative processes. Thrombin is chemotactic for monocytes and lymphocytes [15, 23] and mitogenic for vascular smooth muscle cells, fibroblasts and epithelial cells [43, 180, 225]. It also plays a role in other biologic processes, for example carcinogenesis [110].

Thrombin has a variety of effects on the endothelium and endothelial cells possess high-

affine receptor sites that bind thrombin in a reversible manner. It is also described that human endothelial cells in culture produce PAF when stimulated with thrombin [114, 220]. Concerning its interaction with endothelial cells, thrombin also increases transendothelial permeability in cell monolayer [36, 87]. The mechanism involved Rho-kinase (RhoK) [36, 63] and the Ca^{2+}/calmodulin-dependent MLCK [80, 81, 82]. Activated RhoK and MLCK activation enhances actin-myosin interaction, central stress fibre formation, EC contraction and gap formation. These are indicators of barrier disruption, and increased vascular permeability [60, 82]. However, when applied to isolated perfused lung, edema is only at constant flow prefusion [308] and thrombin does not cause permeability alteration in the vasculature.

1.6 Sphingolipids

Sphingolipids are a class of lipids, which are essential for the structure and function of cells in particular in the membrane. There are three main types of sphingolipids: ceramide, sphingomyelins and glycosphingolipids, which differ in the substituent on their head group. On the next page figure 1.7 shows chemical structure of all three noted sphingolipids. Formerly considered to serve only as structural components, sphingolipids are emerging as an important group of signaling molecules involved in many physiological and (patho)physiological cellular events, including proliferation, apoptosis, stress and inflammation responses [13, 96, 108, 315].

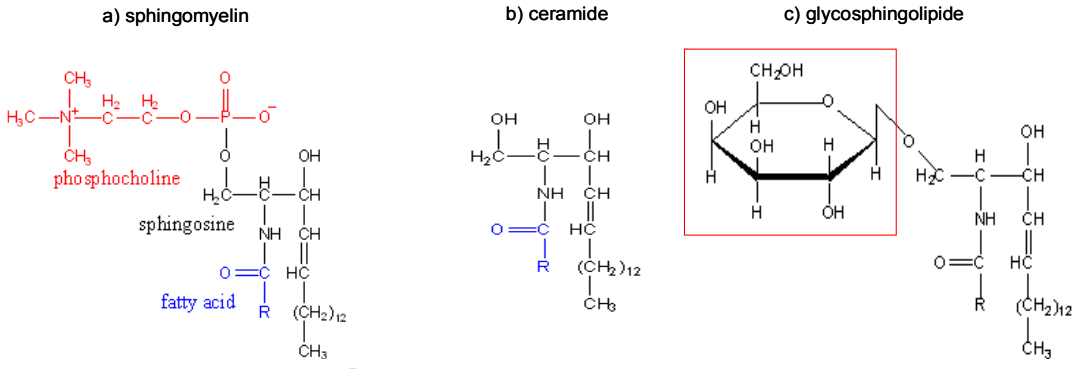


Figure 1.7: ***Overview of sphingolipids****: Sphingomyelin has a phosphocholine or phosphoethanolamine head group (red) and is a common constituent of plasma membranes. Ceramide has a polar "head group", esterified to the terminal hydroxyl of sphingosine. Glycosphingolipide, that including cerebrosides and gangliosides, are commonly found in the outer leaflet of the plasma membrane. The lipid cerebroside has a monosaccharide such as glucose or galactose as the polar head group (red border) and ganglioside has a polar head group that is a complex oligosaccharide.*

1.6.1 Ceramide

Ceramide is a sphingosine-based lipid signalling molecule involved in the regulation of many biological processes, including cellular differentiation [20], proliferation [205], apoptosis [144, 145, 200] and growth inhibition [21]. The amount of ceramide within cells is regulated by intracellular production and degradation, is triggered by various stresses and controlled by ceramide-associated enzymes such as sphingomyelinase isoforms (two different forms exist: acid- and neutral sphingomyelinase; ASM and NSM), ceramidase, glucosylceramide synthase (GCS) and sphingomyelin synthase (SMS). The activity of each form of sphingomyelinase is dependent on the local pH. The ASM acts extracellularly as well as in acidic compartments like lysosomes whereas NSM generates ceramide intracellularly close to the inner membrane. Ceramide can be also generated by *de novo* synthesis, catalysed by the enzyme ceramide synthase [106]. The *de novo* pathway of ceramide has been implicated in chemotherapy-induced apoptosis of some cancer cells [207].

Other mediators which activate sphingomyelinase are TNFα, IL1β, nerve growth factor, FAS and CD40 which are typical mediators of inflammation, stress, heat shock, irradiation, UV light, bacterial or viral infections [102, 104, 120, 144, 145, 252]. The mechanisms by which these mediators stimulate ASM and ceramide remain poorly understood. In order to explain the effect of ceramide, different mechanisms were disscused such as ceramide-binding proteins [119, 203], channel formation by C_2 and C_{16} ceramides [264] and the

organisation of membranes into microdomaines [57, 78, 161].

1.7 Membrane microdomains

1.7.1 Cell membrane

The cell membrane is a selectively permeable bilayer which forms the outer layer of a cell. It consists of, among other components, phospholipids and protein molecules which separate the cell interior from its surroundings and control the input and output.

The membrane as a whole is often described as a *´fluid mosaic model´* a two-dimensional fluid of freely diffusing lipids, embedded with proteins. This membrane-model was first proposed by Singer *et al*. (1971) [262] and extended to include the fluid character in a publication of Nicholson *et al.* in 1972 [195]. The biological membrane is now better described as a *´mosaic of lipid domains´* rather than as homogeneous fluid domains. A growing body of evidence has shown that specialized lipid domains exist in membranes. Until now two different classes of lipid microdomains were characterized in the cell membrane: lipid rafts and caveolae [78].

The lipid rafts, which are believed to be floating substructures on the cell surface, contain primarily sphingolipids such as sphingomyelin (SM) and glycosphingolipids (GSL), and cholesterol. Caveolae represent a subset of lipid rafts and contain the marker protein caveolin, which is essential for invagination of the membrane and involved in a variety of cellular processes.

1.7.2 Ceramide in membrane microdomains

Many references support the notion that ceramide is synthesised in lipid-microdomains where it is involved in several signalling events [57, 78, 161]. Liu *et al.* reported that in response to IL1β, ASM locates to caveolae of human fibroblasts and generates ceramide within the membrane microdomains [161]. A variety of cell receptors were shown to require clustering within lipid rafts for their activation. For example, FAS (CD95) mediates programmed cell death via clustering of the Fas-ligand receptor that involves translocation of ASM to the extracellular membrane surface and depends on ceramide generation within

membrane rafts [66, 102]. Recently, a clinical relevant finding supported the model of ceramide-induced changes within membrane microdomains. *Pseudomaona aeruginosa* infection of epithelial cells triggers activation of ASM and the release of ceramide in sphingolipid-rich rafts. Ceramide reorganizes these rafts into larger signalling platforms that are required for the internalization of pathogen and essential for host defense against it [101].

1.7.3 Caveolae

Caveolae were first identified by electron microscopic examination in the mid 50' by Palade and Yamada, as 50-100nm `*flask shaped*` invagination of the plasma-membrane [207, 314]. A electron microscopic images of caveolae was shown in figure 1.8.a and b. Yamada *et al.* found these vesicle like structures in communication with the extracellular space in gall bladder epithelium [314]. Palade and another group described the same discovery in endothelial cells [32, 207].

Since then, caveolae have been identified in a wide variety of tissues and cell types. Particularly, caveolae have been well described in adipocytes, fibroblasts, where they are extremely abundant, endothelial cells (10.000-30.000 caveolae/cell) [8], type I pneumocytes, striated and smooth muscle cells. Ultrastructural analysis of adipocytes has shown that as much as 20% of the total plasma membrane is occupied by caveolae [49, 65].

Caveolae are distinctive organelles which are rich in lipids, such as sphingolipids, glycosphingolipids and cholesterol [8]. Figure 1.8.c shows the structural and chemical composition of caveolae. The high cholesterol and sphingolipid content of caveolae leads to insolubility in non-ionic detergents such Trition X-100 at 4°C. These properties are the basis for their purification and biochemical characterisation. Caveolae contain a large number of signalling molecules, enzymes and receptors. Table 1.3 shows a selection of proteins associated with caveolae.

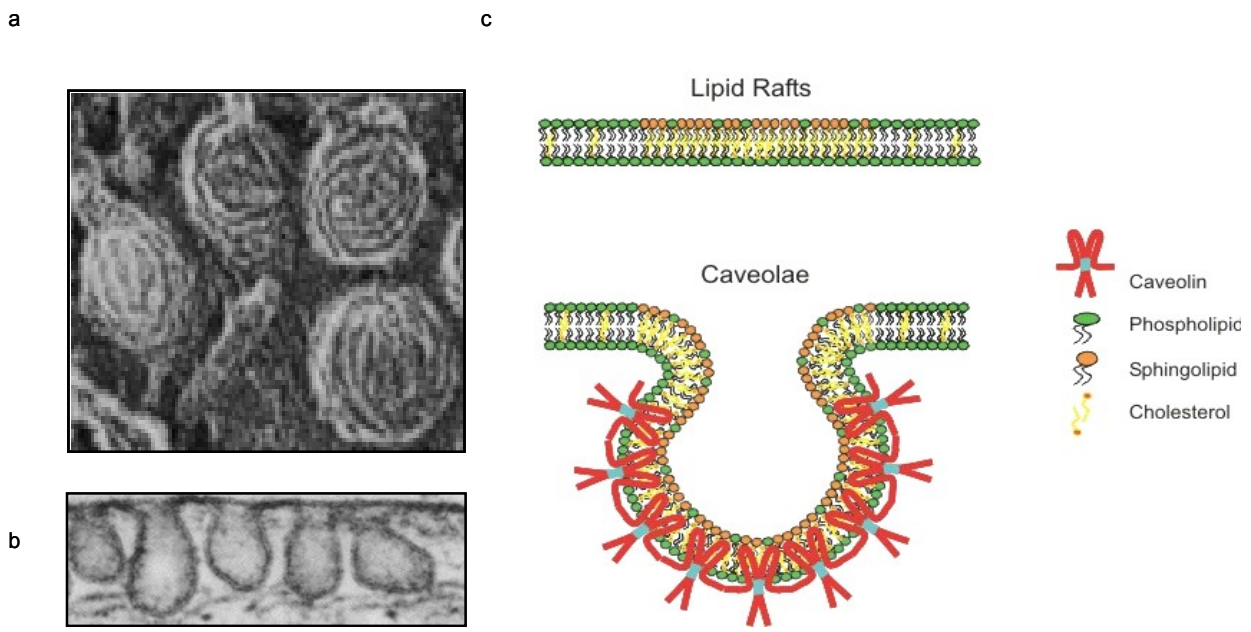


Figure 1.8: ***Electron micrograph of an endothelial cell showing caveolae,*** *50-100 nm structures that are either direct invaginations or in close proximity to the plasma membrane, a) bottom view and b) side view of caveolae. c) The structural and biochemical composition: Lipid rafts formed via a coalescence of cholesterol and sphingolipids; as a result, these microdomains have vastly different biochemical properties than the bulk phospholipid bilayer. Caveolae are generally considered to be ´invaginated´ lipid rafts primarily due to enrichment in a family or proteins known as the caveolins. Adapted from Galbiati* et al. *2001 [78]*

Table 1.3 ***Proteins associated with caveolae in endothelial cells****. Adapted from Frank* et al. *2003 [70]*

Protein	***Function and reference***
Structural proteins	
• *Caveolin-1, 2, 3*	• *Major strutural protein [8, 158]*
• *Flotellin-1 and 2*	• *Signalling and structur [19, 301]*
• *Actin*	• *Involved in cell motility [158, 201]*
• *Annexin II and VI*	• *Promotes membrane fusion, involved in exocytosis [158, 201]*
• *Dynamin*	• *Involved in vesicular trafficking [109, 201]*
• *SNAP*	• *Involved in vesicular transport [245]*
• *VAMP-2*	• *Involved in the targeting/fusion of transported vesicles to their target membrane [245]*
G proteins	
• $G_{\alpha s}$, $G_{\alpha i1}$, $G_{\alpha i2}$,	• *Regulates G protein–coupled receptor activity [158]*
• $G_{ß\,\gamma}$	• *Regulates G protein–coupled receptor activity [158]*
• G_q	• *Regulates G protein–coupled receptor activity [158, 202]*
Transmembrane receptor	
• IP_3 *receptor (IP3R)*	• *Involved in calcium flux [75, 246]*
• *Mannose-6-phosphate*	• *Receptor for mannose 6-phosphate, also definied as insulin-like*

Protein	*Function and reference*
receptor (M6PR)	*growth factor receptor typ II (IGF II-R) [39]*
• *60 kDa glycoprotein (gp60)*	• *Albumin-binding protein [243, 244, 248]*
• *EGF receptor*	• *Endothelail growth factor [186]*
• *VEGF receptor*	• *Vascular endothelial growth factor[67]*
Non-receptor Tyrosine kinases	
• *Src*	• *Regulation of growth factor response [158, 160]*
• *Fyn*	• *Regulation of growth factor response [158, 160]*
• *STAT3*	• *Signal transducers and activators of transcription [131, 160]*
Non-receptor Ser/Thr kinases	
• *MEK*	• *Signal transduction of mitogenic signals [62]*
• *PI3 kinase*	• *Phosphorylation of phosphatidylinositol [158, 160, 322]*
• *PKC α, ß*	• *Protein kinase C, a Ser/Thr kinase [158, 160]*
• *ERK 1/2*	• *Mitogen activated protein kinase [70, 137, 316]*
• ***Other***	
• *eNOS*	• *Production of NO, endothelial form [86, 256]*
• *nNOS*	• *Production of NO, neuronal form [54, 299]*
• *PLC γ*	• *Phospholipase C [160]*
• *Cox 2*	• *Cyclooxogenase, AA metabolism [157]*

With so many different molecules, proteins and receptors located in this membrane structure, it is clear that caveolae must play an important role in many cellular processes [70]. Examples are transport of macromolecules (albumin [91], insulin [16] and native LDL [140, 297]), generation of nitric oxide (NO) by eNOS [183, 256], ion-channel regulation [3, 47, 283]. regulation of angiogenesis [103], shear stress [228] and regulation of vascular permeability [59, 224].

1.7.4 Caveolin

One of the first members of this protein family, caveolin-1 was identified as a v-src phosphorylation substrate in *Rous Sarcoma Virus*-transformed fibroblasts and independently cloned as VIP21, a component of the trans-Golgi-derived vesicles [93, 150]. The 21 to 24 kDa protein caveolin-1 is together with cholesterol and sphingolipids the major structural component of caveolae. Therefore, caveolin-1 is essential for the formation and the stabil-

ity of caveolae. In its absence no caveolae are present, conversely, when this molecule is expressed in cells that lack caveolae, they induce formation of caveolae [30, 59, 212].

Molecular cloning studies have identified three distinct caveolin (CAV) genes caveolin-1, 2 and 3 [204]. Cav-1 the first gene which was discovered, is derived by alternative translational initiation and/or mRNA splicing of two different isoforms [74], termed caveolin-1α consisting of residues 1-178 and the caveolin-1β containing residues 32-178, resulting in a protein of ~ 3 kDa smaller size. Cav-2 has three identified isoforms, the full-length caveolin-2α and two truncated variants, termed caveolin-2β and -2γ [142]. Caveolin-2 is closely related to caveolin-1 as it colocalizes and heterooligomerizes with caveolin-1. In absence of caveolin-1, caveolin-2 is not transported to the plasma membrane [142, 204].

The caveolin isoforms have been identified in a wide variety of tissues and cell types. Caveolin-1 and 2 are coexpressed in many different tissues. They are most abundantly expressed in endothelial cells, type I pneumocytes, adipocytes and fibroblasts. Caveolin-3 is present in all muscle cell types (cardiac, skeletal, smooth muscle), but is also found in other cells like astrocytes and condrocytes [198, 250].

Many cellular functions of caveolin-1 have been discovered. Important for this diversity of caveolin-1 is its particular structure. On the next page figure 1.9 shows a schema of the structure of caveolin-1, which is described below. Caveolin-1 protein has a central 33 amino acid (AA) long hydrophobic domain that is thought to adopt a hairpin conformation into the membrane, leaving both NH_2- and COOH-termini (N- and C-terminus) of the facing the cytoplasm [109]. The C-terminus of caveolin protein is palmitoylated on three conserved cyteine residues (133, 143 and 156 AA) and the N-terminus is tyrosine phosphorylated. Both are post-translational and reversible modification that may be regulated and that in turn may regulate conformation, membrane association, protein-protein interactions, and intracellular localization of the target protein.

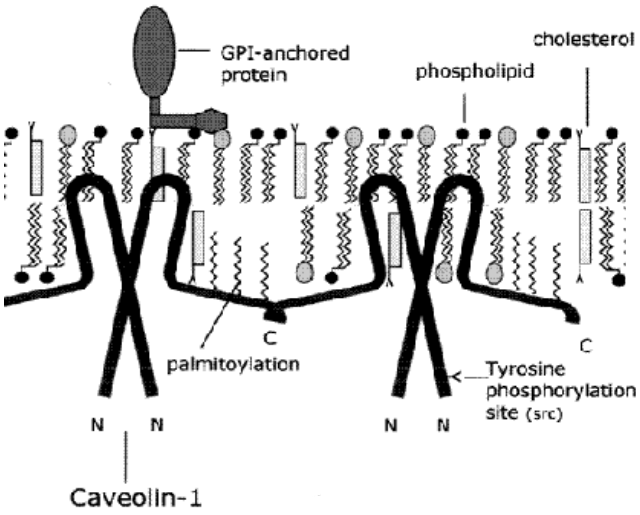


Figure 1.9:Structure of caveolin-1. N-NH2-terminus, C-COOH-terminus. For further details see text.

Caveolin-1 has also different functional domains; the oligomerization domain and a scaffolding domain [52]. The oligomerization domain (residues 61-101 AA) can form oligomers or heterooligomers. These oligomers can interact with lipids like glycosphingolipids or cholesterol, which is required for insertion into model lipid membranes [69]. Within the oligomerization domain, there also exists a short cytosolic domain called scaffolding domain (residues 82-101 AA). The scaffolding domain was originally defined by a series of experiments, including deletion mutagenensis of caveolin-1 GST fusions proteins. By using a GST-fusions protein containing the caveolin-1 scaffolding domain as a receptor to select peptide ligands from a bacteriophage display library, two related but distinct caveolin-binding motifs were identified in most proteins (including heterotrimeric G-proteins, Src family tyrosine kinases and eNOS). These different signalling proteins, on the other hand, contain the caveolin binding motif (FxxxxFxxF, or FxFxxxxF, where F is aromatic amino acid: tryptophan (Trp), phenylalanine (Phe), or tyrosine (Tyr)) and association with caveolin-1 leads to inhibition of their activity [204].

1.7.5 Functional roles of caveolae/caveolin

1.7.5.1 Transcytosis, endocytosis and pinocytosis (potocytosis)

The observation that caveolae can exist as invaginations of the plasma membrane, as completely enclosed vesicles, or as aggregates of several vesicles suggests that this structures are involved in the vesicular transport of micro- and macromolecules (or fluids)

into the cells via endocytosis/potocytosis and across the cells via transcytosis [5, 7, 69, 189, 212, 219, 261, 275]. Figure 1.10 shows a schematic overview of the different mechanisms of caveolar vesicle transport.

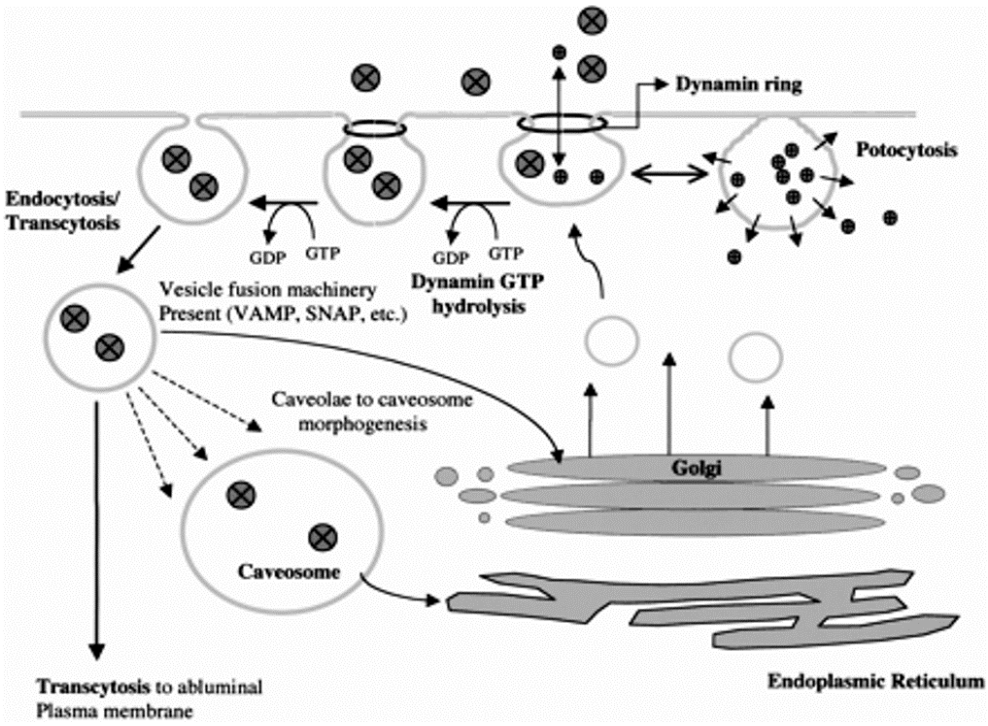


Figure 1.10: ***Caveolar vesicular trafficking:*** *transcytosis, endocytosis and potocytosis. Caveolae appear to mediate the selective uptake and transport of several molecules via different processes. Adapted from Razani B.* et al., 2002 *[223]*

Traditionally, endocytosis has been almost entirely associated with clathrin-coated vesicle [7, 275]. Numerous receptors and their cognate ligands are taken up via this pathway leading to termination or desensitization of signalling cascades. However, it has become clear that certain receptors and extracellular macromolecules are exclusively transported via caveolae rather than clathrin-coated pits. This alternative caveolar pathway was first recognised by the observation that cholera toxin was preferentially bound and internalized via caveolae [189]. Additional observations suggest a role for caveolae in the process of endocytosis included first the identification of several molecules involved in vesicle docking and fusion (SNARE protein, GM1) and later the detection of the small GTPase dynamin, which is known to be involved in internalization process [109, 177, 245]. Perhaps the best documented infectious agent that selectively uses caveolae to enter cells is *Simian Virus 40* (SV40) [5, 266]. A growing list of pathogens, including viruses (e.g. SV40, HIV, *respiratory*

syncytial virus (RSV)) bacteria (e.g. *Eschericha coli* [61]*, Chlamydia trachomatis, Mycobacterium bovis*) and their associated toxins [49], fungi (e.g. *Pneumocystis carnii*) and even prions [49], can interact with caveolae membrane domaines and be taken up by internalization [109].

Further biochemical studies demonstrated that caveolae are include in transcytosis. This transport is rapid (~ 30 sec) and shows macromolecules transported in caveolae from the luminal side of blood vessels to the subendothelial space (first described by Simionescu *et al.* [261]). Transcytosis can be both constitutive (fluid-phase transport) or receptor-mediated (the molecule transported requires the presence of its cognate receptor in caveolae). The transport of many macromolecules such as albumin [228, 250], insulin [16], native LDL (as well as modified LDL) [297] has been associated with caveolae-mediated transcytosis. Some of the inital observations for transcytosis by caveolae was performed with inhibitiors of caveolae formation, specific gold-labelled antibodies and gold-labelled albumin. For example, by using filipin, a sterol binding agent, the transcytosis of albumin and insulin was reduced without affecting clathrin-dependent pathways [247]. McIntosh *et al*. used a specific antibody targeted against lung endothelial caveolae [179]. After injection with this specific gold-labelled antibody, particles were observed first at the surface of lung endothelial cells. Subsequently, particles were observed within internalized caveolae (plasmalemmal vesicles) inside the cells and finally, within the subendothelial space [179]. The first direct evidence supporting a role of caveolin-1 and caveolae in transcytosis were shown by perfusion with gold-labelled albumin in wild type and caveolin-1 deficient mouse lungs. The wild type endothelial cells associated with gold-labelled albumin, internalized and transferred it to the subendothelial space through caveolae, but no uptake or transfer was observed in caveolin-1 deficient animals [250].

Caveolae have also been implicated in a unique form of solute uptake, termed potocytosis (or ´cellular drinking´). The potocytic transport was described by Anderson *et al.* [6]. They observed the potocytosis of folate (form of a water soluble B vitamin) as a process by which cells can transport small molecules (< 1 kDa), without endocytotic vesicles, in caveolae to internal endosomal/lysosomal compartments [6]. In previous studies Rothberg *et al.* [265] observed by microscopy that the GPI-linked folate receptor internalizes folate without entering the clathrin-coated pit endocytose pathway. Kamen *et al.* observed that

caveolae facilitate the uptake of folate by closing their necks, albeit remaining associated with the plasma membrane [134]. In this way, a highly concentrated pool of folate is created, which is subsequently fluxed to the cytoplasm by a three-step process:(i) lowering of caveolar pH by a caveolae-localized proton pump, (ii) low pH dissociation of folate from its receptor, and (iii) flux into the cytoplasm by an anion carrier present in caveolae [134]. At present, the mechanism of the dynamic closing and opening of the caveolar *´mouth´* is poorly understood. More detailed analysis of the general applicability of caveolae to the uptake of other small molecules needs to be conducted before potocytosis can be considered a process distinct from caveolar endocytosis.

1.7.5.2 Nitric oxide synthase signaling mechanisms

Endothelium-derived nitric oxide (NO) has a profound effect on vessel tonicity and permeability. The endothelial enzyme responsible for the generation of NO is known as endothelial NO-synthase (eNOS). This membrane associated enzyme is one of three NOS isoforms (iNOS, nNOS and eNOS).

Endothelial NO-synthase is found enriched in caveolae, where it associates with the caveolin-1 by interacting with the scaffolding domain. Binding of eNOS to caveolin-1 holds in the inactive state [70, 86, 256]. After increased intracellular Ca^{2+} $[Ca^{2+}]_i$, eNOS complexes with Ca^{2+}/calmodulin (Ca^{2+}/CaM) and dissociates from caveolin-1, thereby increasing eNOS activity and NO production [77, 183]. Endothelial NO-synthase is also regulated by phosporylation mediated by the Ser/Thr kinase Akt [84]. Phosphorylation was found to both increase and decrease eNOS activity; that is phosphorylation of serine (Ser^{1179}) increases eNOS activity and sensitivity to Ca^{2+}/CaM, whereas phosphorylation at threonine (Thr^{497}) negatively regulates eNOS activity [77, 84]. Thus caveolin-1 is a major regulator of eNOS activity and thereby of vascular tone and permeability.

The role of caveolin-1 as a mediator of eNOS activity was demonstrated in mouse models [33]. Bucci and coworkers observed eNOS colocalisation and coimmunoprecipitation with caveolin-1 and its enrichment in caveolae. Overexperssion of caveolin-1 or delivery of the antennapedia-conjucated caveolin-1 scaffolding domain peptide (AP-cav) decreased eNOS-dependent NO release [85, 132], indicating that binding of eNOS to the caveolin-1 scaffolding domain serves to inhibite the enzyme. To further characterise this phenome-

non, the scaffolding domain peptide (AP-cav) was tested in a mouse inflammation model induced carageenan [33]. Delivery of the caveolin-1 scaffolding domain reduced the vascular permeability response and tissue edema formation by sequestering eNOS [33] indicating that the eNOS activation and NO production are important determinants of the inflammation response. The blockade of the vascular permeability response was similar to that observed in eNOS knockout mice [76]. Fukumura *et al* observed in eNOS and iNOS-deficient mice that eNOS plays a predominat role in vascular endothelial growth factor (VEGF)-induced angiogenesis and vascular hyperpermeability [76]. In addition, VEGF is also known to increase vascular permeability of microvessels to cirulating macromolecules [38, 42, 296] and it indues vasculo- and angiogenesis *in vivo* [68].

Zhu *et al.* demonstrated increased eNOS activity might be a reaction to inflammatory stimuli (PAF) [320]. This study also demonstrated that the eNOS-derived NO is one signalling pathway for inflammatory mediators increases in mesenteric artery permeability. Furthermore, Zhu and his coworkers showed that the scaffolding domain of caveolin-1 plays an important role in PAF-increased eNOS activity, because application of AP-cav had a significant inhibitory effect on the permeability increase during acute inflammation [320].

In line with this observation it was shown that loss of caveolin-1 markedly increases the vascular permeability [251]. Importantly, this permeability is reversed by L-NAME (a important NOS-inhibitor) injection, thus demonstrating the necessity of caveolin-1 for inhibition of eNOS activity.

1.7.5.3 Ion channel regulation

Several ion channels and receptors, which regulate Ca^{2+} concentration, have been shown to be associated with caveolae (e.g. Ca^{2+} channels; store operated channel (SOC) [47], transient receptor potential channel (e.g. TRPC-1 and 4) [3, 37] and inositol 1,4,5-trisphosphate receptor (IP_3R) [75, 122]). These channels and receptors play an important role in the regulation of endothelial barrier dysfunction. They are involved in the regulation of endothelial cell permeability and transcytosis [196]. Preliminary studies have suggested an important role for intact caveolae in the regulation of proper signaling after the activation of channels, such as the volume-regulated anion channel [283]

It is also important to note that several calcium channels are localized to caveolae and, as a consequence, they might play an important role in the regulation of eNOS activation [123]. In fact, recent work by Isshiki *et al.* has shown that Ca^{2+} waves originate in caveolin-rich regions of endothelial cells and can move with caveolae to the trailing edge of migrating endothelial cells [122, 123] .

1.7.6 Models to study edema formation

To study the molecular mechansims by which pro-inflammatory mediators trigger pulmonary edema formation several model systems have been used.

In vitro experiments, using endothelial cell (EC) monolayer are considered useful system to study mechansims of edema formation. The advantages are (i) easy of use, because parameters, such as composition of culture medium and other factors can be easily changed and (ii) the enhanced permeability of EC-monolayers after stimulation with different substances such as thrombin [82, 87], thromboxane [277], hydroperoxide (H_2O_2) [272] or TNF [233] are well established. However, in intact organs most of these mediators do not contribute to permeability edema [51, 308], thus questioning the cell culture model. Another critical point to studying permeability changes in EC-monolayers is that some substances known to induce edema *in vivo* or in isolated perfused lung such as PAF have no effect on permeability in EC-monolayer experiments [283, 58].

One physiologically relevant model that can be used to study edema formation is the isolated perfused rat lung (IPL) [294, 295]. It is a distinct advantage that studies are performed in an intact organ, with physiological cell-to-cell contacts and native intracellular matrixes. Another advantage is that physiological parameters such as airway and pulmonary vascular resistances, edema formation, gas exchange, tidal volume, pulmonary compliance, can be determined by continuous monitoring [288]. Also, interactions with other organs can be excluded, allowing to investigate the responses solely generated by the lungs. In perfusion experiments the investigator may retain control over several experimental parameters such as perfusion pressure and flow or composition of the perfusate. Thus, depending on the perfusions protocol used it is possible to distinguish hydrostatic from permeability edema. In addition, the IPL preperation also offers the opportunity of investigating administration of multiple agents by different routes and in physical forms. The principal limitation

imposed by the IPL is the short duration of study, since lung mechanics deteriorate with time.

To investigate the effect of PAF-induced edema formation in pulmonary endothelial cells, we established a method to purify these cells from the intact organ. In previous studies, an enzymatic mixture (including collagenase, deoxyribonuclease, chymopapain, pronase, and elastase) was used for the purification of cells from intact lungs. For example, Bundschuh *et al.* [34] isolated primary lung cells by recirculating perfusion through the pulmonary artery of intact lungs with enzymatic solution. After perfusion lung tissue was microdissected, resuspended and primary lung cells were cultured for 5 days [34]. Ugele *et al.* also used a proteolytic enzyme mixture and a typical endothelial cell marker (Willebrand factor (vWf)) to purify EC from intact organ [285]. The disadvantages of both methods are (i) the small yield, (ii) the fact that cells must be cultivated before investigations can starts and (iii) that EC are difficult to separate from the many other cell types of the lungs.

To overcome these problems, we used the well established isolated perfused rat lung (as described before) [294, 295] and purified EC from intact lungs by perfusion through the pulmonary artery with cationised colloidal silica beads solution [125]. The positive charged beads bind on the negative surface of the endothelial cell membranes. Therefore it is possible to isolate only EC from the micovasculature of the rat lungs [126]. To purify a high yield of EC, the isolated lungs are perfused after bead solution with natriumpolyacrylate, to generate a strongly adhering coat to the pulmonary EC [124]. Endothelial cells can be used directly, after seperation from the other cell types of the lung, and do need to be cultivated before investigation. Because the EC purification is performed in IPL, the (patho)physiological changes of EC can be directly investigated.

2 Aim of the study

The acute respiratory distress syndrome (ARDS) is characterised by permeability edema in the lungs. One of the most important endogenous mediators of pulmonary edema is the platelet-activating factor (PAF). PAF increase vascular permeability partly through acid sphingomyelinase (ASM) dependent generation of ceramide in a poorly understood manner.

Since membrane microdomains (caveolae) are particularly rich in sphingolipids, it was the aim of the present study to investigate whether PAF acts in the realm of caveolae and which role caveolin-1, the scaffolding protein of caveolae, plays in PAF induced edema formation. Therefore, it was one goal of this thesis to establish a method for purification of caveolae from intact endothelial cells of isolated perfused rat lung (IPL) and characterise these caveolae in PAF-treated lungs.

In the last years it has been recognized, that caveolae are involved in the transcellular transport of albumin (transcytosis). Since transcytosis may increase oncotic pressure and thus increase edema formation, it was the other major aim to investigate the regulation of transcytosis by proinflammatory mediators and the role of ASM-ceramide pathway in this process. For these studies we used thrombin, a mediator that activates ceramide generation by ASM and alters vascular permeability in endothelial cells (EC) monolayers.

3 Material and Methods

3.1 Material

3.1.1 Animals

Female Wistar rats (weight 220 to 250 g) were obtained from Charles River Laboratories, Inc. (Sulzfeld, Germany) and kept under controlled conditions (22°C, 55% humidity, 12 h day/night rhythm) on a standard laboratory chow and water *ad libitum* were used as lung doners for experiments with isolated perfused rat lungs. Pentobarbital sodium (Nacoren®, 400 µl/kg) was purchased from the Wirtschaftsgenossenschaft Deutscher Tierärzte (Garbsen, Germany).

3.1.2 Cells and cell culture reagents

Human Microvascular Endothelial Cells-Lung (HMVEC-L) were obtained from Cambrex Bioscience (Verviers, Belgium). HMVEC-L cells are primary endothelial cells, prepared from human lung tissue by Clontics (San Diego, USA). Cells were grown in Endothelial Cell Basal Medium-2 (EGM-2-MV) complete growth medium with cell specific reagents from Cambrex Bioscience (Verviers, Belgium). Calf Pulmonary Artery Endothelial Cells (CPAE-cells) were obtained from the American Type Culture Collection (Manassas , USA) CCL-209 and CRL-1730. Cells were grown in Minimal Essential Medium (MEM) without L-glutamine. Medium were added with Earle`salts; 1% 1.0 mM sodium pyruvat and 1% 0.1 mM non-essential amino acids and supplemented with 20% foetal calf serum. All substances were obtained from PAA Laboratories (Cölbe, Germany)

3.1.3 Antibodies

All primary antibodies used in the experiments are listed in the table 3.1 below. Secondary antibodies, Alexa-fluorochrome labelled were obtained from Moleculare Probes (Eugene, USA). It was used in a dilution of 1:10.000.

Table 3.1: ***Overview of antibodies*** *that were used in immunoblot analysis*

Primary Antibody	Dilution used	Secondary Antibody	Supplier
Rabbit-anti-rat-acid-sphingomyelinase (ASM)	1:500	Alexa-Fluor 700 nm Goat-anti-rabbit	Santa Cruz Biotechnology, Inc. (Piscataway, USA)
Anti-ASM	1:250	Alexa-Fluor 800 nm Donkey-anti-goat 800 nm	Gift from K. Sandhoff (Bonn, Germany)
Goat-anti-rat-ACE	1:500	Alexa-Fluor 800nm Donkey-anti-goat	Santa Cruz Biotechnology, Inc. (Heidelberg, Europe)
Rabbit-anti-rat-cP450 reductase	1:500	Alexa-Fluor 700 nm Goat-anti-rabbit	Santa Cruz Biotechnology, Inc. (Heidelberg, Europe)
Mouse-anti-rat-Caveolin-1	1:2000	Alexa-Fluor 680 nm Goat-anti-mouse	BD Transduction Laboratories (Heidelberg, Europe)
Rabbit-anti-rat-Ganglioside GM1	1:500	Alexa-Fluor 700 nm Goat-anti-rabbit	Calbiochem (Bad Soden, Europe)
Mouse-anti-rat-Flotillin-1	1:250	Alexa-Fluor 680 nm Goat-anti-mouse	BD Transduction Laboratories (Heidelberg, Europe)
Goat-anti-rat-LAMP-2	1:500	Alexa-Fluor 800 nm Donkey-anti-goat	Santa Cruz Biotechnology, Inc. (Heidelberg, Europe)
Rabbit-anti-rat-eNOS	1:1000	Alexa-Fluor 700 nm Goat-anti-rabbit	BD Transduction Laboratories (Heidelberg, Europe)
Mouse-anti-rat-peNOS	1:1000	Alexa-Fluor 680 nm Goat-anti-mouse	Serva GmbH (Heidelberg, Europe)
Mouse-anti-rat-Phospho-Caveolin-1	1:2000	Alexa-Fluor 680 nm Goat-anti-mouse	BD Transduction Laboratories (Heidelberg, Europe)
Mouse-anti-rat-IGF-IIR	1:500	Alexa-Fluor 680 nm Goat-anti-mouse	BD Transduction Laboratories (Heidelberg, Europe)
Rabbit-anti-rat-Phospho-p38	1:1000	Alexa-Fluor 700 nm Goat-anti-rabbit	Cell Signaling Technology (Boston, USA)
Rabbit-anti-rat-Phospho-ERK 1/2	1:1000	Alexa-Fluor 700 nm Goat-anti-rabbit	Cell Signaling Technology (Boston, USA)
Rabbit-anti-rat-TRPC-1	1:250	Alexa-Fluor 700 nm Goat-anti-rabbit	Alomone (Jerusalem, Israel)
Rabbit-anti-rat-TRPC-4	1:250	Alexa-Fluor 700 nm Goat-anti-rabbit	Alomone (Jerusalem, Israel)

3.1.4 Silica beads

Silica-beads, with a diameter of 0.5 µm, were obtained from Bangs Laboratories, Inc. (Fishers, USA). Aluminium chlorohydroxide for coating of silica beads was obtained from PFATZ & BAUER Inc. (Waterbury, USA) and all other used substances (see 3.2.1.) were obtained from Sigma (Taufkirchen, Germany)

3.1.5 Inhibitors and substances

All pharmacological agents used are listed in the following table.

Table 3.2 ***Overview of used inhibitors, substances and suppliers***

Substances	**solution in**	**Concentration**	**Supplier**
α-thrombin (human)	*glycerol/water 1:1*	*50 U/ml*	*MP-Biomedicals (Eschwege, Europe)*
Acid sphingomyelinase (ASM)	*PBS*	*1 U/ml*	*Sigma (Taufkirchen, Europe)*
Platelet-activating factor (PAF,1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine)	*ethanol*	*0.05 µM*	*Sigma (Taufkirchen, Europe)*
Inhibitor	**Solution in**	**Concentration**	**Supplier**
1400W	*ethanol/water 1:1*	*0,66 mg/250 g rat*	*Axxora GmbH (Grünberg, Europe)*
Acetyl salicyl acid (ASA)	*PBS*	*500 µM*	*Grünthal (Aachen, Europe)*
Actinomycin D (ActD)	*DMSO*	*2 µg/ml*	*Sigma (Taufkirchen, Europe)*
Cyclohexemide (CHX)	*ethanol*	*1 µg/ml*	*Sigma (Taufkirchen, Europe)*
D609 (Tricyclodecan-9-yl xanthate potassium Salt)	*water*	*300 µM*	*Sigma (Taufkirchen, Europe)*
Imipramine hydrochloride (Imi)	*water*	*10 µM*	*ICN-Biomedicals (Ohio, USA)*
L-NMMA (L-N^G-monomethyl Arginine citrate)	*water*	*100 µM*	*Cayman chemicals (Michigan, USA)*

Inhibitor	*Solution in*	*Concentration*	*Supplier*
L-NAME (L-N^G-Nitroarginine methylester (hydrochloride))	*water*	*100 µM*	*Cayman chemicals (Michigan, USA)*
Ly 294002	*DMSO*	*50 µM*	*Alexis Biomedicals (San Diego, USA)*
Mannose-6-phophate Sodium salt	*PBS*	*1000 µM*	*Sigma (Taufkirchen, Europe)*
NPS 2390	*buffer*	*10 µM*	*Sigma (Taufkirchen, Europe)*
SB 203580	*DMSO*	*20 µM*	*Calbiochem (Darmstadt, Europe)*
SKF-96365, hydrochloride	*water*	*30 µM*	*Calbiochem (Darmstadt, Europe)*
U 0126	*DMSO*	*20 µM*	*Cell Signaling Technology (Boston, USA)*
Y-27632	*water*	*10 µM*	*Tocris Cookson Inc.(St. Louis, USA)*

3.1.6 Buffers and chemical solutions

- Destilled water
- Dimethylsulfoxid; DMSO (Merck, Darmstadt, Germany)
- Ethanol (96%)
- Fluorescein isothiocyante (FITC)-labelled albumin (1 mg/ml, water) (Sigma,Taufkirchen, Germany)
- TBS; Tris-Buffered Saline buffer contains 10 mM Tris, 150 mM NaCl (pH 7.6 (Sigma, Taufkirchen, Germany)
- TBS-T, Tris-Buffered Saline buffer contains 10 mM Tris, 150 mM NaCl and 0.1% Tween 20 (Sigma, Taufkirchen, Germany)
- PBS; Phosphate-Buffered saline solution contains 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 1.42 g Na_2HPO_4 x H_2O), (pH 7.2) (Sigma, Taufkirchen, Germany)

- MBS; MES-Buffered Saline solution contains 125 nM NaCl and 20 mM MES (pH 6) (Sigma, Taufkirchen, Germany)
- HBS; HEPES-Buffered Sucrose solution contains 0.25 mM Sucrose, 25 mM HEPES (Sigma, Taufkirchen, Germany) and 2 mM PefaBloc as a protease inhibitor (Roche, Nutley, USA), (pH 7.2)
- Electrophoresis Buffer (Tris-Glycine SDS Buffer, TGS) (Biorad, Munich, Germany)
- Immunoblotting Buffer (Tris-Glycine Buffer, TG + 20% methanol) (Biorad, Munich, Germany)

3.1.7 Plastics material

Cell culture flasks were obtained from Sarstedt (Nürmbrect, Germany). Costar Transwell membrane inserts (12 mm diameter, 0.4 µm pore size, polycarbonate) from Corning Incorporated (Corning, USA). Beckman SW55Ti or SW28 rotor tubes were obtained from Beckmann Instruments, Inc. (Palo Alto, USA).

3.1.8 Equipment

- Isolated perfused rat lung from Hugo Sachs Electronics (March-Hugstetten, Germany)
- Centrifuge Biofuge from Heraeus (Hanau, Germany)
- Centrifuge Megafuge 1.0 R from Heraeus (Hanau, Germany)
- Beckmann L5-77 Ultracentrifuge and SW55Ti rotor and SW28 rotor (Palo Alto, USA)
- Cell culture incubator from Heraeus (Hanau, Germany)
- Beckmann TL-100 Ultracentrifuge (Palo Alto, USA)
- Polytron PT 1200 from Kinematica AG (Littau Luzern, Switzerland)
- Hoefer Electrophoresis system SE660 (Germany) and SemiPhor blotting system from Pharmacia Biotech (Uppsala, Sweden)
- Odyssey® Infrared Imaging system and software from LI-COR Biosciences (Bad

Homburg, Germany)

- Fluorescence Reader FL600 from Bio-Tek Instruments (Winooski, USA)
- Microplate Reader Titertek Multiscan Plus MK II (Pisley, UK).
- Phase contrast microscopes from Leica Mikrosysteme (Bensheim, Germany)

3.2 Methods

3.2.1 Isolated perfused rat lung (IPL)

To study the role of membrane microdomains (caveolae) by PAF induced edema formation in lung we used isolated perfused rat lung (IPL) for purification of membrane microdomains from intact endothelial cells under different conditions.

3.2.1.1 Preparation of isolated, ventilated and perfused rat lung (IPL)

The rat lungs were prepared and perfused essentially as described by Uhlig *et al.* [294, 295]. Lungs were perfused through the pulmonary artery with Krebs-Henseleit buffer (37°C) as a perfusate medium, which contained 1% rat serum, 2% bovine serum albumin (Serva, Heidelberg, Germany), 0.1% glucose and 0.3% HEPES at a constant hydrostatic pressure of 12 cm H_2O. Edema formation was assessed by measuring the weight gain of the lungs.

3.2.1.2 Experimental design of the perfused lung studies

After preparation and 30 minutes perfusion, 5 nmol PAF (dissolved in ethanol) was injected as a bolus directly into the perfusate. Stock solutions of imipramine (10 µM), D609 (300 µM) and several inhibitors (concentration in table 3.2.) were added into the buffer reservoir 10 minutes before PAF was added.

3.2.1.3 **In situ** *perfusion of rat lung with cationic colloidal silica beads*

Silica perfusion was accomplished by a method, described by Schnitzer *et al.* [246]. The method was slightly modified. After a 30 minutes perfusion, 5 nmol PAF was injected as a bolus directly into the perfusate. Then the flow rat was reduced from 20 ml/min to 2-3 ml/min and the lungs were perfused with the following solutions: MBS buffer for 90 sec at

room temperature (RT) and 1 % colloidal silica beads in MBS buffer (10°C); MBS buffer for 90 sec (10°C), to clear free silica beads from lung; 1% sodium polyacrylate in MBS buffer for 90 sec (10°C), to crosslink and shield membrane-bound silica; and finally with 8-10 ml HBS buffer (10°C).

3.2.1.4 Purification of endothelial cell membrane

After silica perfusion lungs were removed and immersed in cold HBS. The lungs were minced with a cutter to small pieces on ice and then transfered to 10 ml HEPES buffer. Minced lungs were homogenized on ice using a Polytron PT 1200 and a Teflon pestle-glass homogenizer (15 strokes) with a high speed motor running at 425 x g. After filtration through a 0.65 µm and 0.45 µm Nytex net (GE Osmononics Inc, Minnetoka, USA) the homogenate was mixed with an equal volume of 1.02 g/ml Nycodenz (Axis-Shield PoC AS, Oslo, Norway) containing 20 mM KCl and then layered over 0.5-0.7 g/ml Nycodenz containing 60 mM sucrose in a centrifuge tube. After centrifugation (5.700 x g, 30 minutes, 4°C in a Beckman SW 28 rotor) the floating tissue debris were removed and the pellet, which contained the silica-coated endothelial membranes fragments was resuspended with 1 ml MBS buffer. Subsequently, the suspension was homogenized by Polytron PT 1200.

3.2.1.5 Isolation of caveolae from silica-coated endothelial cell membrane

The caveolae from purified pulmonary endothelial cell membrane were isolated as described by Melkonian *et al.* [181], and Arni *et al* [10]. 10% cold Triton X-100 (final concentration of 1 %, Boehringer-Mannheim GmbH, Germany) were added to the membranes for 60 minutes at 4°C. After incubation the suspension was homogenized and the homogenate mixed with 80% sucrose to a achieve a 40% membrane-sucrose-solutions. A 30-5% sucrose gradient was layered over the samples in a Beckman SW55Ti rotor tube and centrifuged at 4°C overnight at 14.000 x g for 16-18 h. Fractions of 150 µl were removed from the top to the bottom and collected as five membrane fractions (A-E); the pellet was dissolved in 150 µl MBS (pellet-fraction, P). Figure 5.1 shows an experimental design of the isolation of caveolae from intact pulmonary endothelial cells by the *in situ* perfusion of isolated perfused rat lung with cationic silica bead solution as described in chapter before.

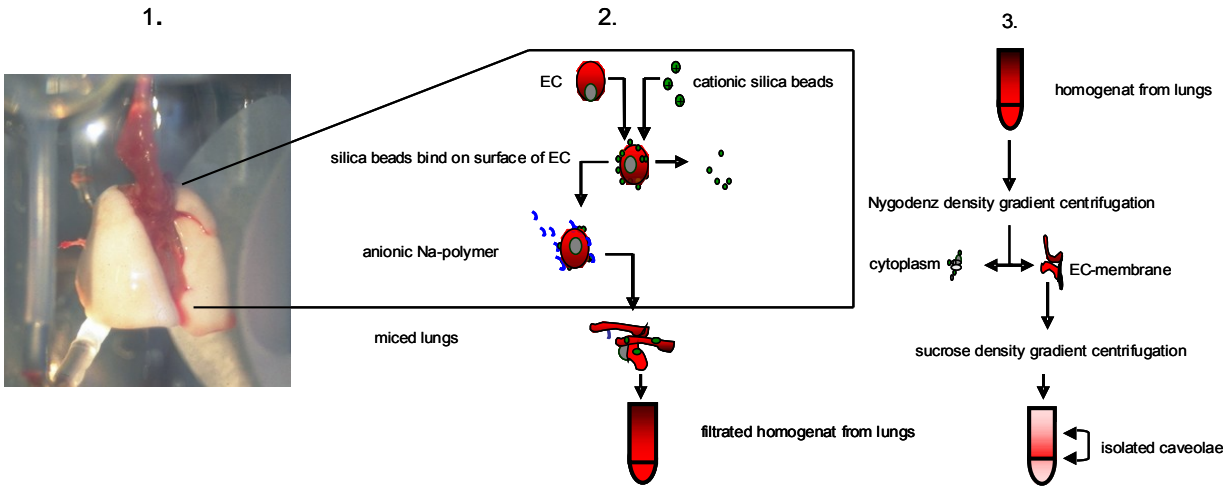


Figure 3.1: Experimental design of the isolation of caveolae from intact pulmonary endothelial cell. **In situ** *perfusion of isolated perfused rat lung (1) with cationic colloidal silica beads (2), purification of endothelial cells and isolation of caveolae from silica bead-coated endothelial cell membrane (3). (black border marked the* **in situ** *perfusion, EC (endothelial cells)*

3.2.1.6 Determination of protein concentration by bicinchoninic acid protein assay (BCA assay)

The protein concentrations of all membrane fraction samples were determined with a BCA assay (Pierce, Rockford, USA). This BCA assay is a biochemical assay similar to the Lowry or Bradford protein assay. A volume of 25 µl from each sample was mixed with 200 µl BCA working solution. The working solution contain BCA-reagent A (1 mg sodium bicinchoninate (BCA), 2 mg sodium carbonate, 0.16 mg sodium tartrate, 0.4 mg NaOH, and 0.95 mg sodium bicarbonate) and 2 volumes BCA-reagent B (0.4 mg cupric sulfate (5 x hydrated) in 10 ml distilled water). Samples were incubated 30 minutes at 37°C. The amount of protein presented in the solution was quantified by measuring the optical intensity at 562 nm and comparing with protein solutions with known concentrations.

3.2.1.7 Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The proteins from membrane fractions samples were separated according to their molecular weight under reducing conditions by electrophoresis as described by Laemmli (Laemmli,1970) in 12% discontinuous polyacrylamide-gels in the presence of 0.02% sodium-dodecyl-sulfate (SDS). The composition of stacking and resolving gels are listed in table 3.3

*Table 3.3:**The composition of SDS-PAGE gels***

Solutions	*Resolving gel (12%)*	*Stacking gel (4 %)*
30% : 0.8% w/v acrylamide:bisacrylamide	*7.0 ml*	*0.66 ml*
1.5 M Tris-Cl pH 8.8	*8.0 ml*	-
0.5 M Tris-Cl pH 6.8	-	*1.26 ml*
10% SDS	*0.2 ml*	*0.050 ml*
H_2O	*7.1 ml*	*3.0 ml*
10% ammoniumpersulfate (APS)	*0.1 ml*	*0.025 ml*
TEMED	*0.010 ml*	*0.005 ml*

Equal amounts of protein (5 µg) for each sample were mixed with a reducing 4 x loading buffer (Biorad, Munich, Germany) and boiled for 5 minutes at 95°C. Samples were added to the stacking gel and run by 100 V for 40 minutes. To separated the proteins in the resolving gel the voltage was increased to 200 V (100 min). The SDS-PAGE was performed of RT in 1 x electrophoresis buffer solution. (1 x TRIS-Glycine SDS Buffer, Biorad, Munich, Germany)

3.2.1.8 Immunoblotting

After SDS-PAGE separation, proteins were transferred to nitrocellulose membranes (Schleicher&Schuell, Marienfeld, Germany). The protein transfer on nitrocellulose membranes was performed with the SemiPhor blotting system (Pharmacia Biotech, Uppsala Sweden) at 0.8 mA/cm² for 75 minutes. The blots were washed with 1 x TBS-T buffer (1min) and blocked by Roti® Block (Roth, Karlsruhe, Germany) for 1 h at RT. The nitrocellulose membranes were incubated overnight at 4°C with different primary antibodies. After washing, nitrocellulose membranes were incubated with a secondary infrared fluorochrome labelled antibody for 1 h at RT and protein bands were detected at 680 or 800nm wavelength with the Odyssey® Infrared Imaging system (LI-COR, Bad Homburg, Germany). All specific protein bands were quantified with the Odyssey® imaging software (LI-COR, Bad Homburg, Germany).

The isolation of caveolae fractions was verified by SDS-PAGE and Western blot analysis

with the specific marker protein caveolin-1. All fractions were tested in immunoblotting system of contamination by proteins from extra-caveolar / extra-rafts membrane. The samples were incubated with specific antibodies of several cell compartments. We used anti-P450 cytochrome, as a microsomal marker, anti-angiotensin-converting enzyme (ACE), as a non-caveolar plasma membrane protein marker and anti-LAMP-2, as a lysosomal marker. All specific protein-bands were visualized and quantified as described below.

3.2.1.9 Coating of silica-beads with aluminium hydroxide (chlorohydrol)

The coating of silica particales were accomplished essentially as described of Jacobson *et al*. [124, 125, 126]. Silica beads, with a diameter of 0.5 µm, were coated with aluminium hydroxide (chlorohydrol, PFATZ & BAUER Inc. Rochester, USA) to generate a positively loaded surface. 10.5 g chlorohydrol was mixed with 90 ml distilled water and blended with 1.5 g beads for 2 min in a high speed blender. The mixture was then stirred manually and incubated in a water bath at 80°C for 30 min. Silica beads were chilled down to RT for 16h. After cooling the pH was adjusted to pH 5.0 with 1 M NaOH. Beads were incubated again for 24h and the pH was once more adjusted to pH 5.0 with 1 M NaOH. Silica bead suspensions were stored at 4°C. Before the coated beads were used for perfusion, bead solution was diluted to 10% silica with attachment buffer (140 mM sorbitol, 20 mM MES) and centrifuged for 5 min at 800 x g. The beads cake was washed and resuspended in MBS-buffer and centrifuged for 5 min at 800 x g for three times. The washed beads were then used for purification of caveolae from intact pulmonary endothelial cells.

3.2.2 In vitro *experiments*

3.2.2.1 Cell culture

HMVEC-L (Human Microvascular Endothelial Cells from lung) primary cells were passaged once a week with change of medium every second day at 37°C and 5.0% CO_2. HMVEC-L cells were used for experiments from the 3^{rd}-8^{th} passage after thawing. For endothelial permeability experiments, all cells were seeded on Costar Transwell membranes inserts (12 mm diameter, 0.4 mm pore size, polycarbonate membrane) at a density of 40.000 cells per cm^2. Media were changed every day, cells were maintained two more days before using them for experiments. For experiments, which included the isolation of

membrane microdomains, HMVEC-L cells were grown to confluence (2.5-3.0 x 10^6 cells/tissue flask) in T175 tissue flasks.

3.2.3 Permeability experiments

3.2.3.1 FITC-albumin diffusion

HMVEC-cells were disseminated and grown (72-96 h) on Transwell membranes at a density of 40.000 cells per cm². For measuring permeability, growth media was changed to low serum media containing normal medium supplemented with 2% heat inactivated foetal calf serum (FCS) 1 h before adding any substance. Cell cultures were preincubated for 1 h at 37°C before adding the inhibitors. After pretreatment, cell monolayers were treated for 2 h at 37°C with α-thrombin (50 U/ml) or in simultaneously with ASM (1 U/ml). In the presence of α-thrombin and other inhibitors, 1 mg/ml fluorescein isothiocyanate bovine-albumin (FITC-labelled albumin) was added into the upper chamber of the Transwell. After diffusion times of 45, 120, 180 and 240 min, the FITC-labelled albumin content of the lower chamber was detected by fluorescence measurement (485 nm excitation wavelength and 530 nm emission wavelength). FITC-labelled albumin diffusion rate across an empty membrane was set to 100% permeability. Diffusion rate across an untreated control cell-monolayer was set to 0% permeability. These two values were determined on every experimental day. Duplicates of every data point were obtained on one day and counted as n=1.

3.2.4 Measurement of caveolin-1 levels in HMVEC-L

3.2.4.1 Preparation and treatment of HMVEC-L cells for caveolin-1 measrument

To measure the caveolin-1 content in total cells and cell membrane fractions, cells were grown confluently in T-175 tissue flasks. For measuring caveolin-1 content, growth medium was changed to low serum medium containing normal medium supplemented with 2% heat inactivated foetal calf serum (FCS). After 1h incubation at 37°C cells were treated with imipramine for 20 h (10 µM) and D609 for 30 min (300 µM) before stimulation with human α-thrombin (50 U/ml) for 2 h. Other cells were treated with ASM
(1 U/ml) for 2 h. Subsequently, the cells were harvested, detached and lysed. The caveolin-1 content was measured after isolation of the membrane microdomains as

described in 3.2.4.2. For determination of the total cell content of caveolin-1 cells were prepared as described in 3.2.4.3.

3.2.4.2 Isolation of membrane microdomaines

Membrane microdomains were isolated essentially as described [10, 181]. Cells were harvested and detached with accutase as detachment agent. The cells (2.5-3.0 10^6/tissue flask) were lysed in lysis-buffer (42 mM KCl, 10 mM HEPES, 5 mM MgCl and protease inhibitors, pH 7.4) for 10 min on ice and by sonification (duty cycle: 30-40, output cont.: 6 strokes). After centrifugation (314 x g, 4°C, 5 min) supernatants were ultracentrifuged (12000 x g, 4°C, 30 min) to purificate membranes. An aliqot of 150 µl supernatant was collected as the cytosolic fraction (C) and the rest of the supernatant was removed. The pellet was suspended in 100 µl Triton X-100 and incubated at 4°C for 60 min. The pellet-triton-suspension was mixed with 100 µl 80% and overlayered with 800 µl 30% sucrose and 200 µl 5% sucrose as a sucrose density gradient to isolate the membrane microdomain. Samples were ultracentrifuged overnight (14.000 x g, 4°C, 16-18h) and 8 samples with volumes of 150 µl were collected as membrane fractions samples (1-8). Pellet fraction (P) was suspended in 150 µl PBS. All fractions were mixed with 50 µl reducing 4x loading buffer and boiled at 95°C for 5 min for SDS-PAGE. The caveolin-1 contents in membrane fractions were measured after SDS-PAGE and immunoblotting by quantifying the protein bands (intensities) of caveolin-1 as described before (3.2.1.8).

3.2.4.3 Caveolin-1 content in whole cells

The amount of caveolin-1 in total cells were determined. 2.5-3.0 x 10^6 cells were stimulated and harvested using accutase for dissolution of cells from tissue flasks. Cells were lysed in lysis-buffer for 10 min on ice and by sonification (duty cycle: 30-40, output cont.: 6 strokes). A volume of 30 µl from each sample was added with 10 µl of 4x loading buffer and incubated at 95°C for 5 min for SDS-PAGE. The total content of caveolin-1 in whole cells was determined after SDS-PAGE and immunoblotting as described in 3.2.1.8.

3.2.4.4 Caveolin-1 transcription and translation-inhibition

The HMVEC-L cells were grown to confluence and treated either with a transcription- or translation-inbititor of caveolin-1. Actinomycin D (ActD, 2 µg/ml) was added as transcripti-on-inhibitor 20h before cells were treated with α-thrombin (50 U/ml) for 3h. Cyclohexemide

(CHX, 1 µg/ml) was added as inhibitor of caveolin-1 synthesis to α-thrombin for 3h. Cells (2.5-3.0 x 10^6 /tissue flask) were harvested, detached and lysed with lysis-buffer and membrane microdomains were isolated as described (3.2.4.2). The content of caveolin-1 measured after isolation of caveolae from endothelial cell membrane and the amount in single fractions and total cells of caveolin-1 were dispositived by caveolin-1 immunoblotting after SDS-PAGE as described before (3.2.1.8).

3.2.4.5 Ceramide content

Lipids were extracted from cell pellets modified to Bligh and Dyer [24], ceramide content was measured by charring densitimetry [234]. In brief, treated and control cell pellets were solved in methanol water emulsion and sonified to extract lipids from the membrane. The lipids were seperated from other membrane components by chloroform/methanol extraction and dried. Subsequently lipids were solved in chloroform and brought onto a high-performance thin layer chromatography (HPTLC) plate (silica gel 60 precoated plate; Merck, Darmstadt, Germany). Ceramide was resolved by running with dichlormethane/methanol/acetate (100:2:5). Thin-layer chromathography (TLC) plates (Merck, Germany) were dried at 180°C, cooled and put into 10% cupric sulfate, 8% phosphoric acid solution. After heating for 2 min at 110°C, lipids bands was made visible with the Fujix-1000 Bioimager (Raytest, Straubenhardt, Germany)

3.2.4.6 Acid sphingomyelinase activity

Acid sphingomyelinase (ASM) activity was determined by modified of the method by micellar *in vitro* assay using ^{14}C-labelled sphingomyelin as a substrate [311]. The volume of the supernantants were reduced from 3 ml to 300 µl using 10 kDa filters, protein content was determined using BCA assay [265]. Cells were suspended in ASM-extraction buffer (250 mM sodiumacetate, 1 mM EDTA, 0,1% Triton X-100, pH 5,0) and homogenized. Samples were centrifuged (20.000 x g, 10 min, 4°C), supernatant was used for the assay, protein content was determined. For all samples, 20 µg protein diluted to 10 µl was incubated at 37°C for 2 h with 40 µl substrate (73 nmol ^{14}C-labelled sphingomyelin + 400 nmol sphingomyelin). Lipids were seperated by chloroform/methanol extraction, 4 ml scintillation liquid was added and radioactivity counted in a β-counter.

3.2.5 Microscopic investigation

3.2.5.1 In situ *fluorescence microscopy*

In situ imaging of endothelial NO production was performed in cooperation with W. Kuebler and his laboratory as described [148].

In brief, lungs were excised and continuously perfused with 14 ml/min autologous blood at 37°C. Lungs were constantly inflated with a gas mixture of 21% O_2, 5% CO_2, balance N_2 at a positive airway pressure (P_{AW}) of 5 cm H_2O. Left atrial pressure (P_{LA}) was set to 3 cm H_2O, yielding pulmonary artery pressure (P_{PA}) of 10±1 cm H_2O. P_{AW}, P_{LA}, and P_{PA} were continuously monitored and recorded (DASYlab 32; Datalog GmbH, Mönchengaldbach, Germany). Lungs were positioned on a custom-built vibration-free microscope stage and superfused with normal saline at 37°C.

For *in situ* imaging of endothelial NO production, membrane-permeant DAF-FM diacetate (5 mol/L), which de-esterifies intracellularly to cell-impermeant, NO-sensitive DAF-FM was infused for 20 min into pulmonary capillaries via a venous microcatheter (SIMS Portex Ltd., Hythe, UK). Intracellular DAF-FM is converted by an NO-dependent, irreversible reaction to an intensely fluorescent benzotriazole derivative with fluorescence intensity linearly reflecting NO concentration. Single venular capillaries were viewed at a focal plane corresponding to maximum diameter (17-28 µm). Endothelial DAF-FM fluorescence was excited at 480 nm by a near monochromatic beam generated by a digitally controlled galvanometric scanner (Polychrome IV; TILL Photonics, Puchheim, Germany) from a 75-watt xenon light source. Fluorescence emission was collected through an upright microscope (Axiotech^{vario} 100HD; Zeiss, Oberkochen, Germany) equipped with an apochromat objective (UAPO 40x W2/340; Olympus, Hamburg, Germany) and dichroic and emission filters (FT 510, LP 520; Zeiss, Oberkochen, Germany) by a CCD camera (Sensicam; PCO, Kehlheim, Germany) and subjected to digital image analysis (TILLvisION 4.0; TILL Photonics, Graefelfing, Germany). Exposure time for each single image was limited to 5 milliseconds. Fluorescence images obtained in 10 sec intervals were background-corrected and fluorescence intensity (F) was expressed relative to its individual baseline (F_0). Since the conversion of DAF-FM to the benzotriazole derivative is irreversible, NO production is reflected by changes of the ratio F/F_0 ($\Delta F/F_0$) over time and was determined in 5 min intervals. At the end of experiments, the exogenous NO donor

SNAP (1 mmol/L) was added to test whether endothelial cells still contained unconverted DAF-FM.

3.2.5.2 Immunhistochemical analysis of caveolin-1 membrane distribution

Cells grown on gelatinized glass cover slips to confluence and stimulated with α-thrombin on the next day, untreated control layers were prepared, too. Glasses were washed and cells fixed with aceton/methanol 1:1 at -20°C. After washing with PBS, cells were incubated with anti-caveolin-1 antibody for 45 min at 37°C in a humid chamber. Washing and incubation was repeated with an Alexa fluorescence labelled secondary anti-goat andibody. All cell layers were embedded in Dabco and visualized with the fluorescence microscope to compare fluorescence intensity of treated and untreated cells.

3.2.5.3 Electron microscopical analysis of caveolin-1 in cells

HMVEC-cells were seeded and grown (24-48 h) on Thermanox coverslips™ (Nunc GmbH, Wiesbaden, Germany) at a density of 50.000 cells per cm². For experiments with α-thrombin, growth media was changed to low serum media containing normal medium supplemented with 2% heat inactivated foetal calf serum (FCS). Subsequently, cells were incubated for 1 h at 37°C. After incubation cell cultures were treated for 2 h at 37°C with α-thrombin. Then Thermanox coverslips were washed in PBS buffer and fixed with 4% formaldehyde solution (contains 0.1% glutaraldehyde in 0.2 M HEPES buffer). The following investigations of caveolin-1 distribution and the albumin-transport by caveolae/caveolin-1 in HMVEC-L cells were performed in cooperation with M.Ochs. The detailed procedure for the electron microscopical images was described previously by H. Fehrenbach and M.Ochs [294]. In brief, the fixed HMVEC-L cells were incubated with caveolin-1 antibody conjugated with 1nm gold particale and with albumin antibody conjugated with 10nm gold particale and observed on a Zeiss EM912 transmission electron microscope and photographed.

3.2.6 Statistics

Unless otherwise stated, all data are presented as mean ± SEM. Data were analysed by unpaired Student`s t-test to test statistical significance. The p-values were corrected for multiple comparisons according to the false discovery rate procedure using the multcomp

module in R 2.1.1 (R Development Core Team, 2005).

4 Results

4.1 Experiments in isolated perfused rat lungs

4.1.1 Role of caveolae

Since sphingolipids in general and ceramide in particular are enriched in caveolae, we started to investigate whether PAF alters the composition of caveolae in pulmonary endothelial cells. Therefore we established a method originally described by Schnitzer *et al.* [246] that permits isolation of caveolae from the endothelial cells of isolated perfused rat lungs. We also investigated the hypothesis that ASM-dependent ceramide production in PAF-induced edema formation is located in the realm of caveolae and that caveolin-1 is involved in signal transduction mediating permeability alterations.

4.1.1.1 Prepration and characterisation of caveolae

Caveolae were isolated from intact endothelial cell of isolated perfused rat lungs (IPL) after perfusion with colloidal silica beads as described in 3.2.1.

Immunoblotting of the resulting fractions showed the typical distribution of caveolin-1 known from caveolae (Fig. 4.1.a) [124, 126, 246]. To increase the amount of material available for subsequent analysis, we pooled the 14 fractions into 6 fractions labeled A-E and P (pellet) (Fig. 4.1.b). Subsequent analysis also showed the presence of flotellin-1, another marker of lipid rafts, in fractions B-E. (Fig. 4.1.c), the presence of flotellin-1 was also demonstrated by mass spectrometry (data not shown). In these fractions we were unable to detect P450 (a microsomal marker), angiotensin-converting enzyme (ACE, a non-caveolar plasma membrane protein) or lysosomal-associated marker protein-2 (LAMP-2, a lysosomal marker) (Tab. 4.1), thus conforming that the membrane fractions prepared by silica coating procedure are not contaminated by proteins from extra-caveolar/extra-rafts membrane.

The presence of other structural proteins, membrane lipids, transmembrane receptors and components of signalling transdruction in caveolae was also tested and the results are shown in table 4.1.

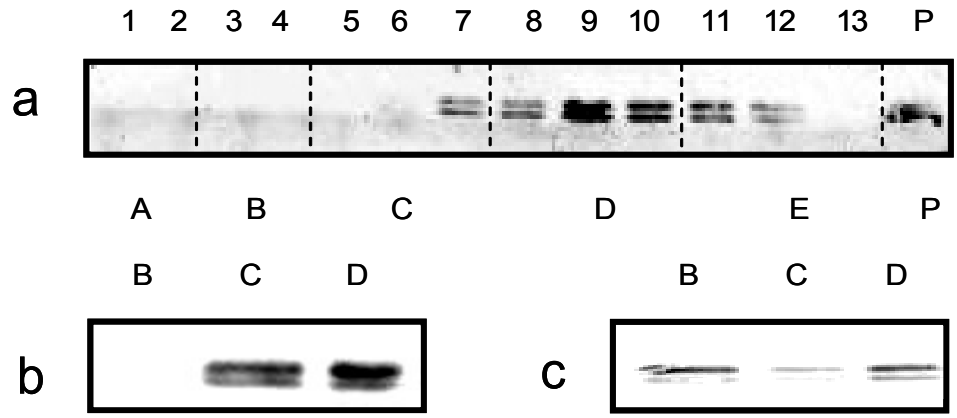


Figure 4.1: Isolated caveolar membrane fractions of intact endothelial cells from untreated rat lungs. Representative immunoblots with anti-caveolin-1 in membrane fractions before (a) and after pooling of samples (b) from 10 independent isolation and immunoblots with antibody against flotellin-1, another marker of caveolae from 5 independent isolations (c).

Table 4.1: ***Summary of all components, which were tested of there presence in caveolae*** *after SDS-PAGE by immunoblots with specific antibodies. All in caveolae detected components were labelled with + and non-detected components were labelled with -.*

Marker-protein	Relevance	Cell compartment	Present in caveolae	Figure
ACE	*angiotensin-converting enzyme*	*non-caveolar plasma membrane*	-	
β-Actin	*structural protein of cytoskelet*	*cytoskeleton*	+	
ASM	*acid sphingomylinase*	*lysosomales, rafts*	+	***4.4***
Caveolin-1	*major protein of caveolae*	*cell membrane, caveolae*	+	***4.1.a***
Cox-1	*cyclooxygenase I,*	*endoplasmatic reticulum*	+	
Cox-2	*cyclooxygenase II,*	*endoplasmatic reticulum*	-	
Cytochrome P450	*microsomal enzyme*	*endoplasmatic reticulum*	-	
$cPLA_2$	*cytoplasmatic phospholipase A2*	*cytoplasma*	-	
eNOS	*endothelial NO-synthase*	*cytosol, caveolae*	+	***4.5***
Flotillin	*integral membrane protein*	*cell membrane, rafts*	+	***4.1.c***
GM1	*membranelipide*	*cell membrane, rafts*	+	
IP3-R	*intrazellular receptor*	*endoplasmatic reticulum*	-	
LAMP-2	*lysosomal-associated membrane protein-2*	*lysosomes*	-	
M6P-R	*mannose 6-phosphate receptor*	*cell membrane,non-rafts*	+	***4.17***
PAF-R	*platelet-activating factor receptor*	*cell membrane,non-rafts*	-	
P-caveolin-1	*phosphocaveolin-1*	*caveolae*	+	
PGE_2-R	*external receptor for prostaglandine E_2*	*cell membrane, non-rafts*	-	

4.1.1.2 PAF increases caveolin-1

Because PAF activates the ASM within minutes [287], caveolae were isolated only 10 min after infusion of 5 nmol PAF into the pulmonary artery. Compared to untreated lungs (Fig. 4.2.a), PAF increased the amount of caveolin-1 in the caveolar fractions B-D of pulmonary endothelial cells; the strongest increase was noted in fraction D (Fig. 4.2 b). The caveolar fractions also contained ASM, but its amount did not change in response to PAF (Fig. 4.3.a, b). However, blocking the ASM pathway with imipramine or D609 prevented the PAF-induced recruitment of caveolin-1 into caveolae (Fig. 4.4.a). In line with our previous study [96], we confirmed that both imipramine and D609 reduce PAF-induced edema (Fig. 4.4.b)

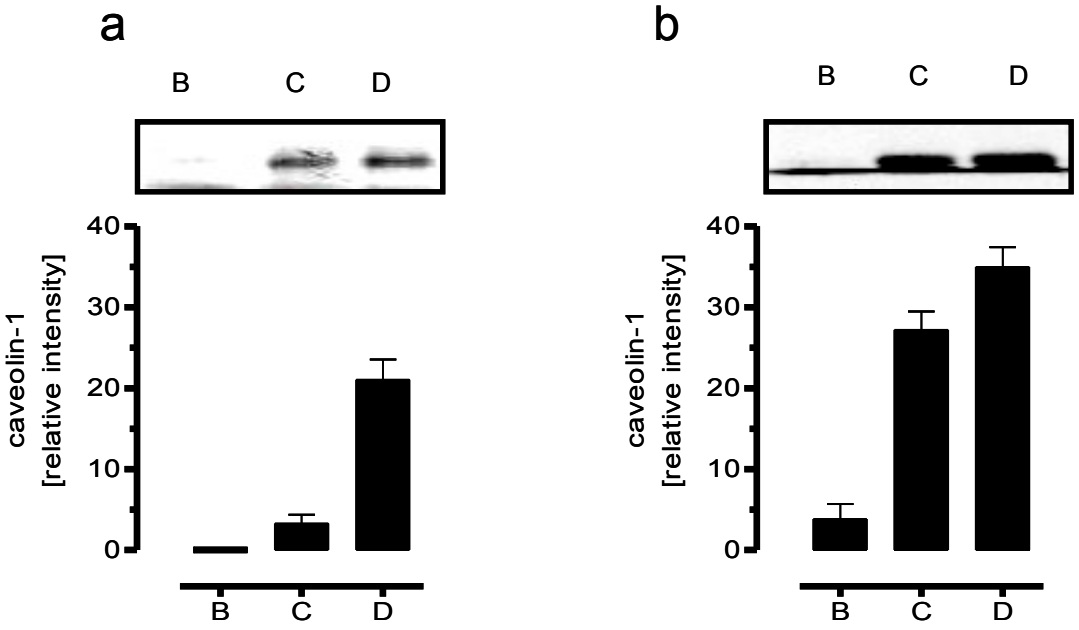


Figure 4.2: Caveolin-1 content in caveolae of pulmonary endothelial cells in control lungs (a) and lungs perfused with PAF for 10 min (b). Caveolae were isolated from endothelial cells of isolated perfused rat lungs and probed by immunoblotting for caveolae. Data are mean ± SEM from 6 independent experiments.

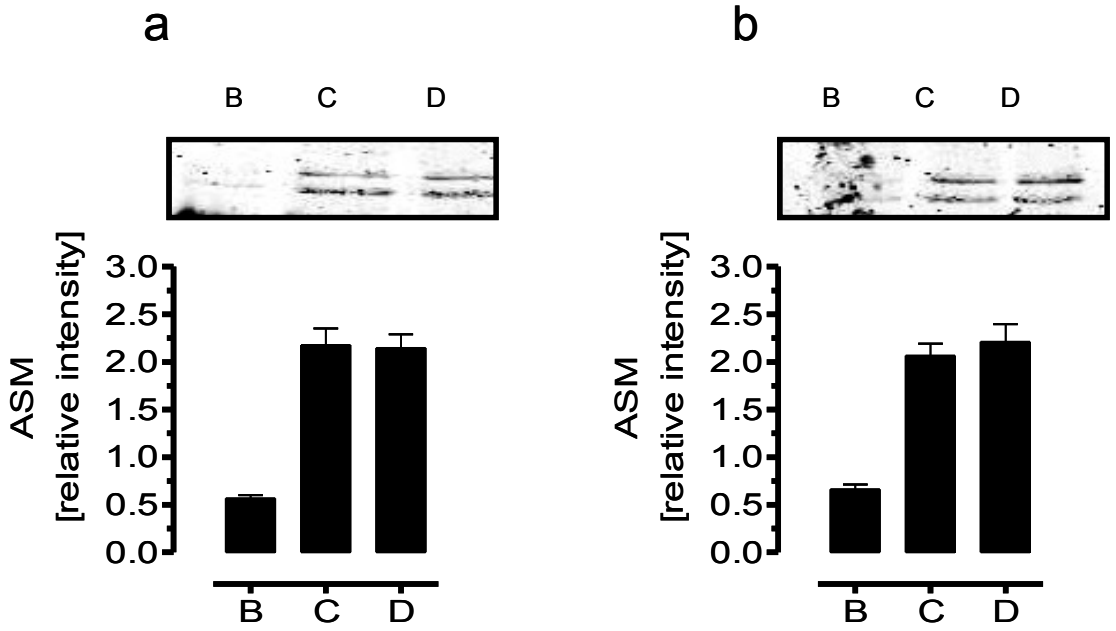


Figure 4.3: ASM content in caveolae of pulmonary endothelial cells in control lungs (a) and lungs perfused with PAF for 10 min (b). Caveolae were isolated from endothelial cells of isolated perfused rat lungs and probed by immunoblotting for ASM. Data are mean ± SEM from 6 independent experiments.

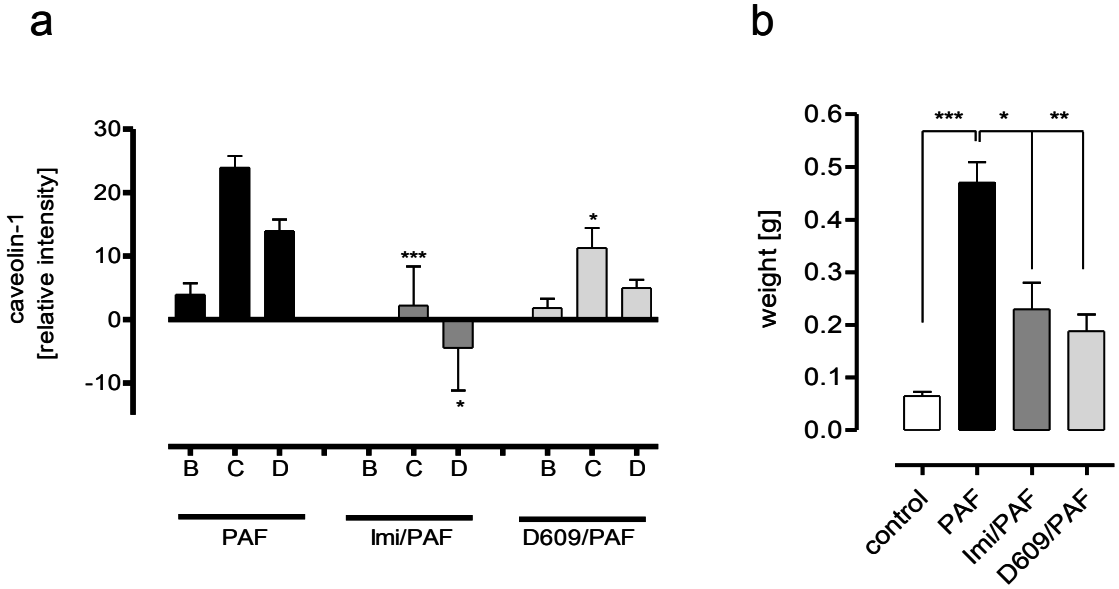


Figure 4.4: Imipramine and D609 reduced caveolin-1 content and prevented PAF-induced edema formation. Lungs were pretreated with imipramine (10µM) or D609 (300µM) 10min before PAF (5nmol) was added as a bolus injection. (a) The content of caveolin-1 in caveolae isolated from pulmonary endothelial cells (b) Edema formation were measured by weight gain of isolated perfused rat lungs; controls, n=22; PAF, n=30; imipramine/PAF, n=4; D609/PAF, n=5. Data represent mean ± SEM. Data were analysed by unpaired Student`s test and corrected by the fdr procedure

4.1.2 Role of endothelial nitric oxide synthase (eNOS)

Because caveolin-1 is known to bind and to downregulate eNOS activity in endothelial cells [33, 84], we investigated the effect of PAF on eNOS and NO formation. Caveolae contained eNOS, and its amount inside the caveolar fractions was not altered in PAF treated lungs (Fig. 4.5).

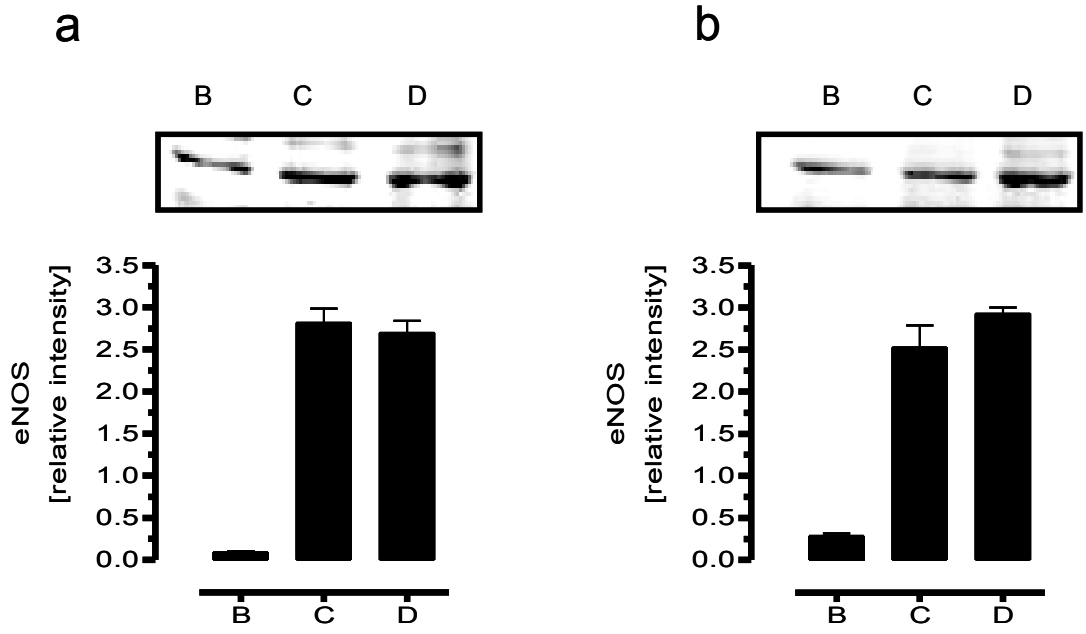


Figure 4.5: Endothelial NO-synthase (eNOS) is present in caveolae of pulmonary endothelial cells in control lungs (a) and PAF perfused lungs (b). Caveolae were isolated from endothelial cells of isolated perfused rat lungs and probed by immunoblotting for eNOS. Data are mean ± SEM from 5 independent experiments.

Imaging of DAF-FM loaded lung capillary endothelial cells *in situ* revealed stable and continuous NO production in control lungs (Fig. 4.6.a) that was completely blocked after addition of the NO-synthase inhibitor L-NAME (data not shown). PAF-treatment markedly reduced basal endothelial NO production for >30 min, while pretreatment with imipramine significantly attenuated this inhibition and thus, reconstituted NO production (Fig. 4.6.a). Imipramine alone had no effect on basal NO production, as demonstrated by similar NO production rates baseline (t = 0 min) (Fig. 4.6.b). The NO production were defined as described in Material and Methods 3.3.5.1. These experiments were performed in cooperation with W. Kübler.

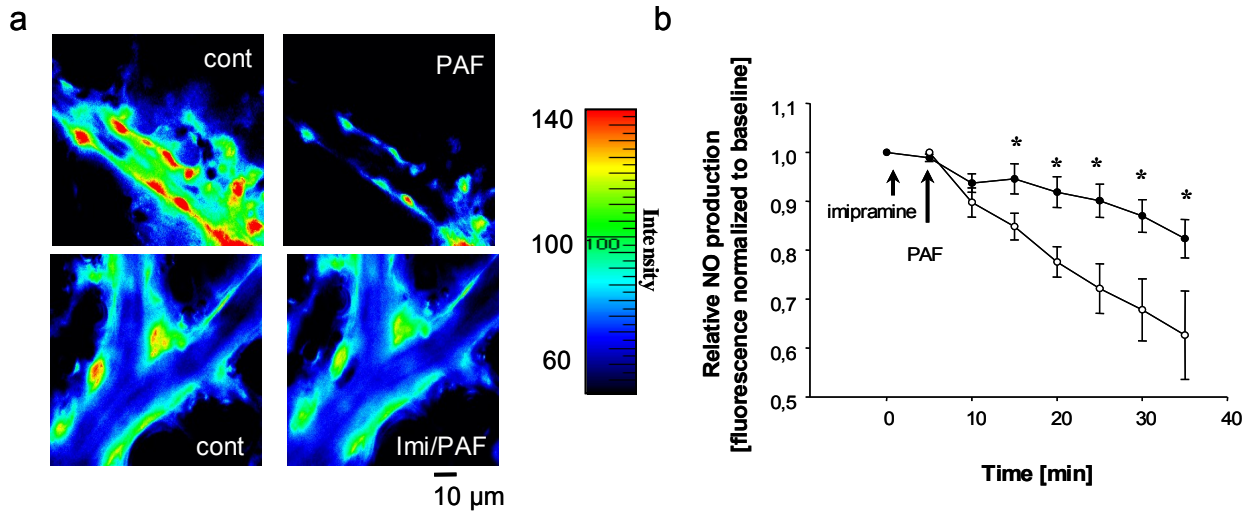


Figure 4.6: Nitric oxide synthesis in endothelial cells **in situ**. *Fluorescence imaging of the NO-sensitive dye DAF-FM in lung endothelial cells* **in situ** *was performed as described before (see ref. 148). Images show NO synthesis before (a, left) and after (a, right) treatment with PAF in the presence (bottom) or absence (top) of imipramine (Imi). Time-dependent of NO production (b) expressed on fluorescence intensity (F) relative to baseline (FO). PAF alone (white circle), imipramine pretreated cells (black circle)*

Next we investigate the effect of several inhibitors of NO-synthases on PAF-induced edema formation in isolated perfused rat lungs. Lungs were pretreated with the following NOS-inhibitors; L-NMMA, L-NAME and 1400W 10 min before PAF was added. Figure 4.7 shows that inhibition of NO-synthases by L-NMMA, L-NAME and 1400W had noeffect on PAF-induced edema.

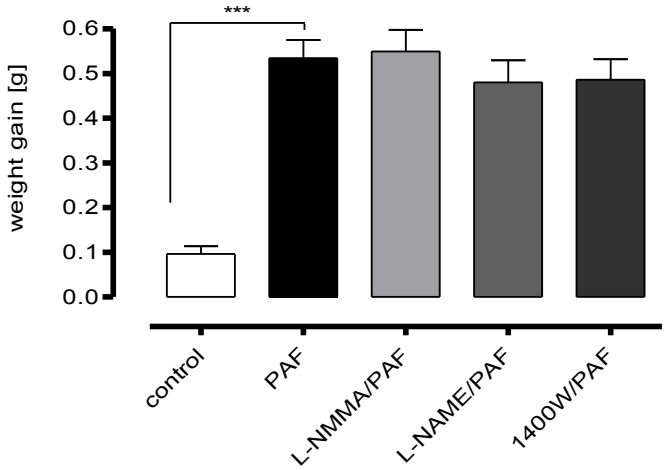


Figure 4.7: Effect of NO-synthases inhibition by L-NMMA, L-NAME or 1400W on PAF-induced edema. L-NMMA (100µM), L-NAME (100µM) and 1400W (100µM) was given 10 min before PAF was added as a blous injection of 5nmol. Edema formation were measured by weight gain of lungs; controls, n=22; PAF, n=30; L-NMMA/PAF, n=6; L-NAME/PAF, n=6; 1400W/PAF, n=9. Data are mean ± SEM and were analysed by unpaired Student`s test and corrected by the fdr procedure

To investigate the involvement of the calcium independent signalling cascade, the phosphatidylinositol-3-OH kinase (PI3K)/Akt-dependent activation of eNOS, we examined the presence of PI3K and Akt in isolated caveolae and blocked PI3K/Akt by Ly294002 [300] perfusion in isolated perfused rat lungs. We identified PI3K (Fig. 4.8.a) and Akt (Fig. 4.8.b) in caveolae and observed that both molecules were attenuated after PAF stimulation (black bar). However in isolated perfused rat lungs pretreatment with Ly294002 had no effect on the PAF-induced weight gain (Fig. 4.9).

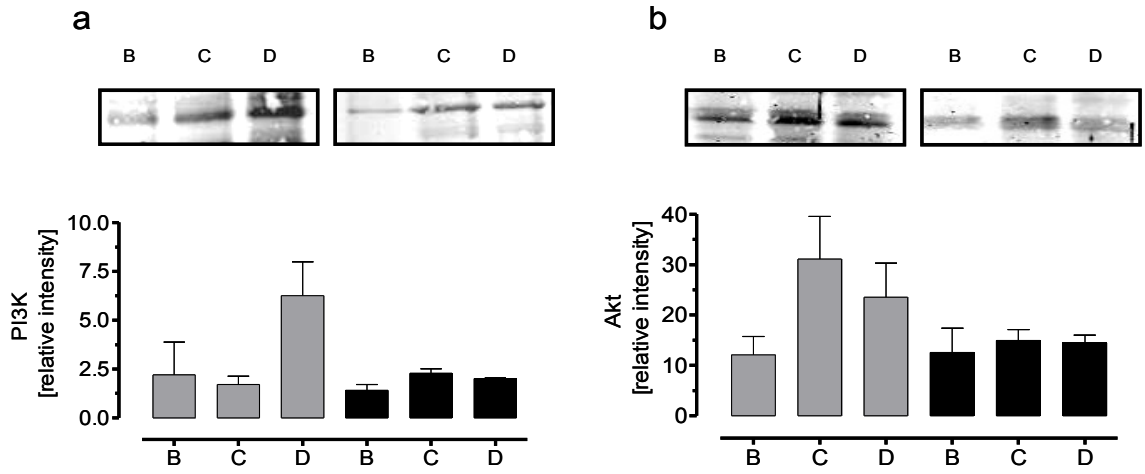


Figure 4.8: PI3-kinase and Akt contents in caveolae of pulmonary endothelial cells in control lungs and PAF perfused lungs. Caveolae were isolated from endothelial cells of isolated perfused rat lungs and probed by immunoblotting for PI3K (a) and Akt (b). Data are mean ± SEM from 5 independent experiments. control (grey bar), PAF treated (black bar)

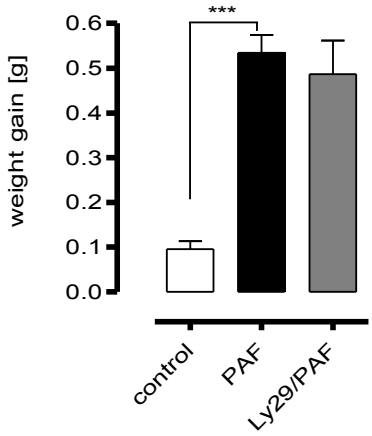


Figure 4.9: Effect of phosphatidylinositol-3-OH kinase inhibition by Ly294002 (Ly29) on PAF-induced edema in rat lungs. Lungs were pretreated with Ly29 (50µM) 10 min before PAF was added as a bolus injection of 5nmol. Weight gain was measured of isolated, perfused rat lungs; control, n=22; PAF, n=30; Ly29/PAF, n=3. Data are mean ± SEM and analysed by unpaired Student`s test and corrected anccording to the fdr procedure.

4.1.3 Role of calcium

4.1.3.1 Intracellular calcium

The important role of calcium for PAF-induced edema formation was described by Göggel *et al.* [95]. He and his co-workers suggested that edema formation is partly dependent on an increase in intracellular calcium concentration and that mechanism is mediated by IP_3R, calcium and MLCK [95]. Since this mechanism is independent of cyclooxygenase inhibition we surmised that this calcium response is evoked by ceramide. We observed that PAF increased intracellular endothelial calcium *in situ* and that this response was prevented by imipramine (Fig 4.10.). These experiments were performed in cooperation with W. Kübler. The intracellular calcium concentration were defined as described by W. Kübler *et al*. [148].

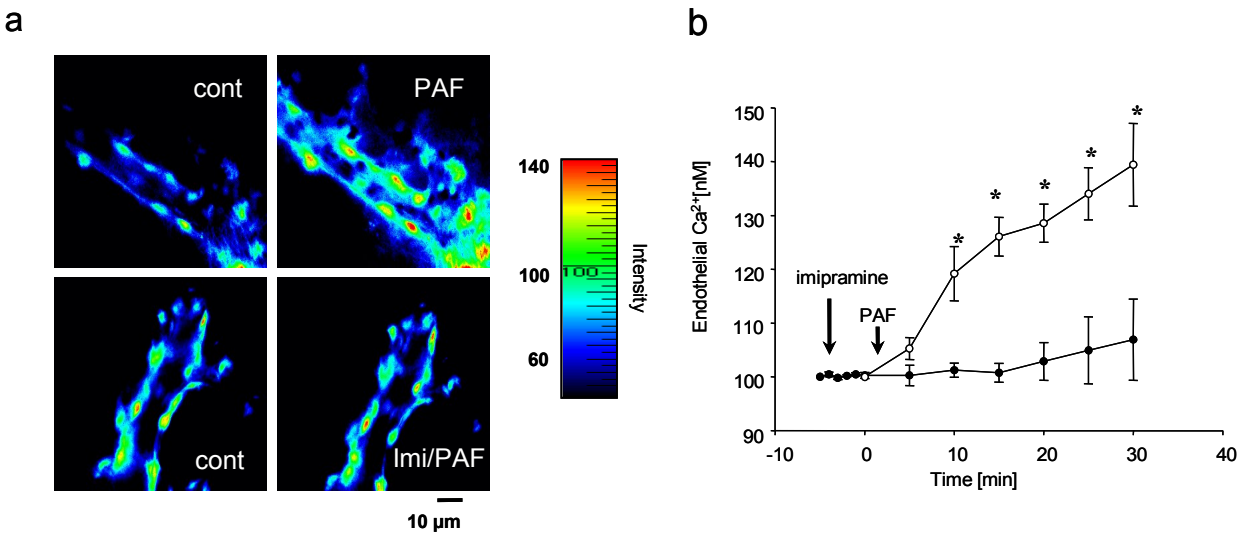


Figure 4.10: Intracellular calcium concentration ($[Ca^{2+}]_i$) in endothelial cells **in situ.** *Fura-2 ratio imaging of endothelial $[Ca^{2+}]_i$ in situ was performed as described before (see ref. 148). (a) Images show endothelial $[Ca^{2+}]_i$ before (left) and after (right) treatment with PAF in the presence (bottom) and absence (top) of imipramine (Imi). (b) Intracellular endothelial calcium concentration was measured by Fura-2 intensity over the time. [PAF alone (white circle, imipramine pretreated cells (black circle)*

4.1.3.2 Extracellular calcium

The rise in calcium is controlled at least in part by both IP_3-dependent Ca^{2+}-release from intracellular stores and influx of extracellular Ca^{2+}; this was also demonstrated for PAF [95]. Recently, TRP channels (some of which may also be regulated by IP_3) have been identified as the long sought calcium channels responsible for the store-operated calcium entry. Also caveolae have been implicated as calcium entry ports and in some instances it was suggested that this might be regulated by TRP channels [3, 47]. In line with this, we identified TRP channel protein-1 (TRPC-1) and TRP channel protein-4 (TRPC-4) inside caveolar fractions by immunoblotting analysis. Figure 4.11 shows representative immunoblots of TRPC-1 and TRPC-4 in caveolae from pulmonary endothelial cells of control and PAF perfused lungs. In fraction B-D protein bands of anti-TRPC-1 and anti-TRPC-4 were detected. However, PAF did not appear to alter the expression of TRPC-1 or TRPC-4 protein in the caveolae.

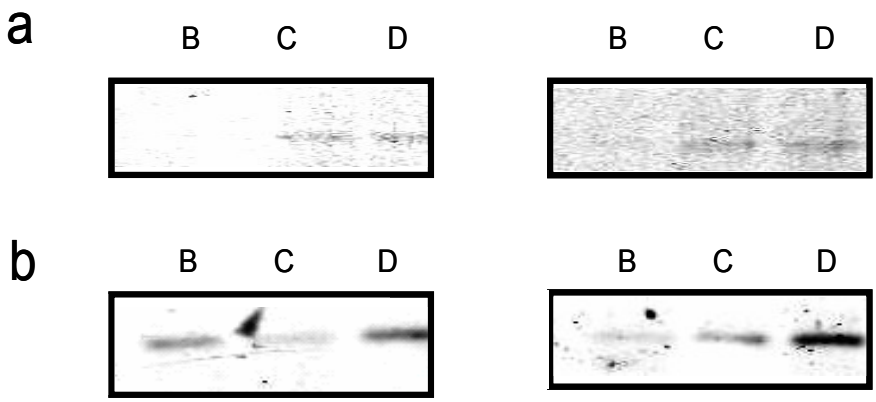


Figure 4.11: Transient receptor potential channels (TRPC) protein 1 and 4 are expresse in caveolae. Representative immunoblots with antibody against TRPC-1(a) and TRPC-4 (b) in caveolae, after purification from endothelial cells membrane from control lungs (left) and PAF perfused lungs (right).

To investigate the effect of inhibition of TRP channels on PAF-induced edema formation, we blocked TRP channels by SKF 96365 (1-[b-[3-(4-Methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole, HCl). SKF 96365 was described as a non-selective inhibitor of store operated channels (SOC) and TPR channels in cell culture [235, 236]. It was described that SKF 96365 inhibits receptor-mediated Ca^{2+} entry in activated endothelial cells [236]. In this study, however, blocking of the TRP channels by SKF 96365 had no effect on

PAF-induced edema formation (see next page Fig. 4.12).

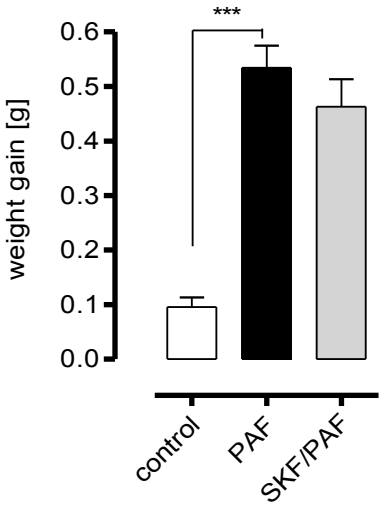


Figure 4.12: Effect of SKF 96365 (SKF) - TRP-channel inhibitor - on PAF-induced edema formation in isolated perfused rat lungs. PAF was given as a bolus injection of 5nmol 30 min after beginning perfusion. SKF (30µM) was given 10 min before injection of PAF. Weight gain was measured in lungs of the following groups: control, n=22; PAF, n=30; SKF/PAF, n=3. Data are mean ± SEM. Data were analysed by Student`s t-test and corrected to fdr procedure.

The calcium sensing receptor (CaSR) plays an important role for local changes in the extracellular calcium concentration and using confocal microscopy the colocalization of CaSR and caveolin-1 at the plasma membrane were demonstrated [133, 235]. Therefore we investigated the role of CaSR on PAF-induced edema formation. We used NPS 2390 (2-quinoxaline-carboxamide-N-adamantan-1-yl), a CaSR antagonist, to blocked CaSR in isolated perfused rat lungs. Figure 4.13 shows that the pretreatment with NSP 2390 had no effect on PAF-induced edema formation in lungs.

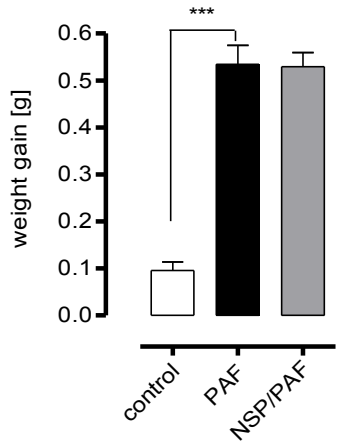


Figure 4.13: Effect of CaSR inhibition by NSP 2390 (NSP) on PAF induced edema formation in perfused rat lungs. PAF was given as a bolus injection of 5nmol 30 min after beginning of the perfusion. NSP 2390 (10µM) was given 10 min before PAF. Weight gain was measured in control lungs, n= 22; PAF-treated lungs,n=30 and lungs pretreated with NPS 2390, n=3. Data are mean ± SEM and were analysed by unpaired Student`s t-test. The p-values were corrected according to the fdr procedure.

4.1.4 Role of MAPK

MAP kinases were identified by virtue of their activation in response to growth factor stimulation of cells in culture, hence the name mitogen activated protein kinases (MAPK). MAPK may be associated with caveolae and have been implicated in the regulation of vascular permeability [137, 293, 316]. In this study we showed the presence of extracellular signal-related kinases 1 and 2 (ERK-1/2) (Fig. 4.14.a) and its amount inside the caveolar fractions was only slightly altered in PAF treated lungs. The caveolar fractions also contained the phosphorylated form, P-ERK-1/2. Compared to untreated lungs, PAF decreased the amount of caveolin-1 inside the caveolar fractions B-D of pulmonary endothelial cells (Fig. 4.14.b).To investigate the functional significance of ERK-1/2 in the mechanism of PAF-induced edema formation a selective inhibitor of MEK, U0126, was used in isolated perfused rat lungs. Figure 4.15 showed that no significant reduction of weight gain was measured in U0126 pretreated lungs. Also, higher concentrations of this inhibitor had no effect (data not shown).

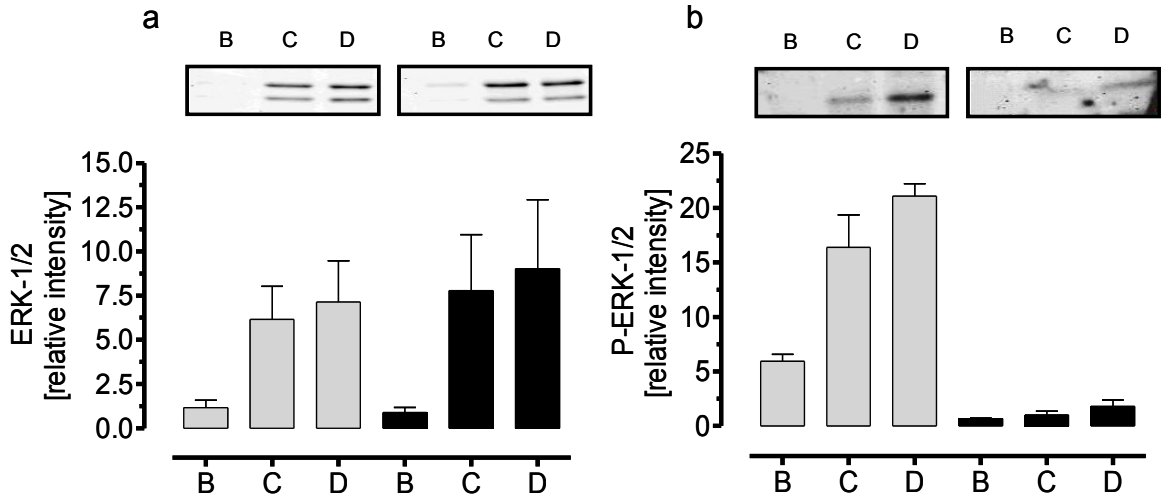


Figure 4.14: Extracellular signal-related kinases 1 and 2 (ERK-1/2) and its phosphorylated form (P-ERK) are present in caveolae isolated from endothelial cells of isolated perfused rat lungs (control and PAF treated lungs) and probed by immunoblotting for ERK-1/2 (a) and P-ERK-1/2 (b). Data are mean±SEM from 3-5 independent experiments. control (with bar), PAF treated (black bar)

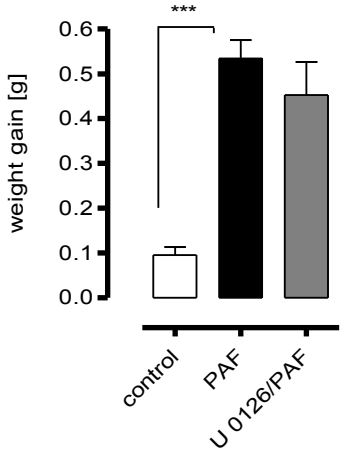


Figure 4.15: Effect of MEK inhibition by U0126 on PAF-induced edema formation in perfused rat lungs. PAF was given as a bolus injection of 5 nmol 30 min after beginning of the perfusion. U0126 (20µM) was given 10 min before PAF was added. Weight gain was measured in control lungs, n=22; PAF perfused lungs,n=30 and lungs pretreated with U0126, n=3. Data are mean ± SEM and were analysed by unpaired Student's t-test and corrected according to the fdr procedure.

4.1.5 Mannose-6-phosphate receptor (M6P-R) is present in caveolae

It has been suggested that extracellular ASM binds to the cells through M6P-R [83, 107]. To investigate the involvement of this receptor in PAF-induced edema Göggel *et al*. perfused isolated rat lungs with M6P to block free receptor binding sits and observed that lungs were partly protected against PAF-induced edema formation. Therefore we investigated the localisation of M6P-R in isolated membrane microdomaines of pulmonary endothelial cells. Figure 4.16 shows that M6P-R was present in samples C-D in caveolae

of isolated membrane fractions from endothelial cells from control lungs (Fig. 4.16.a) and PAF perfused lungs (Fig. 4.16.b). However PAF had no significant effect on the expression of M6P-R.

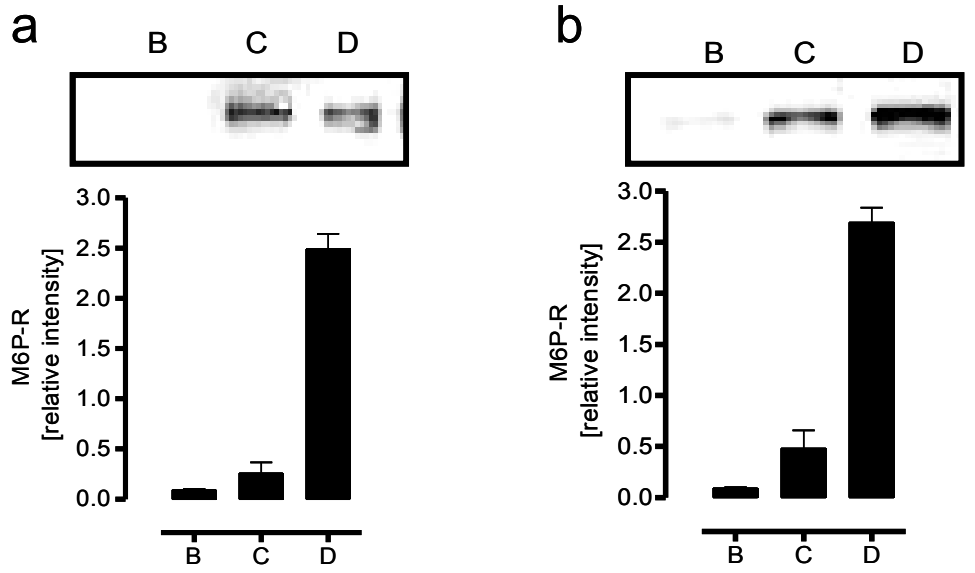


Figure 4.16: Mannose-6-phophate receptor (M6P-R) content in caveolae of pulmonary endothelial cells in control (a) and lungs perfused with PAF for 10 min (b). Caveolae were isolated from endothelial cells of isolated perfused rat lungs and probed by immunoblotting for M6P-R. Data are mean ± SEM from 3 independent experiments each.

4.2 In vitro *experiments*

4.2.1 Thrombin effects in human microvascular endothelial cells from lungs

In our hands, PAF has no effect on endothelial permiability in cell culture model. To obtain further insights in cellular signalling events, which are involved in vascular permeability alterations, we used thrombin as an inducer of increase endothelial permeability in cultured cells. Thrombin was used because it is widely used, so new data can be integrated into a large body of research.

4.2.1.1 Thrombin stimulates transendothelial FITC-albumin diffusion

Before, the mechanisms of thrombin-induced alterations of endothelial cell monolayer permeability were described as vascular leakage mediated by endothelial cell contration [27, 63, 169]. Most of these data were obtained by measuring the electrical resistance of monolayers as a measured of permeability [283]. But this elegant method disregards transcellular transport. In this study, FITC-albumin diffusion (3.4.2.1) was used to investigate thrombin-induced permeability in endothelial monolayer. Figure 4.17.a shows that HMVEC-L responded to thrombin stimulation in a concentration-dependent manner. A thrombin concentration of more than 100 nM produced an increase of the permeability index higher than 100% (Fig. 4.17.b). This finding suggstes that thrombin induces permeability by active transcellular transport of albumin.

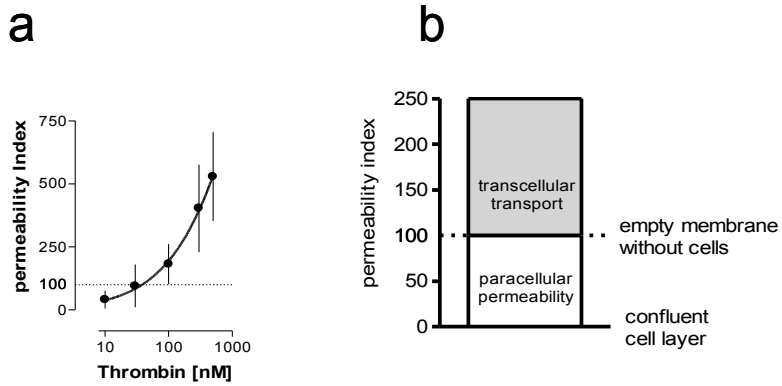


Figure 4.15: Thrombin increase FITC-albumin diffusion across endothelial cell monolayers. Dose-dependent increase in thrombin stimulated cells of FITC-albumin concentration into the bottom chamber below after 240 min (n=3) (a). The permeability index; defines albumin transport across cell monolayers. 0 % denotes untreated intact cell monolayer and 100% denotes empty cell-free monolayer (b).

4.2.1.2 Sphingomylinase activity and ceramide content in HMVEC-L cells

Next we investigated the involvement of ASM and ceramide in the response of endothelial cells to thrombin. In HMVEC-L cells, ASM activity in the cell pellet was elevated from 18.8 nmol (control level) to 24.7±0.35 nmol/2h (SEM, n=3) and 24.5±0.30 nmol/2h (SEM, n=5) substrate turnover after 30 min of thrombin stimulation (Fig. 4.18.a). We observed no increase of ASM activity in the subernatants of HMVEC-L cells (data not shown). The ceramide content after thrombin stimulation was also increased in HMVEC-L cells (Fig. 4.18.b). The ceramide content was highest after 30 min at 2.15±0.23 nmol/10^6 cells. This value corresponds to an increase of 45% compared to control levels.

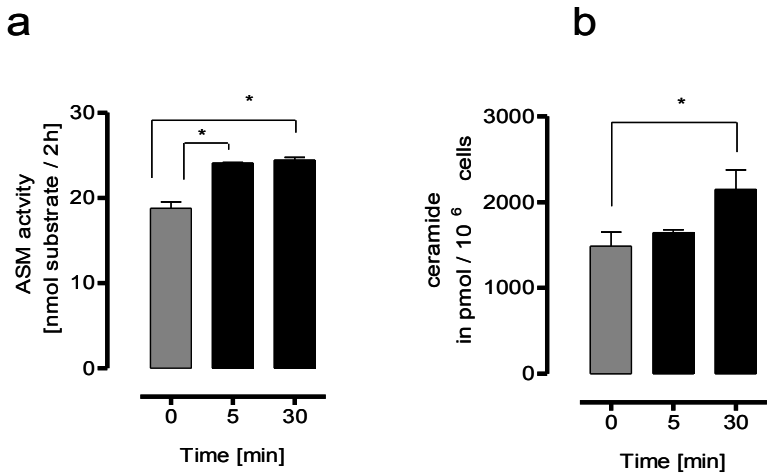


Figure 4.16: ASM activity and ceramide content of HMVEC-L cell monolayers after stimulation with thrombin. ASM activity of HMVEC-L after stimulation with thrombin for 0, 5, 30 min (a). significantly higher than contol (t=0 min), p<0.01 Dunett-test (n=2-5). Ceramide content of HMVEC-L cells after stimulation with thrombin for 0, 5, 30 min (b).* significantly higher than contol (t=0 min), p <0.05 Dunett-test (n=3-5). Cell numbers was 2 x 10^6.*

4.2.1.3 Thrombin-induces transcytosis by the acid sphingomylinase pathway

To investigate wether ceramide and ASM contributes to increase of albumin transport, we added different inhibitory substances, that interfere with the ceramide pathway, before stimulating the cells with thrombin. Both, imipramine and D609 have repeatedly been shown to block the sphingomylinase pathway. In HMVEC-L cells, both substances signifigantly inhibited the increase in FITC-albumin diffusions rate induced by thrombin (Fig. 4.19.a). Imipramine inhibited the albumin transport about 85% and D609 about 150%. Note, that in these experiments FITC-albumin diffusion induced by thrombin was over 200% of the diffusion rate across empty membranes (fig. 4.19.a). These findings suggest that thrombin-

induced increase of albumin transport mediated by ASM-ceramide. To confirm this we used Y27632 (Y27) a specific inhibitor for Rho/Rho kinase (which is involved in the mechanism of thrombin-induced paracellular leakage) togehter with imipramine and antibody against ceramide (cer-Ab). Figure 4.19.b shows that the increase of albumin diffusion rate was partly reduced by preincubation with imipramine, cer-Ak and Y27632 alone. But if cell monolayers were simultaneously pretreated with imipramine and Y27 or cer-Ab and Y27, FITC-albumin diffusion was almost complete reduced. The thrombin increased of FITC-albumin diffusion was attenuated more than 150%. These findings confirmed that thrombin-induced albumin transport beside the established mechanism of thrombin-induced permeability by cytoskeletal rearrangement and cell contraction.

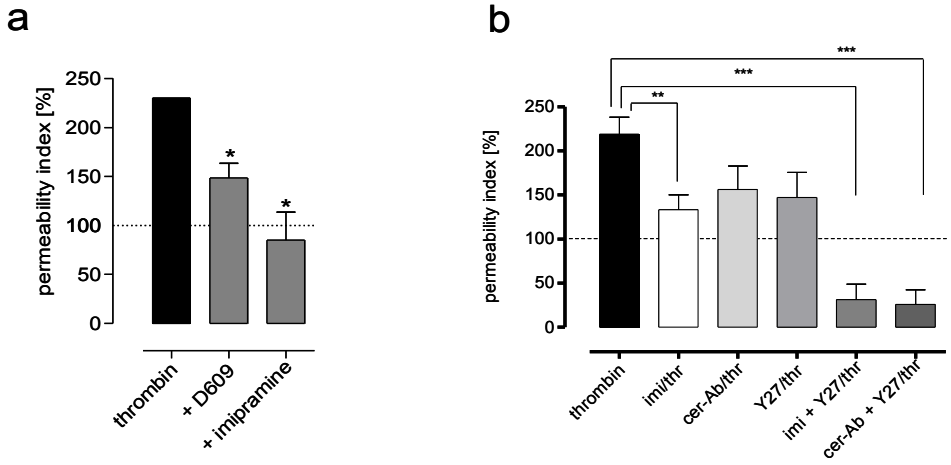


Figure 4.17: Effect of ASM pathway and Rho-kinase inhibiton on thrombin-induced increase of FITC-albumin diffusion across endothelial cell monolayers. 4 x 105 HMVEC-L cells were preincubated with imipramine (10µM) for 20h or with D609 (300µM) for 30 min alone (a) and simultaneous with imipramine and Y27 (10µM) or ceramide antibody (50µg/ml) and Y27 for 30min, before thrombin was added for 180min (b). The permeability was detected by FITC-albumin transmigration from the upper chamber of the Transwell insert system to the lower chamber across the cell monolayer. Permeability index: 0% denotes intact untreated monolayers and 100% denotes cell-free monolayers: >100% indicates transcellular transport. * Significant lower to thrombin; imipramine p<0.007, imi/Y27 and cer/Y27 p<0.0005. Data are mean±SEM from 6 independent experiments. Data analysed by unpaired Students t-test and corrected by the fdr procedure (n=6). imi (imipramine), cer-Ab (antibody against ceramide), Y27 (Y27632)

4.2.1.4 Thrombin increase caveolin-1

Transcellular transport of albumin commences in caveolae [182, 217]. Two molecules are required for this process, caveolin-1 and gp60. Caveolin-1 is needed for formation of caveolae and gp60 acts as a receptor for albumin [243, 244]. Therefore we investigated the presence of the caveolae-signature molecule caveolin-1 in detergent-resistant membrane fractions (caveolae). Figure 4.20.A shows representative immunoblots of caveolin-1 in cell membranes isolated from untreated cells (a) and thrombin stimulated cells (b). Compared to control cells, thrombin increased the amount of caveolin-1 in the membrane fractions 1-4 of pulmonary endothelial cells (c). The increased amount of caveolin-1 in the plasma membrane was confirmed by immune histochemnistry (Fig. 4.20.B) as well as by electron microscopy (Fig. 4.20.C). Electron microscopy was performed in cooperation with Ch. Mühlfeld and M. Ochs as described [294].

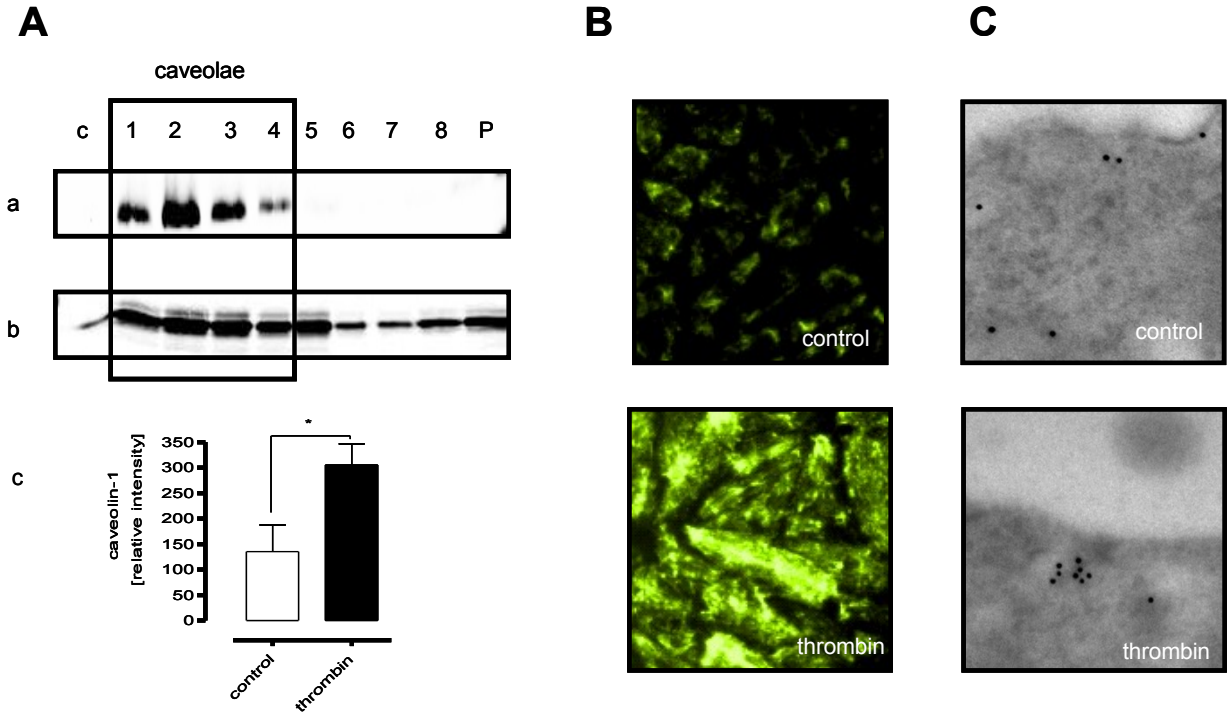


Figure 4.18: Thrombin induced enrichment of caveolin-1 in the plasma membrane. 2.5 x 10^6 cells were treated with 500nM (50U/ml) thrombin for 120 min. (A) Caveolin-1 content in detergent-resistant membrane fractions. Immunoblots of the cytosol (c), membrane fractions 1-8 and pellet (P) with anti-caveolin-1 antibodies of detergent-resistant membrane fractions from (a) control cells and (b) thrombin stimulated cells. (c) Quantification of caveolin-1 amount in caveolae-fractions. Thrombin is significantly higher to control ($p < 0.0431$). Data are mean ± SEM and analysed by unpaired students t-tests (n=4) corrected by the fdr procedure. (B) Representative fluorescence microscopic images (1:400) of HMVEC-L cells stained with an anti-caveolin-1 antibody and an Alexa fluorescence-secondary antibody. Cell layers of control and thrombin stimulated cells are shown after 120 min.(C) Electron micscopy, shown are gold-labelled anti-caveolin-1 antibodies.

4.2.1.5 Thrombin-induced albumin transport in caveolin-1 containing vesicles

To investigate transcellular transport of albumin; HMVEC-L cells were stained with gold labelled antibodies for caveolin-1 and albumin. Thrombin-induced albumin transport was directly visualized by electron-microscopy (Fig. 4.21). Remarkably, albumin was found close proximity to caveolin-1 after incubation with thrombin. These findings indicated that albumin was transported in caveolin-1 containing vesicles. Electron microscopy was performed in cooperation with M. Mühlfeld and M. Ochs as described [294].

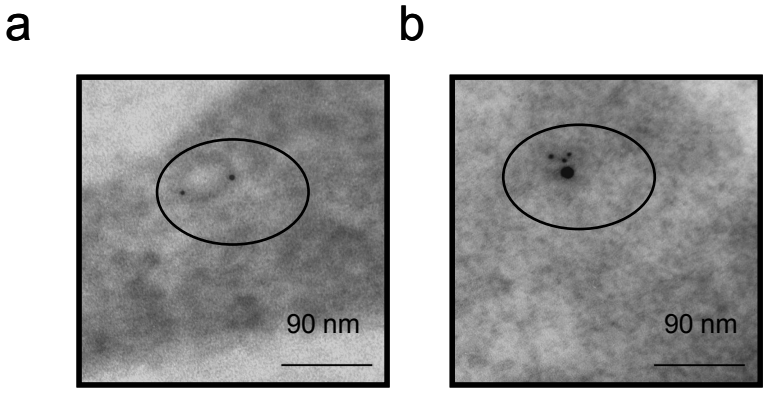


Figure 4.19: Thrombin-induced albumin transport in caveolae. Electron microscopic images, shown are gold-labeled anti-caveolin-1 antibodies (small, particle with diameter 1nm) and gold-labeled albumin antibodies (large, particle with diameter 15nm large) in untreated (a) and thrombin-treated (b) HMVEC-L cells. Thrombin (50U/ml) was added for 60min.

4.2.2 Thrombin regulates caveolin-1 content

4.2.2.1 Inhibition of ASM blocks caveolin -1 increase

To investigate wether ceramide contributes to the increase of caveolin-1, we preincubated HMVEC-L cells with imipramine and D609, before thrombin was added. Figure 4.22. shows pretreatment of HMVEC-L cells with both inhibitors reduced the amount of caveolin-1 in lipid rafts. Representative immunoblots of anti-caveolin-1 in cytosol, membrane fractions 1-8 and pellet fraction of control cells (a), thrombin-treated cells (b) and imipramine and D609 preincubated cells (c) were shown in Figure 4.22.A. The content of caveolin-1 was elevated from 85.55±24.36 counts in the control to 192.0±40.58 counts after 120 min thrombin stimulation (Fig 4.22.B), but reduced more than 100% in imipramine and D609 pretreated HMVEC-L cells.We also investigated the content of caveolin-1 in whole cell lysate of endothelial cells. On the next page figure 4.23.a shows the characteristic caveolin-1 immunoblot of cell lysates under different conditions. Thrombin increased caveolin-1 and

preincubation with D609 and imipramine decreased the content of caveolin-1. Figure 4.23.b shows the quantification of the protein bands and confirmed the findings that that thrombin-induced increase of caveolin-1 was reduced by imipramine and D609. The intensity of caveolin-1 protein band was reduced from 136.7±13.09 counts in thrombin stimulated cells to 86.30±6.82 counts in imipramine pretreaded cells and to 67.06±13.28 counts in D609 pretreated cells

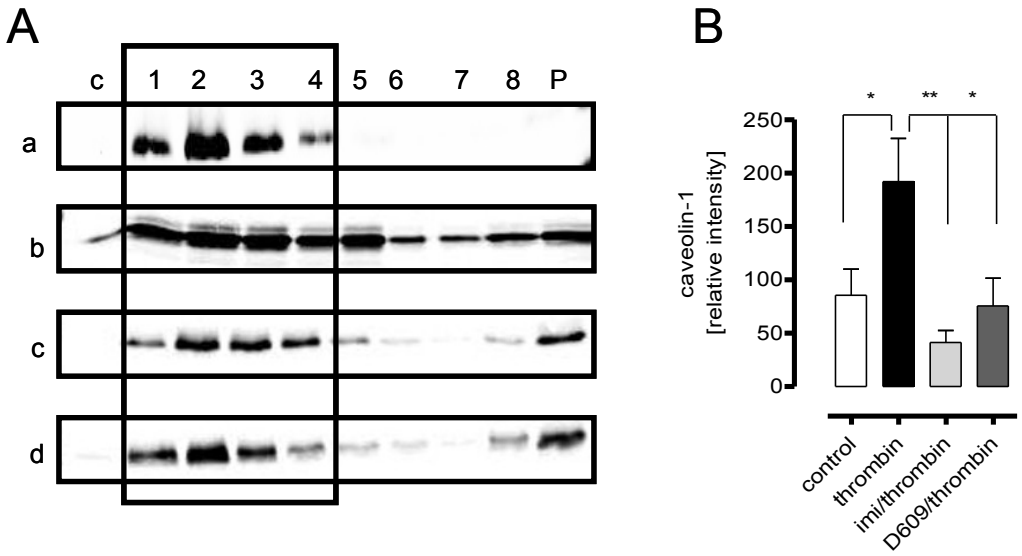


Figure 4.20: Figure 4.20: Inhibitory effect of imipramine and D609 on thrombin-induced increase of caveolin-1 in HMVEC-L cells. 2.5 x 106 cells were pretreated with imipramine (10µM) for 20h and D609 (300µM) for 30 min before 500nM (50U/ml) thrombin was added for 120min and caveolae were isolated. Representative immunoblots of caveolin-1 in isolated fractions of cytosol (c), membrane fractions 1-8 and pellet (P) from control (a) thrombin treated cells (b) and imipramine (c) D609 (d) pretreated cells (A). The caveolin-1 content in caveolae was significantly lower than thrombin; imipramine p<0.006 (n=10) and D609, p<0.04 (n=10) and higher than to control, thrombin p<0.04 (B). Data are mean±SEM and analysed by unpaired Students t-test. P-valus were corrected by fdr procedure. Frame shows caveolar fractions; imi (imipramine).

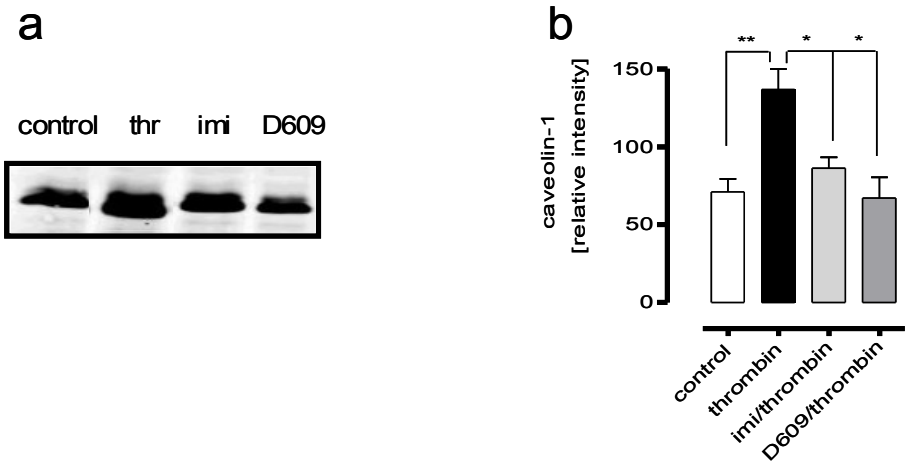


Figure 4.21: Caveolin-1 content in whole cell-lysate. 2.5 x 10^6 HMVEC-L cells were preincubated for 20h with imipramine (10µM) or for 30 min with D609 (300µM) before 500nM (50U/ml) thrombin was added for 120min. Representative immunoblots stained with anti-caveolin-1 antibody (a). Quantification of caveolin-1 protein bands in the whole cell-lysate (b). Compared to thrombin stimulates cells the content of caveolin-1 signifigantly lower than in controll cells and in cells pretreat with imipramine p<0.03 (n=3) or D609 p<0.02 (n=3) to p<0.009. Data are mean±SEM and are analysed by unpaired Students t-test and corrected by fdr procedure. Imi (imipramine), thr (thrombin).

4.2.2.2 Blocking transcription and translation during thrombin stimulation

To investigate whether thrombin stimulated the protein synthesis of caveolin-1, we blocked the transcription with actinomycin D (ActD) [56] or the translation with cycloheximide (CHX) [155] during thrombin stimulation. Figure 4.24. shows that both inhibitors attenuated the content of caveolin-1 in the whole endothelial cells lysate. Subsequently, we examined the effect of protein synthesis inhibition on caveolin-1 content only in caveolae of endothelial cell membrane. Figure 4.25.A shows the characteristic distribution of caveolin-1 in cytosol, membrane fraction 1-8 and pellet fraction of control cells (a), thrombin stimulated cells (b), ActD pretreated cells (c) and CHX pretreated cells (d). The content of caveolin-1 inside caveolae enhanced by thrombin stimulation (Fig. 4.25.B). In the present of both inhibitors, the content of caveolin-1 was attenuated by CXH about 30%, but not completely and effectively as on transcription levels (Fig. 4.25.B). These findings suggest that thrombin could be involved in *de novo* synthesis of caveolin-1.

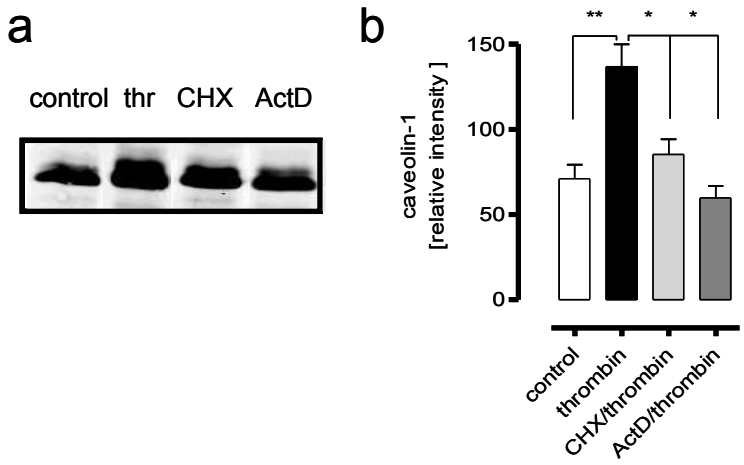


*Figure 4.22: Effect of protein biosynthesis inhibition by actinomycin D or cycloheximide on the thrombin-induced increase of caveolin-1 in whole endothelial cell lysates. 2.5 x 10^6 HMVEC-L cells were incubated simultaneous with 500 nM (50U/ml) thrombin and ActD (2µg/ml) or thrombin and CHX (1µg/ml) for 180 min. Representative immunoblot of caveolin-1 in the whole cell lysate (a).Caveolin-1 content in caveolae of whole cell * significantly lower to thrombin; ActD (p<0.03) and CHX (p<0.03) and higher than control, thrombin p<0.009. Data are mean±SEM from 5 independent experiments. Data analysed by unpaired Students t-test and corrected by the fdr procedure. ActD (actinomycin D), CHX (cycloheximide, thr (thrombin)*

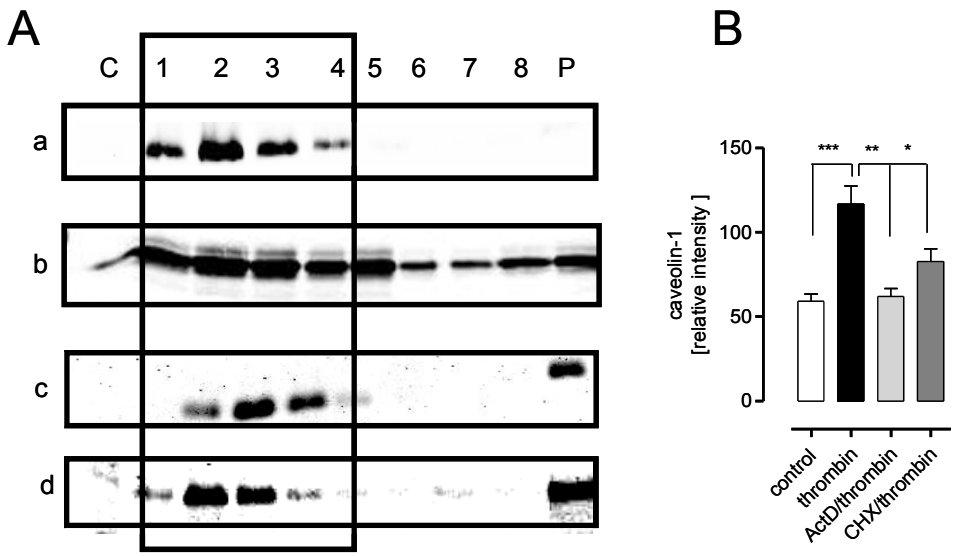


*Figure 4.23: Effect of protein biosynthesis inhibiton by actinomycin D or cycloheximide on the thrombin-induced increase of caveolin-1 in caveolae of endothelial cell membrane. 2.5 x 10^6 cells were incubated simultaneously with 500nM (50U/ml) thrombin and ActD (2µg/ml) or thrombin and CHX (1µg/ml). (A) Caveolae were isolated from endothelial cell membranes of control cells (a), thrombin-treated cells (b), trombin/ActD-treated cells (c) and thrombin/CHX-treated cells (d). (B) Caveolin-1 content in the membrane fractions (1-4). * Significant lower than thrombin; ActD p<0.002 (n=3), CHX p<0.02 (n=3) and higher than control, thrombin p<0.0006 (n=3). Data are mean±SEM analysed by unpaired Student`s t-test and corrected by the fdr procedure. Frame shows caveolar fractions, ActD (actinomycin D), CHX (cycloheximide), c (cytosol), p (pellet)*

4.2.3 Thrombin contributes transport in intact vessels

To show that thrombin contributes transytosis also in intact vessels were performed in co-operation with W.Kübler in isolated rat lungs. Vascular endothelial cells were labelled with the highly fluorescent, calcium-sensitive dye, fura-2 (green). Microscopic investigation of FITC-albumin diffusion in aortic endothelial cells is shown in figure 4.26. In control endothelium FITC-albumin (red) was observed on the outside, but not in the inside of the endothelial cells (Fig. 4.26, left). In thrombin-treated cells FITC-albumin was located in vascular endothelial cells as is indirected by red fluorecence the signal in the green marked cell wall. These findings indicate that thrombin-induces transcellular transport of albumin in also in intact vessels.

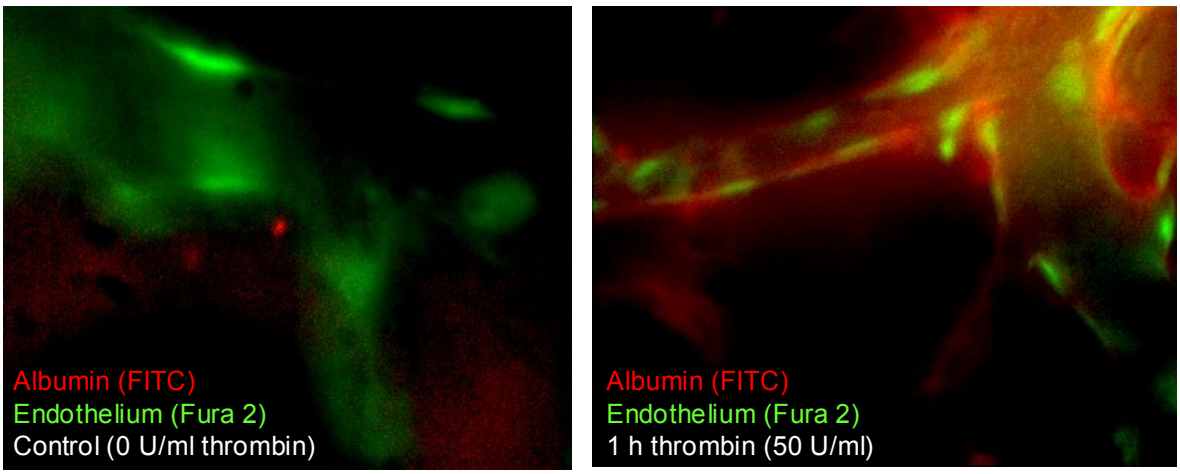


Figure 4.24: Representative fluorescence images of vascular endothelail cells in rat lungs. Endothelial cells stained with Fura-2 fluorecent dye (green colour) and albumin labelled with FITC fluorescent dye (red colour). Endothelial cells of vessels under control and after thrombin stimulation are shown (n=2)

4.2.4 Extracellular acid sphingomyelinase

Endothelial cells can secrete lysosomal acid sphingomylinase in the subendothelial space after incubation with inflammatory cytokines (IL 1β, INFγ) [273]. In was observed that inhibition of this enzyme reduces the mortality in sepsis models and the extent of pulmonary edema in acute lung injury [290]. Therefore we investigated the effects of extracellular acid sphingomylinase (ASM) in HMVEC-L cells.

4.2.4.1 Ceramide measurement after extracellular ASM treatment in cells

To investigate whether extracellular ASM generates ceramide from sphingomyelin (which is located in the outer leaflet of the cell membrane) HMVEC-L cells were treated with ASM or thrombin. Figure 4.27.a shows that both lipids, C_{16} ceramide and C_{18} ceramide were located in lysates from control, thrombin- and ASM-treated HMVEC-L cells. Compared to control cell lysate (2.76±0.281nM), the amount of ceramide in ASM-treated cell lysate (2.887±0.1803 nM) was almost equal (fig.4.27.b). In the presence of thrombin the content of both lipids, C_{16} ceramide and C_{18} ceramide, was elevated from 2.76±0.281nM (control level) to 3.583±0.393 nM. Figure 4.27.c shows the percentages of ceramide. The amount of ceramide after thrombin stimulation was 25% higher that in the control cell lysate. These findings indicate that external administration of ASM did not change the content of ceramide in the cell lysate.

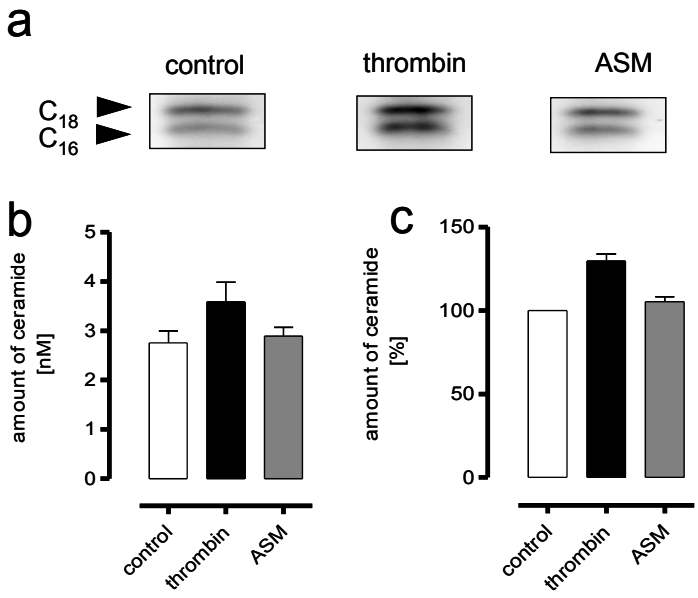


Figure 4.25: Ceramide content of HMVEC-L cells after stimulation with acid sphingomyelinase (ASM) (1 U/ml) and thrombin (50 U/ml). Cell number was 3 x 10^6 cells. Stimulation time for both mediators was 120min.Visualisation of lipid bands; C_{16} ceramide (C_{16}) and C_{18} ceramide (C_{18}), from control cells, thrombin or ASM-treated cells. Concentration of ceramide in control, thrombin and ASM-treaded cell lysat samples (a,b) and calculated increase of ceramide as precent (n=3) (c).

4.2.4.2 External ASM increased caveolin-1

Next we investigated whether treatment with external ASM increases the caveolin-1 content in cells. We incubated HMVEC-L cells with ASM for 120 min and subseqently the caveolae were isolated from endothelial cell membranes. ASM increased the content of caveolin-1 in the caveolae fractions 1-4. Characteristic immunoblots of cytosol (c), membrane fractions and pellet (P) from control (a) and ASM-treated cells (b) are shown in figure 4.28.A. Figure 4.28.B shows the content of caveolin-1 in caveolar membrane fraction 2 isolated from ASM or thrombin stimulated cells in comparison to control cells. The caveolin-1 content was stronger elevated in response to thrombin.

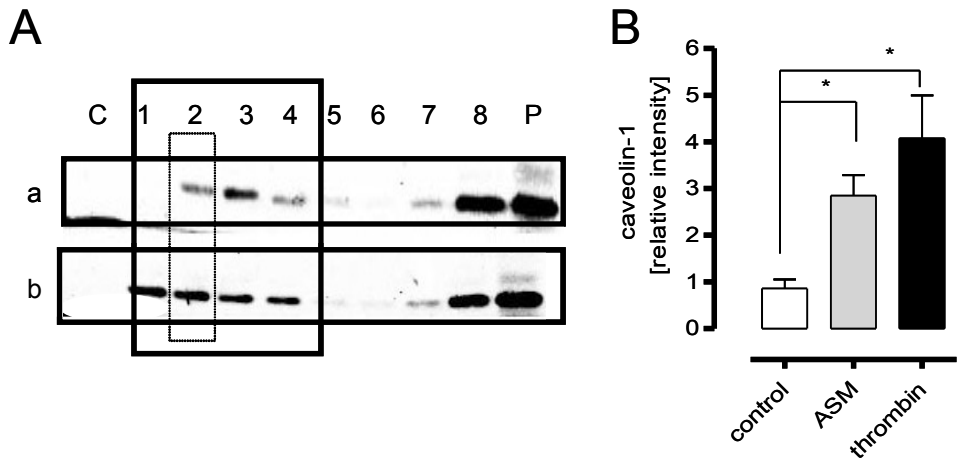


*Figure 4.26: Extracellular ASM increases caveolin-1. 2,5 x 10^6 HMVEC-L cells were incubated with thrombin (50U/ml) or ASM (1U/ml) for 120min. (A) Representative immunoblots of cytosol, membrane fractions and pellet were isolated from control cells (a) and ASM-treated cells (b). (B) Caveolin-1 content in caveolar fraction 2 * significantly higher than in control, ASM p<0.02 (n=3) and thrombin p<0.03 (n=3). All data are mean±SEM analysed by unpaired students t-test and corrected by the fdr procedure. Frame shows caveolar fraction and dashed frame the caveolar membrane fraction 2. ASM (acid sphingomyelinase), c (cytosol), pellet (P)*

4.2.4.3 External ASM increase FITC-albumin diffusion

Next we examined if administration of extracellular ASM increased the transport of albumin across endothelial cells. We measured FITC-albumin diffusion rate across HMVEC-L cell monolayers in the Transwell insert system. Figure 4.29.a shows that the FITC-albumin concentration was elevated by ASM from 80.56 ng/ml (control level) to 128.33 ng/ml in ASM-treated cells. Figure 4.29.b shows that ASM increased FITC-albumin diffusion by more than 100%. However, compared to thrombin the effect by ASM was lower.

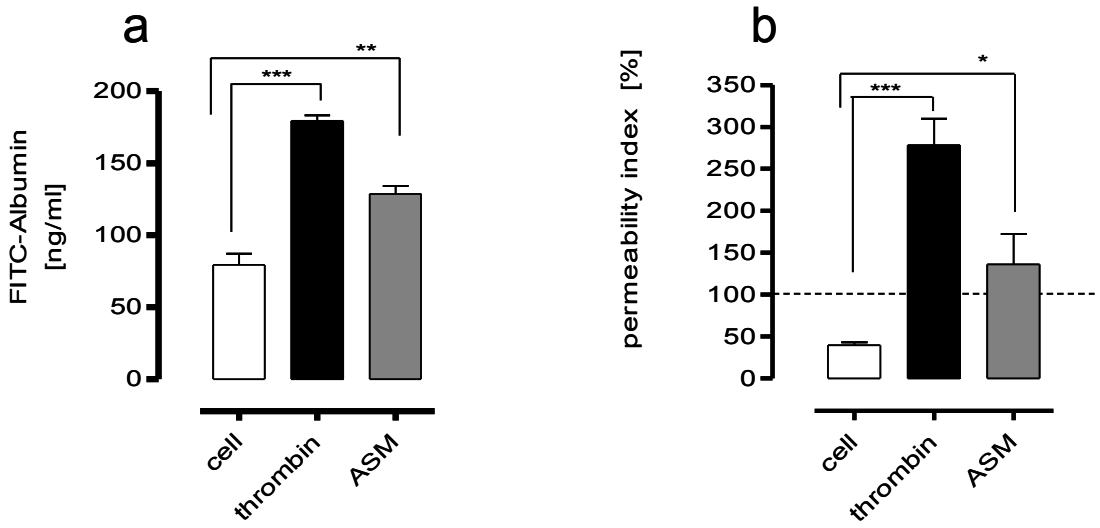


*Figure 4.27: External ASM increases FITC-albumin diffusion across HMVEC-L cell monolayers. The FITC-albumin diffusions rate was measured in control, thrombin or ASM stimulated endothelial cell monolayers (a) Concentration of albumin, * significantly higher than control, thrombin p<0.001 and ASM p<0.004. (b) Permeability index: 0% denotes intact untreated monolayers and 100% denotes cell-free monolayers: >100% indicates transcellular transport. * significantly increased vs. control; thrombinp<0.0003 and ASM p<0.0338 (n=6). Data are mean ± SEM from 6 independent experiments. Data analysed by unpaired Students t-test and corrected by the fdr procedure*

5 Discussion

5.1 PAF in isolated perfused rat lung

Platelet-activating factor (PAF) is a potent proinflammatory mediator that elevates vascular permeability and leads to edema formation in pulmonary inflammation. Recently our group has shown that PAF-induced edema formation is partly mediated by acid sphingomyelinase (ASM)-derived ceramide [96]. The mechanisms by which ceramide alters vascular permeability are poorly understood.

The presence of sphingolipids in caveolae is well documented, where they contribute to the enhanced rigidity of membrane, but may also assume signalling functions [8]. Caveolae are enriched in caveolin-1 and endothelial NO synthase (eNOS), to the effect that eNOS is bound and thereby kept in its inactive state by caveolin-1 [54, 70, 91]. The alleged role of sphingolipids in caveolae, the localisation of eNOS within caveolae and the potential role of NO in the regulation of vascular permeability let us to investigate whether PAF administration in the lungs would alter the composition of caveolae and affect NO synthesis.

5.1.1 Regulation of caveolin-1 by PAF

Here we provide direct and indirect evidence that PAF causes translocation of caveolin-1 to the caveolae of endothelial cells.

- PAF increased the amount of caveolin-1 in Triton X-100-resistant membrane fractions isolated from PAF treated lungs (Fig. 4.2). According to previous studies these fractions most likely represent caveolae [10, 124, 125, 126, 181]. This conclusion is further supported by the presence of flotillin-1 (another marker of caveolae [19] in these fractions and by the absence of lysosomal, nuclear and microsomal markers (Fig. 4.1, Tab. 4.1).
- Indirect evidence for the recruitment of caveolin-1 in response to PAF is provided by the fact that PAF decreased endothelial NO production (Fig. 4.6). Caveolin-1 inhibits eNOS activity by a direct interaction with eNOS through its “scaffolding domain,” located between amino acids 82 and 101 within endothelial plasmalemmal caveolae

[77, 84, 183, 256].

- While the present study did not demonstrate a link between caveolin-1 activation and edema formation, such a mechanism seems still feasible given the fact that inhibition of ASM does prevent not only caveolin-1 recruitment, but also edema formation induces by PAF (Fig. 4.4). Future studies will have to clarify the underlying mechanisms.

Recruitment of caveolin-1 to the plasma membrane has also been described for rat pulmonary microvascular endothelial cells in response to shear stress [229]. In that study caveolin-1 redistribution was associated with an increased number of cell surface caveolae. Whether this is true in our model as well remains to be shown. The fact that caveolin-1 increased while eNOS did not (Fig 4.2, 4.5) which is in contrast to the effect of shear stress [229], suggests that if new caveolae were recruited to the plasma membrane they contained more caveolin-1 than eNOS protein. The alternative explanation is that caveolin-1 was recruited to sphingolipid rich membrane regions produced by the ASM. Such a scenario would be supported by the finding that pretreatment with two pharmacological agents, which disrupt the hydrolysis of sphingomyelin by ASM (D609 and imipramine) also reduced the amount of caveolin-1 in caveolae (Fig. 4.4). How caveolin-1 recruitment to the plasma membrane into caveolae are controlled requires more investigations.

5.1.2 Caveolin-1 regulates eNOS activity

Since caveolin-1 is well known to inhibit eNOS, the increased caveolar levels of caveolin-1 explain the reduction of endothelial NO production in response to PAF. A similar mechanism has been suggested in models of portal hypertension [33, 320] and liver cirrhosis [255] where elevated caveolin-1 levels were associated with reduced NO-synthesis. Under these conditions caveolin-1 competes with Ca^{2+}-calmodulin (Ca^{2+}/CaM) for eNOS binding and accordingly the block by caveolin-1 can be overcome by providing extra calmodulin [254]. The calcium independent activation of eNOS can occur through phosphorylation of the eNOS by PKB/Akt-kinase which in turn is activated by phosphatidylinositol-3-OH kinase (PI3K). The influence of caveolin on the PI3K/Akt pathway is not well investigated. However, PI3K was found in caveolae from fibroblasts [159], myeloid-derived cells [319] and endothelial cells [163] and this suggests that PI3K activity might be controlled by cave-

olin under certain conditions. In support of this, we also identified PI3K and Akt in caveolae and observed that both were attenuated after PAF stimulation (Fig. 4.8). This finding suggests that the increased amount of caveolin-1 in caveolae may have blocked PI3K activity and the subsequent activation by Akt-kinase in response to PAF. Interestingly, it has been shown that ceramide may inactivate PI3K by caveolin-1 overexpression [321, 322]. A similar effect of caveolin-1 overexpression was described for the downstream effector molecule Akt in fibroblasts and epithelial cells [159]. Both studies indicate a possibility of how ASM activity may diminish NO activity. Thus inhibition of PI3K should not attenuate PAF induced pulmonary edema, which is exactly what we observed (Fig. 4.9).

In other studies it was observed that PAF-induced anaphylactic shock depended on PI3K signaling and on NO produced by the constitutive enzyme eNOS [40] and that PAF activated the PI3K/Akt pathway on binding to its transmembrane receptor, a typical G-protein-coupled receptor (GPCR) [41, 190], which can interact with the regulatory subunit (p85) of PI3K [319]. This interaction causes an increase in the activity of Akt (downstream effector molecule of PI3K activation) and a direct phosphorylation of eNOS followed by increased NO production [84]. In comparison to this, our findings suggest that PAF reduced NO-production mediated by caveolin-1, maybe by binding the downstream effector molecules PI3K/Akt in the pathway of the eNOS activation. This could be an explanation for the fact that Ly294002, a potent and specific inhibitor of PI3K [300], had no effect in our model.

Taken together, our findings suggest (i) that caveolin-1 plays a major role in the PAF-triggered reduction in endothelial NO levels, (ii) the increase of caveolin-1 by PAF-induced recruitment of caveolin in ASM-ceramide dependent manner, (iii) reduced the NO production through direct binding of eNOS and (iv) indirectly through inhibition of the downstream signal molecules PI3K/Akt from the Ca^{2+}-independent pathway of eNOS activation.

5.1.3 Role of NO in edema formation

The role of NO in the development of pulmonary edema is still controversial. While in the systemic circulation most, but not all of the evidence indicates that increased NO production mediates vascular hyperpermeability [185, 186], this situation is more confusing with respect to the lungs. Unspecific inhibition of NO synthesis did on the one hand attenuate pulmonary edema induced by PMA [238]; ischemia/reperfusion [135] and IL-2 [12, 206, but

on the other hand it aggravated or had no effect against pulmonary edema induced by LPS [45, 113] or pancreatitis [274]. One possible conclusion from these studies is that both too much and too little NO can increase vascular permeability [218]. However, it should also be noted that many studies assessed vascular permeability by tracers [136, 260] or indirectly by methods such as the Landis technique [151]), but not edema formation. This may not be the same as is illustrated by the eNOS knock-out-mice that do not show pulmonary edema despite an altered tracer distribution [218]. The question of how to interpret tracer studies is also highlighted by the fact that the result of such studies may even depend on the fluorescence dye to which the tracer (in that case albumin) is coupled [306]. In the lungs, edema formation is not only a function of vascular permeability, but also of hydrostatic pressure, lymphatic drainage and alveolar fluid clearance which are all differentially regulated by nitric oxide. Thus, nitric oxide may have pro- and anti-edematous effects depending on rate, site, and duration of NO synthesis as well as on activation of interrelated signalling pathways. It should also be noted that in many models the mode of action is difficult to dissect, because NO does not only regulate vascular permeability but in addition also attenuates leukocytes activation/adhesion and reduces vascular pressure and hence hydrostatic edema [113, 165, 194]. Therefore, we would like to emphasize that our isolated perfused lung model was designed to exclude extrapulmonary leukocytes and hydrostatic edema. The former is achieved by blood-free perfusion and through rinsing of the lungs at relatively high perfusion rates (> 25 ml), the latter by using constant pressure perfusion [294, 295]. Thus, this model is well suited to study PAF-induced alterations in pulmonary vascular permeability independent of many confounding factors. Using this model, in the past we have shown that PAF increases vascular permeability by the concomitant action of PGE_2 and ceramide [94, 96]. While PGE_2 is not known to stimulate NO synthesis, for ceramide this has been described [121. However, in those cases where ceramide did stimulate NO production, the ceramide was generated by the neutral sphingomyelinase (NSM) in response to shear stress [148]. In our model PAF activates the acid (ASM) rather than the neutral sphingomyelinase [96] and the experiments with imipramine indicate that this is causally related to decreased endothelial NO levels (Fig 4.10). Taken together we propose that in endothelial cells depending on the stimulus either ASM or NSM are activated leading to decreased or increased NO synthesis respectively. This hypothesis shall be tested by contrasting the effects of PAF-induced NO decrease to that of stretch in-

crease NO.

Recently it was shown in eNOS-deficient or L-NAME-treated mice that NO is critical to maintain endothelial junctional permeability [218]. From these findings, a drop in endothelial NO, as reported here, would be expected to enhance vascular permeability. And in fact, if the PAF-induced drop in endothelial NO levels was prevented by imipramine (Fig. 4.6), the PAF-induced edema was reduced (though not completely, because this treatment did not affect the PGE_2-dependent pathway [305]). In the study of Predescu and colleagues [218] however, L-NAME increased junctional permeability only after 30 min rather than within a few minutes, suggesting that along the PAF-ASM-axis additional mechanisms such as increased intracellular calcium levels are likely to come into play as well [121]. Such an additional mechanism appears also likely on the grounds that eNOS deficiency alone does not cause pulmonary edema. In addition to the observation of Predescu *et al*. [218], other have observed that inhibition of NO synthesis with L-NAME or L-NMMA increases vascular permeability dependent on changes in the endothelial cytoskeleton [247]. These findings are in line with our observation that perfusion with L-NAME or L-NMMA in isolated rat lungs did not prevent the PAF-induced edema formation (Fig. 4.7). To exclude the possibility that PAF induced edema through inducible NOS (iNOS) generated NO, we pretreated rat lungs with 1400W, a potent inhibitor of iNOS, but a reduction of edema formation was not detected (Fig 4.7) [287]. Previous studies had show that PAF activates the inducible form of NOS in response to cytokines (e.g. TNFα, IL 1β, IFγ) in cell culture of immortalized astrocytes (DITNC) [302]. Peng *et al.* showed in the mice model of ventilator-induced lung injury (VILI) that iNOS rather then eNOS gene expression and activity was significantly upregulated by mechanical high ventilation (HV_T, 20 ml/kg for 2 hours) and that this contributes to pulmonary edema in wild type mice [213]. Taken together these findings, in caveolae isolated from pulmonary endothelial cells of isolated perfused rat lungs we made the novel observation that PAF increases caveolin-1 levels within minutes, suggesting that PAF causes reorganization or formation of new caveolae in endothelial cells. Because caveolin-1 is well known to block eNOS activity, we conclude that PAF treatment reduces NO production. Pretreatment with the ASM-inhibitor imipramine prevented the PAF-induced decrease in endothelial NO levels and reduced edema formation indicating that both effects are mediated by ASM. Inhibition of NOS with L-NAME, L-NMMA or 1400W had no effect on PAF-induced edema, suggesting that along the PAF-ASM axis

additional mechanisms such as increased intracellular calcium levels are likely to come into play as well. Future investigations to examine employing perfusion C_2 ceramide in isolated lungs and experiments with ASM-knockout mice and caveolin-1-deficient mice will provide further insights into this novel pathway.

5.1.4 Role of the mitogene-activated protein kinase (MAPK)

A variety of extracellular stimuli can target transmembrane molecules to activate intracellular signal transdruction. These signals are then transduced through the cell by signalling cascades to regulate cellular processes such as differention, proliferation, apoptosis and inflammation. One specific intracellular event is the activation of mitogen-activated protein kinases (MAPK) which are divided into four distinctly regulated groups : extracellular signal-related kinases (ERK)-1 and 2, c-Jun amino-terminal kinases (JNK 1,2 and 3), p38 MAPK and ERK-5. Since in previously it was observed that ERK-1/2 activation regulates PAF-induced hyperpermeability [317, we investigated the ERK-1/2 pathway in this thesis. The presence of MAPK, particular ERK-1/2, in caveolae is well established [79, 137, 316]. In keeping with this we identified the native unphosphorylated form of ERK-1/2 (Fig. 4.14.a) in caveolae from pulmonary endothelial cells. Interestingly we detected only a slightly increase of the native form of ERK-1/2 in caveolae in response to PAF (Fig. 4.14.a).

The essential events in the regulation of MAPK are phosphorylation and dephosphorylation on amino acid residues of threonine, tyrosine and serine. Phosphorylation is catalyzed by various specific protein kinases, whereas phosphatases dephosphorylate. The activation of the ERK-pathway is well established and is illustrated by the following example: epidermal growth factor (EGF) induced ERK activation (next page Fig. 5.1). The binding of EGF induces receptor dimerisation and autophosphorylation (P) on tyrosine residues **(1)**. These phosphotyrosines function as docking sites for signalling molecules including the Grb2-SOS complex, which activates the small G-protein Ras by stimulating the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) **(2)**. This exchange elicits a conformational change in Ras, enabling it to bind to Raf-1 and recruit it from the cytosol to the cell membrane, where Raf-1 activation takes place. Raf-1 activation is a multi-step process that involves the dephosphorylation of inhibitory sites by protein phosphatase 2A (PP2A) as well as the phosphorylation of activating sites by PAK (p21*rac/cd-*

c42-activated kinase), Src-family and yet unknown kinases **(3)**. Activated Raf-1 phosphorylates and activates MEK (MAPK/ERK kinase), which in turn phosphorylates and activates extracellular-signal-regulated kinase (ERK) **(4)**. Activated ERK has many substrates in the cytosol e.g. cytoskeletal proteins, phospholipase A2, signalling proteins including tyrosine kinase receptors, SOS, signal transducer and activator of transcription proteins (STATs) and others **(5)**. ERK can also enter the nucleus to control gene expression by phosphorylating transcription factors **(6)** such as ETS-like protein-1 (Elk-1), activation protein-1 (AP-1) or others, which are involved in inflammation, proliferation and cell survivale.

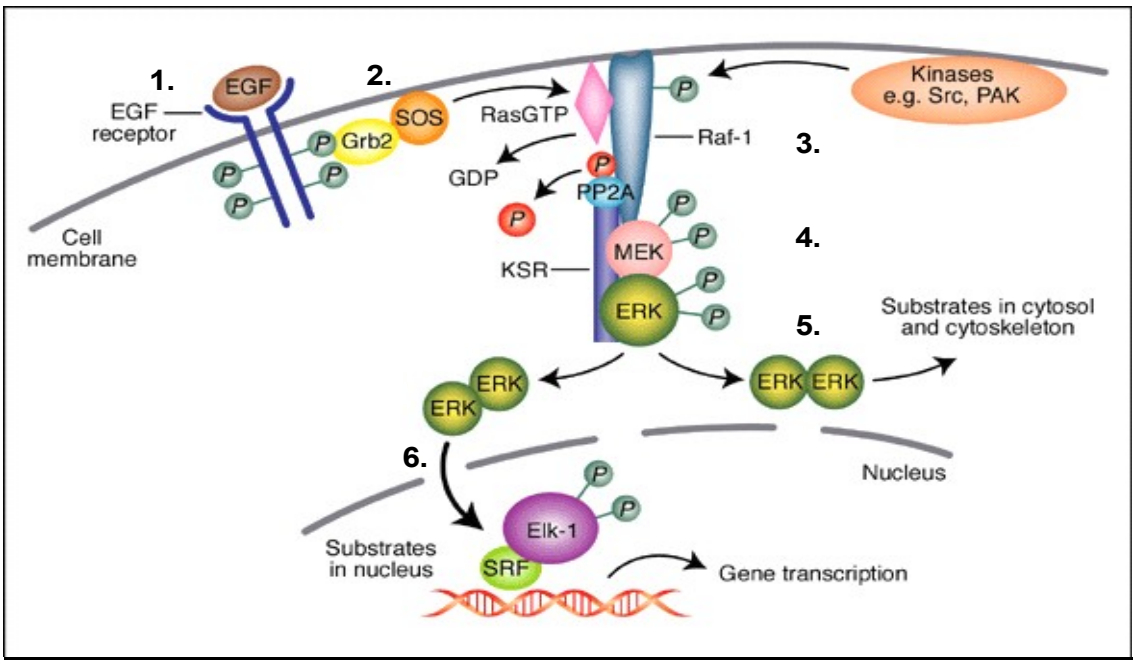


Figure 5.1:The organisation and function of the ERK pathway in response to epidermal growth factor. For more details see text. EGF (epidermal growth factor), MEK (mitogen activating protein kinase/ERK kinase), ERK (extracellular signal-regulated kinases), ELK-1 (ETS-like protein-1). Adapted from Kolch W.* et al *[143]

Here we detected the phosphorylated form of ERK-1/2 in caveolae and its amount was decreased in response to PAF (Fig 4.14.b). These findings suggest PAF inhibites the ERK-1/2 signalling pathway, maybe by dephosphorylation. Other groups discovered that overexpression of caveolin-1 dramatically inhibits the ERK-1/2 signalling pathway [62] whereas targeted downregulation of caveolin-1 increased basal ERK-1/2 activity [79]. It has been speculated that caveolin inhibits ERK-1/2 by stabilising the molecules in an inactive conformation within caveolin [137, 204]. This is in line with our own data that PAF induced an increase of caveolin-1 (Fig 4.2) and decrease of P-ERK-1/2 in caveolae (Fig. 4.14.b). Therefore we assume that caveolin might act as a negative regulator for the ERK-1/2 sig-

nalling in response to PAF.

Furthermore, administration of U0126, which inhibits both active and inactive MEK1/2, (phosphorylates ERK-1/2) showed no effect on PAF-induced edema formation in rat lungs indicating that ERK-1/2 is not involved in this process (Fig. 4.15). In contrast to our own data, other investigators have documented that the effect of PAF increased microvascular permeability in mouse mesenteric fat tissue by the ERK-pathway [317]. In future studies it will be important to study whether different mechanisms are operation in lungs and the gut.

5.1.5 Caveolae are sites of calcium entry

We demonstrated that PAF increases endothelial calcium levels *in situ* (Fig.4.10) and also the amount of caveolin-1 (Fig.4.2). Both effects were blocked by imipramine (Fig 4.4, 4.10). Thus, we surmised that the calcium response must be evoked by PAF-derived ASM-metabolites and calcium.

The important role of calcium for edema formation is well established. In previous studies our group observed that PAF-induced edema formation is partly dependent on an increase in intracellular calcium concentration and also on extracellular calcium [95]. In that case the rise of intracellular calcium is controlled by IP_3 dependent Ca^{2+} release from intracellular stores (e.g. endoplasmic reticulum; ER). This is supported by the findings that inhibition with L-108 (inhibit PI-PLC, which in turn activates IP_3) and Xestospongin C (specific IP_3R antagonist) attenuated PAF-induced edema formation [95]. Since edema formation in the IPL is also dependent on extracellular Ca^{2+}, this argues for an important role of a capacitive Ca^{2+} entry (CCE) through store operated calcium channels (SOC`s) [122] or receptor activation of Ca^{2+} channels. This is also supported by the finding that PAF-induced edema formation is reduced by lanthanum chloride, an unspecific calcium channel blocker [95]. These findings are further corroborated by the observation that thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, which to emptied intracellular Ca^{2+} stores and activation of SOC`s, induces edema formation in isolated rat lungs which is dependent on extracellular Ca^{2+} [44].

In the present context it is important to note that IP_3R, Ca^{2+}-ATPase have been described in the plasma membrane and especially in caveolae [70, 75, 246]. In many reports it is also described that IP_3R, Ca^{2+}-ATPase, PLCβ, $G_{aq/11}$ and SOCs are found in a multicomplex with caveolin-1 in membrane microdomains. These observation confirme our assump-

tion, that caveolae and SOC´s are involved in PAF increased vascular permeability [164, 196].

The mammalian homologues of the *Drosophila* transient receptor potential (TRP) gene family of channels are expressed in the plasma membrane of endothelial cells, function as SOC´s and are involved in the regulation of vascular permeability [30, 72, 162, 164, 196, 279, 280]. Here we identified TRPC-1 and TRPC-4 in caveolar fractions of pulmonary endothelial cells (Fig. 4.11). These findings support our hypothesis, that in response to PAF caveolae act as calcium entry ports and that might be regulated through TRPC-1 and/or TRPC-4.

The role of TRPC in store-operated entry has studied in reports of TRPC-4 being activated by G-Protein receptor coupled (as is the PAF-receptor) [30, 237]. A direct evidence for this and the TRPC-dependent Ca^{2+} entry, which is a key determinant of increased microvascular permeability, was provided in studies with TRPC4-knockout ($TRPC4^{-/-}$) mice and lung vascular endothelial cells (LECs) [279].

To examine the role of TRPC-1 and TRPC-4 for PAF-induced edema formation, the extracellular calcium entry through transient calcium channels in isolated perfused rat lungs was blocked with SKF 96365 (Fig 4.12). Recent studies have reported that the lanthanides lanthanum (La^{3+}) and gadolinium (Gd^{3+}), which are commonly used to block non-selective cation channels and other Ca^{2+}-permeable channels, have a differential effect on TRPC subtypes [237]. Unlike most other TRP-related channels, which are inhibited by La^{3+} and Gd^{3+} currents, TRPC-4 is potentiated by La^{3+} in a concentrations-depend manner [288]. Therefore we used SKF 96365, a relatively new non-specific blocker of TRPC, whose application *in vivo* has not be described so far, that was effectively used in cell culture [235, 236. However, SKF 96365 had no effect on PAF-induced edema formation in isolated perfused rat lungs (Fig. 4.12). In several studies it has been observed that the inhibitory effect of SKF 96365 differs in magnitude depending on the cell type and concentration [127, 149]. Whereas a concentration of SKF 96365 between 3 and 10 µM is responsible for inhibition of Ca^{2+} entry in cells, it has been observed that concentrations above 10 µM induce apoptosis or activate a novel cation entry pathway. In contrast to these findings it was found in human microglia cells that PAF induced entry of Ca^{2+} was attenuated in the presence of 20 µM SKF 96365 [236]. Neverthess, the action of SKF 96365 is poorly un-

derstood and this our findings should be interpreted with caution.

The calcium-sensing receptor (CaSR) plays an important role for sensing local changes in the extracellular calcium concentration and has emerged as an important mediator of a wide range of Ca^{2+}-dependent physiological responses Ca^{2+} signalling in various tissues. Therefore we examined the functions of CaSR in pathophysiological processes in lungs. However inhibition with NSP 2390 had no effect on PAF induced edema formation in lungs. This finding indicates that CaSR may not be important in this animal model of pulmonary edema (Fig. 4.13).

Taken together, our data demonstrate that PAF increases endothelial calcium levels and that this response is prevented by imipramine. This provides evidence for ASM-dependent ceramide mediating increased calcium levels. Whether the extracellular calcium entry is regulated through TRPC-1 and TRPC-4, remains to be shown. Further studies are required to clarify the role of TRP-channels in PAF-induced edema formation. To study the effects of PAF on permeability and endothelial calcium transients one could used various TRP-channel-deficient mice or pretreat the lungs with antibodies against TRP-channels.

5.1.6 Hypothetical mechanism of how PAF reduces NO production and increases calcium by the ASM-ceramide pathway

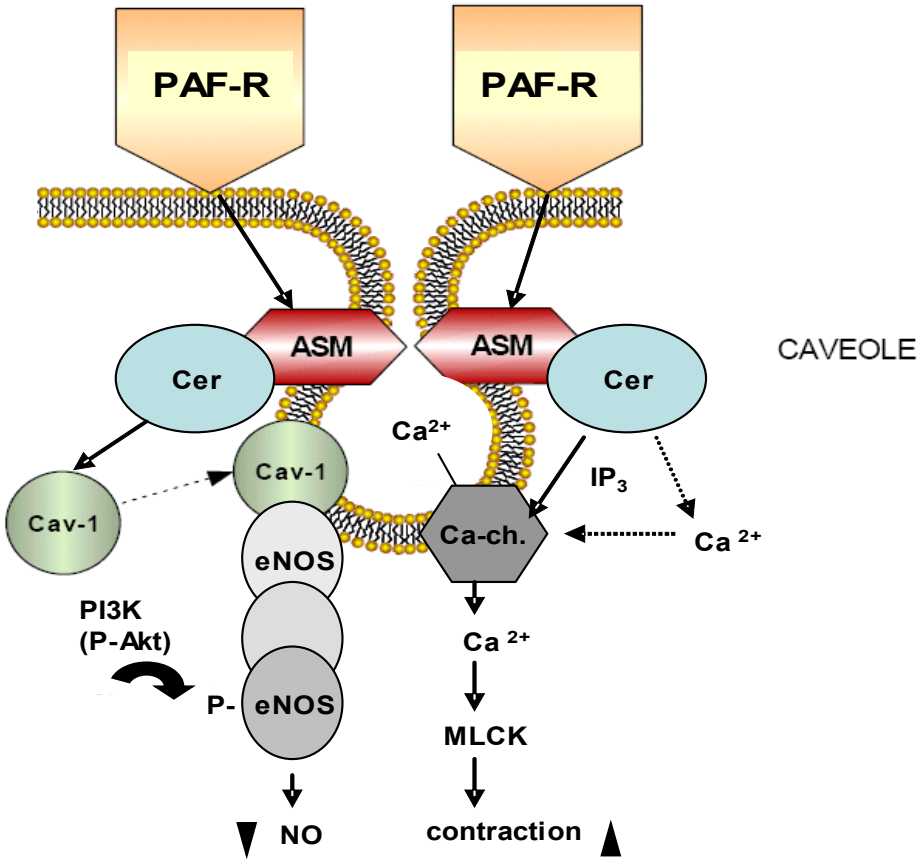
Figure 5.2 gives a schematic overview of the hypothetical mechanisms of how PAF attenuates NO-production by eNOS inhibition and increases calcium levels in pulmonary endothelial cells by ASM-dependent recruitment of caveolin-1 into caveolae of pulmonary endothelial cells.

PAF treatment recruits caveolin-1 into caveolae of pulmonary endothelial cells. This action is regulated by acid sphingomyelinase generated ceramide. Ceramide mediates an increase of caveolin-1 either by reorganization of present caveolae or formation of new caveolae in endothelial cells. The overexpression of caveolin-1 reduces NO production by direct binding of eNOS and indirectly through inhibition of downstream signal molecules PI3K/Akt from the Ca^{2+}-independent pathway of the eNOS activation. Whether the PAF-mediated NO reduction promotes or aggravates edema formation remains to be shown.

Pretreatment of the lungs with the ASM inhibitor imipramine prevented the PAF-induced increase in endothelial calcium levels and caveolin-1 indicating that caveolae could act as

a calcium entry port. Furthermore, transient receptor potential channels (TRPC-1 and TRPC-4) which regulates Ca^{2+} entry dependent on intracellular store depletion of Ca^{2+} are present in caveolae. Activation of TRPC possibly by IP_3 triggered release of Ca^{2+} out from intracellular stores may enhance the calcium signal. Enhanced Ca^{2+} concentration then leads to increased MLCK activity and hence to MLC phosphorylation, which leads to endothelial cell contraction thus enhancing vascular permeability. The role of TRPC in this hypothetical mechanism remains elusive.

Figure 5.2: Hypothetical mechansims of how PAF reduces NO production and increases calcium levels in pulmonary endotheilal cells. *For details see text. ASM (acid sphingomyelinase, Ca^{2+} (calcium, Ca-ch. (Ca^{2+}-channel, cav-1 (caveolin-1), cer (ceramide), eNOS (endothelial NO synthase, NO (nitric oxide), IP_3 (inositol 1,4,5-trisphosphate, MLCK (myosin light chain kinase, PAF (platelet-activating factor, P-Akt (phosphorylated Akt kinase, PI3K (phosphatidylinositol-3-OH kinase*



5.2 Thrombin mediated transcytosis

Previous studies demonstrated that an increase of albumin diffusion is not only the result of barrier "leakiness" but, rather, also an active process occurring primarily an endothelial vesicular pathway, named transcytosis [91, 185, 186]. In this study we report the novel observation that thrombin induced albumin transport in human microvascular endothelial cell (HMVEC) is mediated by sphingomyelinase-induced caveolin-1 recruitment into lipid rafts.

5.2.1 Thrombin activates albumin transcytosis

Thrombin is widely used to study the mechanisms of increased endothelial cell (EC) monolayer permeability *in vitro* [154, 168, 168]. Most of these data were obtained by measuring the transendothelial electrical resistance (TER) of monolayers as a measure of permeability [53]. This method provide a high temporal resolution but disregards transcellular transport [283]. The present study differs from these studies to thrombin-induced endothelial cells permeability in two aspects. First we used FITC-albumin diffusion rather than TER to detect changes in permeability and second, we measured albumin diffusion at time points (3-4 h) considerably later than usual (< 2 h). These modification enabled us to differ between thrombin increased albumin diffusion rates either by transcellular transport or paracellular leakage. These mechanisms are important in the development of permeability edema, because increased protein concentration on one side of the endothelial barrier lead to fluid flux driven by the oncotic gradient. Since albumin is essential for maintaining the oncotic pressure, it is important to clarify whether transcytosis can be activated in response to inflammatory mediators. Furthermore albumin is the major plasma protein of blood (composition of plasma protein: 60% albumin and 40% globulins) and has also an important cargo chaperone function. It binds to many water-insoluble substances (hormone, ions, fatty acid) in the plasma and facilitates their delivery across the vessel wall barrier. Albumin also plays an important role in the transport of drugs.

Using FITC-albumin diffusion to investigate endothelial monolayers permeability, we discovered that thrombin raised albumin diffusion to an extent that largely exceeds that of empty, i.e. cell-free, membranes. Note, that in these experiments FITC-albumin diffusion induced by thrombin was 230% of the diffusion rate across empty membrane (Fig. 4.17). This elevation of albumin diffusions rates across confluent endothelial monolayers by

thrombin to levels above the diffusion rate of an empty membrane could only be explained by active transcellular albumin-transport. Albumin transcytosis has been visualized before in mouse diaphragm [185] and in perfused lung capillaries [222] where transcellular gold-labelled-albumin transport was directly shown in electron microscopic images. Various studies of the endothelial barrier to define the transport pathway at an ultrastructural level showed the involvement of vesicles or vesicle-derived structures, particularly caveolar vesicle (caveosome), in the transport of macromolecules through continuous endothelial cells under physiological conditions [90, 91, 185, 187, 222, 208, 217]. In this thesis we report for the first time albumin transport under influence of inflammatory mediator, in this case thrombin. Thrombin-induced transcytosis was directly visualized by immune-electron microscopy (Fig. 4.21). Remarkably, albumin was found in close proximity to caveolin-1, indicating that albumin was transported in caveolin-1 containing vesicles by thrombin treatment of the cells (Fig 4.21). Fluorescence microscopy images of FITC-albumin diffusion in aortic endothelial cells showed that in thrombin treated cells FITC-albumin was in the inside of the vessel and also within endothelial cells. But in control cells FITC-albumin was detected only on the outside indicating that thrombin induced transcytosis (Fig. 4.26). These results demonstrate that albumin transcytosis is activated in response to the inflammatory mediator thrombin.

The mechanism of thrombin-induced permeability in endothelial cells that have been commonly defined depend on paracellular leakage by endothelial cell contraction and stress fibre formation [27]. Thrombin activates myosin light chain kinase (MLCK) and inactivates myosin phosphatase (through the Rho/Rho-kinase pathway) leading to stress fibre formation and actomyosin contraction and this results in endothelial gap formation by cytoskeleton rearrangement and increase permeability [82, 178]. Similar responses have also been observed in human pulmonary artery and human microvascular endothelial cells of the lungs (HMVEC-L) [138]. However, our findings show that endothelial contraction is not involved in thrombin-induced increases in albumin transcytosis. This is suggested by observation in HMVEC-L cells that simultaneous pretreatment with pharmacological inhibitors of Rho-kinase (Y-27632) signalling pathway and the ASM-ceramide pathway (imipramine, antibodies against ceramide) show an addition and completely inhibition of the thrombin-induced albumin translocation (Fig. 4.19.b). Therefore we propose that thrombin on the one hand induces cell contraction by Rho-kinase pathway, and on the other hand activates

albumin transcytosis possibly per ASM mediated activation of downstream signalling molecules.

5.2.2 Thrombin activates ASM and causes ceramide production

The sphingolipid ceramide is an important second messenger involved in apoptosis [142, 144], regulation of differentiation, growth suppression and cell senescence [145]. A new functional role of ceramide was observed by investigation of downstream signalling pathways mediateing changes in transendothelial permeability in HMVEC cells [88]. Lindner *et al* characterised the effects of ceramide on endothelial cells with the respect to endothelial cell permeability, apoptosis and necrosis [156]. It was demonstrated that the permeability-enhancing effects of ceramide is a non-apoptotic mechanism, which is independent of cytoskeleton rearrangements and which is mediated by unidentified PLC and Ser/Thr kinase isoenzymes in confluent EC-monolayers [156]. Furthermore, it is established that ceramide can be generated from sphingomyelin (SM, 90% are present in the outer leaflet of cell membrane) by ASM as a consequence of cell stimulation in membrane microdomains (caveolae) [25]. These findings and the findings, described below, supported our hypothesis that ceramide might be involved in thrombin-induced albumin transcytosis. Former our findings proposed a role of ceramide in the mediation of thrombin-induced transendothelial albumin diffusion: (i) ASM activity in the cell pellet was elevated after 30 min of thrombin stimulation which was accompanied by an increased ceramide content in cells (Fig 4.18) and (ii) the two pharmacological inhibitors of the ASM pathway, D609 [121, 177] and imipramine [1, 118], strongly attenuated thrombin induced transcellular albumin transport (Fig 4.19.a). The xantogenate D609 is an agent that was shown to inhibit ceramide production [167, 252], although its mechanism is discussed controversially [146, 166]. Imipramine is structurally unrelated to D609 and also described to inhibit ASM by destabilizing the enzyme [1, 118]. The only known common effect of both substance is to down-regulate ceramide generation. Both substances reduced thrombin-induced albumin diffusion to 100%, but not below (Fig.4.19.a) in HMVEC-L cells. Simultaneous pretreatment with imipramine and Y-27632 (Rho-kinase inhibitor) inhibited the thrombin-induced increases in FITC-albumin translocation to far below 100% (Fig. 4.19.b). We also observed that (i) pretreatment with an antibody against ceramide alone and in combination with Y-27632 inhibited thrombin mediated albumin translocation in HMVEC-L cells (Fig 4.19.b).

The effect of the antibody indicated that an extracellular form of ASM may be existent. In the last few years a lot of evidences for a secretory form of ASM, called S-ASM was described in the literature. Tabas *et al* characterised a S-ASM which is derive from the ASM gene and which under certain conditions can operate at neutral pH [240, 273]. A wide variety of cell types e.g. peritoneal macrophages, skin fibroblast, murine microglia cells, human monocytes, are established to secrete lysosomal ASM in the subcellular space [241]. The first evidence that endothelial cells secrete ASM was reported by Marathe *et al* [172]. He and his co-workers observed in human coronary artery and umbilical vein endothelial cells (HUVEC) after incubation with inflammatory cytokines (IL-1β and INF γ) an increase of S-ASM secretion, whereas lysosomal ASM activity was decreased. Our findings are some what different from this observation. In HMVEC-L cells ASM activity was not found in the supernatant of thrombin-stimulated cells, but only in the cells themselves together with enhanced ceramide production (Fig. 4.18). Thus with respect to the role of extracellular ASM the present data are somewhat conflicting: The effectiveness of the ceramide antibody and the increased albumin translocation by external ASM (Fig.4.29) speak in favour of a contribution of extracellular ASM in mediating thrombin-induced albumin transcytosis. On the other hand, the absence of extracellular ASM following thrombin and the absence of increased ceramide levels after addition of external ASM (Fig. 4.27) argue against a role of external ASM in this model. Possible explanations - that need to be further investigated - to reconcile these findings are that ASM is extracellular active, but is not released from the cells, and that the effect of extracellular ASM is too local to result in a detectable increase of ceramide. Taken together, our data demonstrated thrombin induced activation of sphingomyelinase (ASM), ceramide production and activates albumin transcytosis in a ASM-ceramide dependent fashion.

5.2.3 Thrombin-induced albumin transcytosis via caveolae

5.2.3.1 Caveolae and albumin transcytosis

Transcytosis was one of the first functional roles proposed for caveolae. In this process, caveolae transport macromolecules in caveosomes (caveolar vesicles) from the luminal side of the capillary endothelial cells to the subendothelial space. The functional role of caveolae in transcytosis is established. Transcytosis by caveolae was proven by (i) inhibition of caveolae formation, (ii) the use of caveolae-specific tracer molecules, such as gold-la-

belled-albumin, ratio-iodianted albumin, (iii) the development of caveolin-1 knockout mice. For example: it was observed that methyl-β-cyclodextrin, a caveolae disrupting agent, inhibits albumin transport through pulmonary microvascular endothelial monolayers, evidence for the fact that caveolae are involved in the transport process [128]. Caveolin-1 knockout mice, which lack detectable caveolae in endothelial cells, show no interstitial accumulation of gold-labelled albumin in contrast to wild type mice [250]. Similarly, when incubated with ratio-iodianted albumin, isolated aortic rings from caveolin knockout mice showed no uptake of this tracer, while aortic rings from wild-type animals showed temperature- and time-dependent albumin uptake [250]. The first clues that albumin was internalized via receptors came from morphological studies where albumin association with endothelial cells was concentrated in caveolae open to the blood vessel lumen [90, 91, 185]. Four groups of albumin-binding proteins (ABP) were identified (molecular mass 18, 31, 60, and 72 kDa) which specifically bind to albumin and are expressed on the endothelial cell surface [89, 128, 187, 244, 259]. Further biochemical and functional studies have shown that the glycoprotein gp60 is the albumin-binding receptor and mediates albumin-internalization in caveolae: (i) gp60 association with caveolin-1, (ii) depletion of gp60 reduced albumin uptake, (iii) activation of gp60 by cross-linking using an antibody against gp60 caused up to a threefold increases in uptake and transcellular transport of albumin [182]. Thus it now seems clear that caveolae do indeed mediate transcytosis (i.e. transcellular transport) of specific macromolecules in endothelial cells. The mechanism how transcytosis of albumin is regulated is shown in figure 5.3 [33]. In brief: clustering of gp60 and albumin **(1)**, induced G_i-protein coupled activation of Src **(2)**, Src in turn phosphorylates caveolin-1, dynamin-2 **(3)**, indicating caveolae formation and endocytosis of albumin **(4)**, trafficking of caveolae by interaction with $v_{vesicle}$-SNARE **(5)**, interaction of caveolae with t_{target}-SNARE on target membrane **(6)** induced albumin exocytosis **(7)**. But the signalling pathways that regulate formation and release of caveolae from the plasma membrane are poorly understood. Therefore, a major aim in this study was to investigate the regulation of these pathways by proinflammatory mediators.

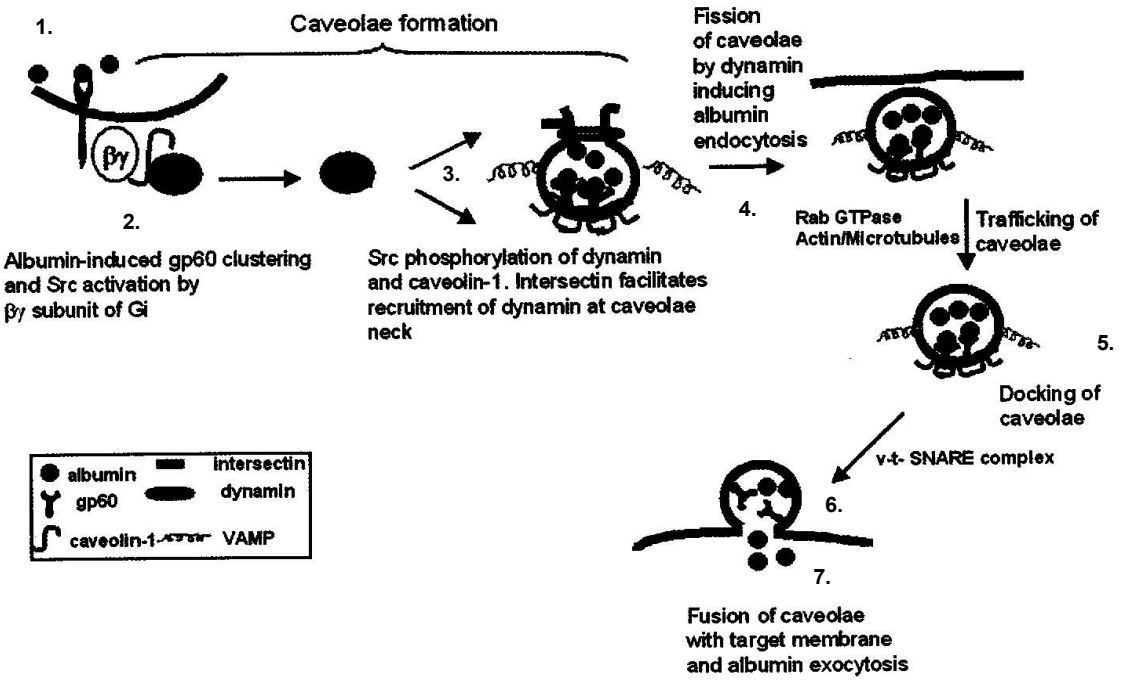


Figure 5.3: Signalling mechanisms regulating albumin transcytosis. For detail see text. Adapted from Minshall et al. [187]

5.2.3.2 Thrombin increase caveolin-1

Some biochemical properties of membrane microdomains such as (i) resistance to Triton X-100 solubilisation at 4°C, (ii) richness in glycosphingolipids and cholestrol as well as (iii) enrichment with lipid anchored membrane proteins are useful for their isolation from endothelial cell membranes [8]. We successfully isolated membrane microdomains from endotheilal cell membranes by methods described from Melkonian *et al.* and Arni *et al.* [10, 108]. First the whole membrane is seperated and then caveolae from the membrane itself are from the cytosol isolated. So we were able to quantify both caveolin-1 content in the cytosol, membrane and within membrane microdomains. The abudance of caveolin-1 in the isolated membrane microdomains suggests that caveolae and not lipid rafts were isolated form HMVEC-L cells [30]. The content of caveolin-1 within caveolae of HMVEC-L cells significantly increased within 2 h after thrombin treatment (Fig 4.20.A). The increased amount of caveolin-1 in the plasma membrane was confirmed by immune histochemistry as well as by electron microscopy (Fig. 4.20.B, C). Electron microscopy further indicated that in untreated cells caveolin-1 resided dispersed in the cytoplasm, whereas in thrombin-treated cells it was found clustered in the membrane (Fig.4.20.C) These findings demonstrated that thrombin-induced increase of caveolin-1 in endothelial cell membrane.

5.2.3.3 Thrombin-induced activation of caveolin-1 de novo *synthesis*

Measurements of cav-1 in whole cell lysates show an elevation of caveolin-1 in thrombin treated cells in comparison to control cell lysates (Fig.4.23). Therefore, we examined if thrombin-induced this increase of caveolin-1 either through activation of *de novo* synthesis or by translocation of present caveolin. We blocked the protein biosynthesis by inhibition of transcription with actinomycin D (ActD, a commonly used blocker [56]) and disrupted the translation-step of protein biosynthesis with cycloheximide (CHX) [155]. With both inhibitors, we observed a reduction of the cav-1 content in membranoes caveolae fraction (Fig. 4.25) as well as in the whole cell lysates (Fig. 4.24) in thrombin treated HMVEC-L. These findings and the fluorescence- and electron microscopic images from the caveolin-1 protein distributation in cells (Fig. 4.20.B, C) let as assume that thrombin-induced the activation of *de novo* synthesis of caveolin-1 in cells and thereafter cav-1 translocation to the cell membrane. Evidence for thrombin-induced *de novo* synthesis was reported in HUVEC-cells by Huang *et al* [117]. He and his co-workers also used actinomycin D to block the expression of angiopoietin-2 (Ang-2, necessary for remodeling in angiogenesis) and demonstrated that the thrombin-mediated Ang-2 mRNA synthesis was blocked by actinomycin D [56]. It has been reported that endothelial cells, when stimulated by thrombin via protease-activated receptors (PAR), express various mediators and proteins including cytokines, chemokines, growth factors, and adhesion molecules, therefore it seems likely that thrombin stimulates protein biosynthesis. In any can, mechansim of thrombin-induced increase of caveolin-1 in caveolae from cell membrane require further investigation.

5.2.3.4 Thrombin-induced increase of caveolin-1 is mediated by ASM-ceramide pathway

Our data suggest a role for ASM in the mediation of increased albumin transcytosis induced by thrombin: (i) ASM and ceramide synthase activity were increased within 30 min of thrombin stimulation, which was accompanied by an increased ceramide content (Fig. 4.18), (ii) two structurally inhibitors of the ASM pathway, D609 [137, 252] and imipramine [1, 118], attenuated thrombin-induced albumin transcytosis (Fig. 4.19.a), (iii) external addition of ASM activated transcytosis and increased cav-1 in caveolae isolated from cell membranes (Fig. 4.29, 4.28), (iv) ceramide antibodies did block the effect of thrombin-induced transcytosis (Fig. 4.19.b), indicating a role for extracellular ASM. How exactly sphin-

gomyelinase activates transcytosis remains unknown. Our findings suggest that ASM somehow leads to *de novo* synthesis of cav-1 (Fig. 4.22, 4.23), which is known to be involved in albumin transcytosis. Pretreatement of HMVEC-L cells with D609 and imipramine reduced the thrombin-induced increase of cav-1 in caveolar fractions and in whole cells (Fig. 4.22, 4.23). Blocking protein synthesis with ActD and CHX had a similar effect (Fig. 4.24, 4.25), suggesting *de novo* synthesis of caveolin-1. Subsequently cav-1 is moved to the plasma membrane where it forms clusters in what presumably are caveolae, as demonstrated by the histological figures togehter with the immunoblots from membrane fractions (Fig. 4.20, 4.22). Similar mechanism was proposed by Rizzo *et al*. He and his group reported in bovine arortic endothial cells (BAEC) that chronic shear stress stimulates caveolae formation by cav-1 translocation from the Golgi apparatus to the luminal plasma and alters cell signalling responses [229]. Other groups observed in cultured HUVEC cells by using indirect immunofluorescence and double fluorescence labelling that in control cells, cav-1 was primarly localized on the cell surface, but also inside the cells. And in cells, which was exposed to laminar flow, an increase of cav-1 expression was observed [270, 271]. It is also important to note that cav-1 binds different lipids (or proteins) and this protein-lipid interaction is described as the driving forces for caveolae fromation [69]. Taken together, a potential mechanism for increase caveolin-1 in plasma membrane could be that thrombin mediates recruitment of newly formed caveolin-1 ceramide production, it froms new caveolae. Since caveolae are required for transcytosis of albumin [217], this hypothesis explain how thrombin manages to increase albumin transcytosis.

5.2.4 (Patho)physiological importance of thrombin activates albumin transcytosis

In the past years publication of several systematic reviews assessed the effect of albumin (or synthetic colloids) versus various crystalloids on mortality of critically ill patients. An overview of this studies is shown in table 5.1. There are several choices of colloid and there is an ongoing debate about the relative effectiveness of colloids compared to crystalloid fluids. Our study adds to this controversial discussion. We describe a mechanism which may explain why albumin administration does not fails to decrease the risk of acute lung injury or to improve survival.

For 70 years albumin solutions are widely used in fluid resuscitation of critically ill patients

(such as severe burns, sepsis, trauma, acute circumstances (ARDS)) to treat low serum albumin concentrations. The advantages of albumin are (i) it has effectively replaces volume and supports colloid oncotic pressure (COP), (ii) unlike synthetic colloids (dextran, hydroxyethylstarch (HES or HAES), gelantine), it has transport functions and binds reversibly to anions, cations, and some substances that are active or toxic only in the free form, (iii) it has anticoagulant properties, inhibiting platelet aggregation and enhancing the inhibition of factor Xa by antithrombin III [129, 130]. However, albumin is pooled from human donors, is in short supply and remains expensive compared to synthetic colloids, thus it should be used with caution.

Based upon Starling`s equation, a reduced colloid osmotic pressure gradient between the intravascular space and the interstitium can promote edema formation, a characteristic feature of acute respiratory distress syndrome (ARDS). Re-establishment of this gradient through colloid administration might serve to diminish edema. Increasing osmotic pressure with colloidal products has remained an attractive theoretical premise for volume resuscitation. However, clinical studies demonstrated that infusion of albumin alone, indeed increased colloid osmotic pressure in clinical practice, but did not improve the critical situation of patients [129, 130]. The main drawback to colloid therapy lies in the pathological states with endothelial injury and capillary leak, in other words the clinical scenario where colloids are commonly given. The colloid solution may leak into the interstitium and remain there exerting an oncotic gradient, pulling additional water into the interstitium, particulary if the capillary walls become much more permeable under the influence of inflammatory mediators. Another plausible explanation results from the findings in this thesis. We observed that an added inflammatory mediator, i.e. thrombin, induced an increase of transcellular albumin transport via caveolae in human pulmonary endothelial cells. This transcellular transport of albumin, also referred to as transcytosis, could increase COP. For the first time we demonstrated that inflammatory mediators may activate albumin transport through the endothelium suggesting that transcytosis via caveolae may play an important role in the development of pulmonary edema. Whether caveolae will also transport HES and gelantine, synthetic colloids, is not esthablished. But, dextran, another commonly used synthetic colloid, is found in caveolae, although it is unknown whether caveolae meditate transcytosis of dextran. To clarify the possibility of dextran transcytosis, Measurements of FITC-labelled dextran diffusion, in the established EC monolayer system, would be helpful.

Another possible significance of transcytosis activated by inflammatory mediators becomes clear by the following exemplary considerations. It is established that neutrophils are adhering to the injured capillary endothelium, and imigrate through the interstitium into the air space. Activated neutrophils release various cytokines (TNF) and other proinflammatory mediators, such as platelet-activating factor. Here we have shown that PAF increases cav-1 in caveolae isolated from pulmonary endothelial cells. Thus PAF-induced edema formation may be also involve transcytosis of albumin. Further studies are required to study this hypothesis in *in vivo* model of inflammation (administration of LPS, TNF α and subsequent purification of caveolae from pulmonary EC to measure the cav-1 content in comparison to control animals). To investigate the importance of transcytosis in inflammation one could also use our established *in vitro* model (FITC-albumin diffusion in EC-monolayer) under influence of LPS or TNF α.

Table 5.1: ***Summary of randomised trials comparing colloid and crystalloid fluid resuscitation*** *that met criteria for inclusion.*

Author	***Critical illness***	***Number of trials (Patients)***	***Treatment***	***Conclusions***
Velanovich (1989) [298]	*Trauma*	*8 (826)*	*Colloid vs crystalloid (?)*	*In trauma patients colloid resuscitation is equal to crystalloid* *In non-trauma patients treatment with colloids is efficacious.*
Bisonni et al. *(1991) [22]*	*Surgery, hypovolemia, and* ***ARDS***	*7 (354)*	*Colloid vs crystalloid (?)*	*No significant mortality-rate advantage in using colloids; the cost-effectiveness ratio for crystalloids is much lower compared to colloids; crystalloids should always be used in resuscitation efforts.*
Wade et al. *(1997) [304]*	*Trauma*	*13 (1233)*	*HDS vs HS*	*Treatment with HSD resulted in a survival until discharge of 37.9% compared with 26.9% with standard care*
Schierhout and Roberts (1998) [239]	*Trauma, Surgery, Burns, Sepsis,* ***ARDS***	*19 (1315)*	*HA vs HC, IC*	*Resuscitation with colloids was associated with an increased absolute risk of mortality of 4% or 4 extra deaths for every 100 patients resuscitated*
Cochrane Injuries group (1998) [48]	*Heterogeneous, seriously ill;* *Surgery, Trauma, Sepsis (Hypovolemia)* *Burns* *Hypoalbuminemia*	*24 (1204)* *13 (534)* *3 (163)* *8 (507)*	*HA vs RL*	*No evidence that HA administration reduces mortality in critically ill patients with hypovolaemia, burns, or hypoalbuminaemia and a strong suggestion that it may increase mortality*
Choi et al. (1999) [46]	*Trauma*	*17 (814)*	*HA vs IC*	*No difference was observed between crystalloid and colloid resuscitation with respect to mortality and* ***pulmonary edema****; but crystalloid resuscitation associated*

Author	*Critical illness*	*Number of trials (Patients)*	*Treatment*	*Conclusions*
				with a lower mortality in trauma patients.
Wilkes and Navicks (2001) [312]	*Burns, Hypoalbuminaemia, premature infant*	*55 (2958)*	*HA* vs RL*	*No effect of albumin on mortality was detected; this finding supports the safety of albumin.*
Boldt et al. *(2003) [28]*	*Heterogeneous, seriously ill;* *Trauma, cardiac surgery, ICU* *non-trauma*	*40 (2454)* *35 (2341)* *5 (113)*	*HA vs HES*	*HA is the preferred first-line volume therapy in patients at risk for coagulation disorders. With respect to platelet function, volume replacement with low-molecular-weight HES solutions can be recommended in this situation without any risk.*
Rizoli et al. *(2003) [227]*	*Trauma*	*6 (?)*	*Colloid vs crystalloid (?)*	*The choice of fluid may have a small or no effect on mortality.* *Trauma; use of colloids is associated with a trend toward increased mortality, patients should continue to be resuscitated with crystalloids.*
Lang et al *(2003) [152]*	*Surgery*	*1 (36)*	*HES vs RL*	*Replacement with HES compared to crystalloid solutions reduces the inflammatory response in patients undergoing elective major abdominal surgery.*

*Abbreviations: ARDS (acute respiradory destress syndrome), HA (human albumin), HC (hypertonic crystalloid), HES (hydroxyethylstarch), HSD (hypertonic saline/dextran), HS (hypertonic saline (7.5 % NaCl), IC (isotonic crystalloid), ICU (intensive care unit), RL (Ringer lactate solution), (?) no statements of particulars, * used concentration of albumin 2.0-20 %, fat-marked; studies include data from patients with lung injury, pulmonary edema or ARDS.*

5.2.5 Hypothetical mechanism of thrombin induced transcytosis

Next page figure 5.4. summarises a hypothetical mechanism of thrombin-induced transcytosis in endothelial cells. Taken together our findings demonstrated that albumin transcytosis can be activated by thrombin. We have described a novel functional role of thrombin and explain how thrombin manages to increase albumin transport through the sphingomyelinase pathway, including activation of sphingomyelinase (ASM) and which generating of ceramide. Increased ceramide levels may lead to enrichment of caveolae with caveolin-1, the moving from the cytosol into the cell membrane, were its may form new caveolae. Inhibition of ASM with two structurally dissimilar inhibitors (D609 and imipramine) attenuated both cav-1 recruitment and albumin transport. This mechanism appears to depend on protein synthesis, because cav-1 recruitment was also prevented by blocking transcription with actinomycin and translation with cycloheximide.

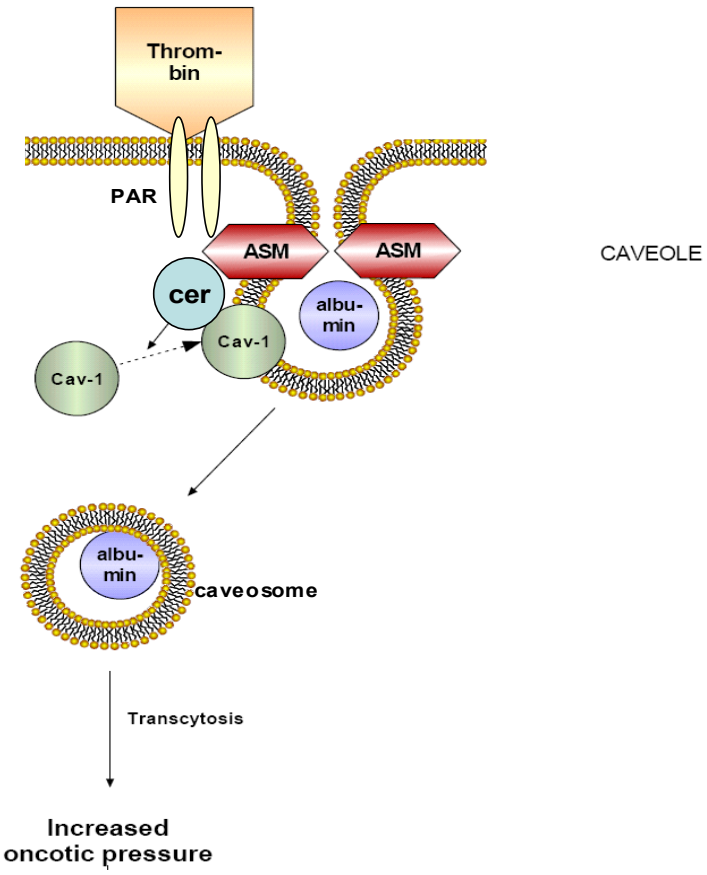


Figure 5.4: Hypothetical mechanism of thrombin-induced transcytosis. For details see text. ASM (acid sphingomyelinase), cav-1 (caveolin-1), cer (ceramide), PAR (protease-activated receptors)

6 Summary

In the present study, the mechanisms of increased vasculary permeability were investigated in isolated perfused rat lungs and in endothelial cell monolayers. In particular, the role of membrane microdomains (caveolae) was investigated. Our data indicate that caveolae and caveolin-1 play an important role in PAF-induced edema formation in the lungs. In addition, the activation of transcytosis by the inflammatory mediator thrombin represents a novel potentially pro-edematogenous mechanism.

To investigate the role of caveolae and caveolin-1 in PAF-induced pulmonary edema, we established a method to isolate caveolae from pulmonary endothelial cells from intact rat lungs. Subsequently, these caveolae were analysed by immunoblotting. PAF treatment increased the expression of caveolin-1 in caveolae of endothelial cells within five minutes. Imipramine and D609, two structurally dissimilar inhibitors of the ASM-pathway, reduced the PAF-induced increase of caveolin-1 and also the development of pulmonary edema. Because of the widely discussed role of NO in the regulation vascular permeability and because of the established negative regulation of endothelial NO synthase (eNOS) by caveolin-1, PAF-induced NO-production of the pulmonary vasculature was investigated in isolated rat lungs by *in situ* fluorescence-microscopy. PAF reduced NO-production in pulmonary endothelial cells and this was blocked by imipramine. In addition, PAF attenuated the activation of Akt kinase, another possible activator of eNOS. These findings suggest that PAF inhibits eNOS by recruitment of caveolin-1 to caveolae, and by interfering with the Ca^{2+}-independent eNOS activation, i.e. the Akt kinase pathway. Our pharmacological data further suggest that all these effects of PAF do depend on the activation of ASM.

Based on the important role of intracellular calcium in the regulation of vascular permeability, we used *in situ* fluorescence microscopy to demonstrate that PAF increases intracellular Ca^{2+}-concentrations in pulmonary endothelial cells. Inhibition of ASM with imipramine largely prevented the PAF-induced Ca^{2+} rise. Taken together with the other effects of imipramine, in particular its effects on caveolin-1 recruitment and edema formation, and in view of the presence of TRPC1 and TRPC4 Ca^{2+}-channels in caveolae we hypothesize that in PAF-induced permeability changes caveolae may serve as Ca^{2+}-entry ports and that this process is initiated by ASM activation. In summary, the PAF-activated ASM-path-

way leads to enrichment of caveolin-1 in caveolae, downregulation of eNOS and upregulation of cytosolic Ca^{2+}.

In another major part of this thesis we showed that thrombin increases FITC-albumin transfer across confluent human microvascular endothelial cell (HMVEC-L) monolayers to an extent that largely exceeds that of empty layers, suggesting active transcellular transport. Thrombin-induced albumin transcytosis was directly shown by immune-electron microscopy. Thrombin induced activation of ASM, ceramide production and enrichment of lipid rafts with caveolin-1. The latter was observed to move from the cytosol into lipid rafts. Inhibition of ASM with two structurally dissimilar inhibitors (D609, impriamine) attenuated both cav-1 recruitment and albumin transport. This mechanism appears to depend on protein biosynthesis, because cav-1 recruitment was also prevented by blocking transcription with actinomycin D and translation with cycloheximide. Our findings indicate that thrombin stimulates transcellular albumin transport in a sphingomyelinase-dependent fashion by inducing *de novo* synthesis of cav-1, which is then transferred to lipid rafts. This mechanism may be important for edema development, because increased protein concentrations on the basolateral side of the endothelial barrier may lead to fluid flux driven by the oncotic gradient.

The present work describes the critical role of caveolae and caveolin-1 in PAF-induced pulmonary edema formation. We also show for the first time that inflammatory mediators like thrombin may induce albumin transcytosis. Apparently, ASM plays a central role in the initiation of these processes. Possibly, ASM may serve as a novel therapeutic agent in the treatment of pulmonary edema.

7 Deutsche Zusammenfassung

In der vorliegenden Arbeit wurden Mechanismen der erhöhten vaskulären Permeabilität in isoliert perfundierten Rattenlungen und kultivierten Endothelzellen untersucht. Insbesondere wurde die Rolle von Membranmikrodomainen (Caveolaen) überprüft. Die erhalten Daten unterstützen die Hypothese, daß Caveolen und Caveolin-1 eine wichtige Rolle bei der Ödembildung in der Lunge (ausgelöst durch den Plättchen-aktivierenden Faktor) spielen. Ebenfalls konnte die Aktivierung der Transzytose durch den Entzündungsmediator Thrombin, als ein neuer potentiell pro-ödematogen wirksamer Mechansimus aufgezeigt werden.

Um die Rolle von Caveolen/Caveolin-1 beim PAF-induzierten Lungenödem zu untersuchen, wurde eine aufwendige Methode zur Aufreinigung von Caveolen aus Endothelzellen intakter Rattenlungen etabliert. Anschließend wurden mittels Immunoblots die Veränderungen in den Caveolen unter dem Einfluß von PAF analysiert. PAF erhöhte die Expression von Caveolin-1 in den Caveolen pulmonärer Endothelzellen. Imipramin und D609, zwei chemisch verschiedene Hemmstoffen der Saure Sphingomyelinase (ASM) reduzierten sowohl die PAF-induzierte Erhöhung von Caveolin-1 also auch das Lungenödem. Da die negative Regulation der endothelialen NO-Synthase (eNOS) durch Caveolin-1 gut bekannt ist und NO auch die vaskuläre Permeabilität reguliert, wurde die PAF-induzierte NO-Bildung in Endothelzellen im intakten Organ mit Hilfe der *in situ* Fluoreszenzmikroskopie untersucht. Unsere Daten legen nahe, daß PAF die NO-Bildung in pulmonalen Endothelzellen durch direkte Bindung von eNOS an Caveolin-1 und indirekt durch die Blockade der Downstream-Signalmoleküle PI3K/Akt (Ca^{2+} unabhängigen Signalweg der eNOS Aktivierung) unterdrückt. Auch diese Wirkung von PAF ist abhängigt von der Aktivierung der ASM.

In weiteren Experimenten konnte mittels *in situ* Flureszenzmikroskopie ein Anstieg der intrazelluläre Ca^{2+} Konzentration nach PAF-Gabe demonstriert werden. Vorbehandlungen der Lunge mit Imipramin reduzierte den PAF-induzierten endothelialen Ca^{2+} Anstieg. Da Imipramin auch den Caveolin-1 Gehalt von Caveolen reduzierte und die TRP-Kanäle 1 und 4 (Ca^{2+} Kanäle) in Caveolen nachgewiesen wurden, stellen wir die Hypothese auf daß Caveolen als Ca^{2+} „Eintrittspforte“ fungieren können. Zusammengefaßt, zeigen diese Befunde, daß der durch PAF-aktivierte ASM-Weg zur Anreicherung von Caveolin-1 in Caveo-

len führt, zur herrunterregulierung der eNOS und zur Heraufregulierung des zytosolischen Kalzium.

Unsere *in vitro* Untersuchungen an Zellen zeigen eine ganz neue Funktion von Thrombin. In konfluenten Endothelzellmonolayern beobachteten wir, daß Thrombin die FITC-Albumin Diffusion so starkt erhöht, daß nur ein aktiver Albumin-Transport in Frage kommt. In immuno-elektronenmirkoskopischen Aufnahmen konnte der Thrombin-induzierten Transport von Albumin durch die Endothelzelle direkt gezeigt werden. Thrombin induzierte die Aktivierung von ASM, die Bildung von Ceramid und Caveolin-1 in pulmonären Endothelzellen. Fluoreszenz- und elektronenmikroskopischen Aufnahmen zeigten , daß Thrombin den Transfer von Caveolin-1 aus dem Zytosol in die Zellmembran induzierte. Hemmung des ASM-Weges mit Imipramin und D609 verringerte sowohl den Caveolin-1 Gehalt als auch den Albumintransport. Dieser Mechansimus scheint abhängig von der Caveolin-1 *de novo* Synthese zu sein, da die Caveolin-1 Rekrutierung durch Actimomycin D und Cycloheximide (Hemmstoffe der Proteinbiosynthese) blockiert wurde. Unsere Daten zeigen, daß Thrombin den transzellulären Transport von Albumin stimuliert, indem es ASM-abhängig die Caveolin-1 Neubildung und dessen anschließenden Transfer in die Caveolen der Endothelzelle vermittelt. Da die Transzytose von Albumin den onkotischen Druck erhöhen kann, kann es auch über den Mechanismus zu einer verstärkten Ödembildung kommen.

In der vorliegenden Arbeit wurde die zentrale Rolle von Caveolen und Caveolin-1 im Hinblick auf die PAF-induzierte Ödembildung in der Lunge dargelegt. Weiterhin zeigen wir erstmals, daß ein Entzündungsmediator wie Thrombin auch Transzytose auslösen kann. Eine zentrale Rolle bei der Vermittlung der hier beschrieben (patho)physiologischen Mechansimen in der Zelle spielt dabei die ASM. Möglicherweise bietet daher die ASM einen therapeutischen Ansatz für die Behandlung von Lungenödemen.

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Eike Reppien

Gerichtstr.45
22765 Hamburg
Telefon: 040-38699396

Persönliche Angaben

Geburtstag:	23.10.1972
Geburtsort:	Rostock, Mecklenburg-Vorpommern
Staatsangehörigkeit:	deutsche Staatsbürgerschaft

Ausbildung

1979-1989	10. Klassige Allgemeinbildende Polytechnische Oberschule, in Rostock
1989-1992	Höhere Medizinische Berufsfachschule, Ausbildung zur Medizinisch-technischen Laborassistentin (Note: „gut“), in Greifswald
1992-1995	Abendgynasium, Abitur, in Rostock
1995-1998	Universität Rostock Studium der Biologie/Vordiplom
1998-2002	Freie-Universität Berlin Studium der Biologie/Diplom (Note: „gut“)
08/01-03/02	Diplomarbeit am Robert Koch-Institut bei Prof. Dr. R. Burger, Abteilung „Zelluläre Infektabwehr“, Thema: „Charakterisierung von Differenzierungsantigenen auf Leukozyten des Meerschweinchens mit Hilfe von monolklonalen Antikörpern“ (Note: 1,8), in Berlin
seit 01/2003	Promotion bei Prof. Dr. S. Uhlig Abteilung Lungenpharmakologie Forschungszentrum Borstel, Leibniz Institut für Medizin und Biowissenschaften