Stig Sverre Tyvold

Bronchial Microdialysis

Thesis for the degree of Philosophiae Doctor

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Norwegian University of Science and Technology
Faculty of Medicine
Department of Circulation and Medical Imaging

NTNU – Trondheim
Norwegian University of
Science and Technology

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Norsk sammendrag

Bronkial mikrodialyse

I denne avhandlingen beskrives utviklingen av en ny metode for kontinuerlig overvåkning av sammensetningen av væskefilmen som kler luftveiene i de minste bronkiene i store dyr og mennesker (bronkial mikrodialyse). Utgangspunktet var ideen om at eksisterende metoder hadde vesentlige begrensninger. Ideen var å finne en metode for kontinuerlig måling som ikke skadet pasienter. Metoden skulle evalueres under overvåking av lungepåvirkning etter eksperimentell avklemming-gjenoppretting av tarm sirkulasjonen og under aortakirurgi.

Det første arbeidet evaluerte mikrodialyse i bronkiene og blod i et eksperimentelt oppsett med kontrollert blod-konsentrasjonen av et lite molekyl og et større molekyl. Vi konkluderte med at bronkial microdialyse kunne måle konsentrasjonen av molekyler i væskefilmen som kler luftveiene i de minste bronkiene. Bruken av en endogen korreksjonsfaktor (urea) forbedret nøyaktigheten.
I det andre arbeidet ble bronkial mikrodialyse brukt for å beskrive den inflammatoriske responsen i bronkiene i en grise-modell med avklemming-gjenoppretting av tarm sirkulasjonen. Vi fant en økning i den bronkiale konsentrasjonen av inflammatoriske molekyler i løpet av de første 60 minuttene av perioden med gjenopprettet tarm sirkulasjon.
Det tredje arbeidet var en studie av inflammatorisk respons hos pasienter som gjennomgikk planlagt kirurgi for utposning på hovedpulsåren. Et bredt spektrum av inflammatoriske molekyler ble målt i bronkiene og i blod under og umiddelbart etter operasjonen. Inflammatoriske molekyler økte i løpet av de første 60 minutter av gjenopprettet sirkulasjon etter at pulsåre-graftet var på plass.

Avhandlingen konkluderer med å svare på forskningsspørsmålene: Bronkial microdialyse er gjennomførbart hos gris og mennesker og er i stand til å måle dynamikken i inflammatoriske molekyler i bronkiene uten registrerte skader. Men metoden trenger videre evaluering og utvikling for å støtte riktige klinisk beslutninger på et tidligere tidspunkt enn det som er mulig med de metoder som er tilgjengelige i klinikken i dag.

Cand.med. Stig Sverre Tyvold
Institutt for sirkulasjon og bildediagnostikk, NTNU

Hovedveileder: Professor Petter Aadahl
Biveileder: Førsteamanuensis Erik Solligard

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Original papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I Continuous monitoring of the bronchial epithelial lining fluid by microdialysis.
Tyvold SS, Solligård E, Lyng O, Steinshamn SL, Gunnes S, Aadahl P.
Respir Res. 2007 Nov 1;8:78.

II Bronchial microdialysis of cytokines in the epithelial lining fluid in experimental intestinal ischemia and reperfusion before onset of manifest lung injury.
Tyvold SS, Solligård E, Gunnes S, Lyng O, Johannisson A, Grønbech JE, Aadahl P.
Shock. 2010 Nov;34(5):517-24.

III Bronchial microdialysis monitoring inflammatory response in open abdominal aortic aneurysm repair.
Tyvold SS, Dahl T, Dragsund S, Gunnes S, Lyng O, Damås JK, Aadahl P, Solligård E.
Manuscript.

Summary

This thesis describes the stepwise development of a new method for monitoring the dynamic composition of the epithelial lining fluid in bronchi in large animals and humans. The starting point was the belief that existing methods had significant limitations. Existing methods were invasive and their use created lung injury or failed to measure substances of interest. The idea was to find a method for continuous measurement that did not harm patients. The method was to be evaluated monitoring lung involvement in organ crosstalk after experimental intestinal ischemia-reperfusion injury and during aortic surgery.

The first paper evaluated microdialysis in the bronchi and blood in an experimental setting with controlled blood-concentration of one small substance (lactate) and one larger substance (fluorescein-isothiocyanate-dextran 4000Da). We concluded that bronchial microdialysis could measure concentration of substances within the bronchial epithelial lining fluid. The use of the concentration gradient of an endogenous correction factor (urea) improved the accuracy. In the second paper we used bronchial microdialysis to describe the inflammatory response in the bronchi in a pig model of intestinal ischemia-reperfusion injury. We found an increase in the bronchial concentration of inflammatory mediators within the first 60 minutes of intestinal reperfusion after intestinal ischemia. The third paper was a study of the inflammatory response in patients undergoing aortic surgery. A broad spectrum of inflammatory markers was measured in bronchi and blood during and immediately after surgery. Inflammatory mediators increased within the first 60 minutes of reperfusion of the lower body when the aortic graft was in place. The concentrations of seventeen out of eighteen inflammatory mediators, as measured by bronchial microdialysis, were significantly different in patients suffering organ failure directly after surgery.

The thesis concluded by answering the thesis questions and claims that bronchial microdialysis is feasible in pigs and humans, can be done with no registered complications and is able to measure the dynamics of inflammatory mediators in the bronchi. However the method needs further evaluation of methods to ease secure placement of the probe, measure the absolute concentration of substances in the bronchial epithelial lining fluid. The delay in analysis of cytokines needs to be significantly reduced to take advantage of the results in support for expedited clinical judgement.

Abbreviations

AAA	abdominal aortic aneurysm
AECC	American European Consensus Conference
ATP	adenosine triphosphate
ALI	acute lung injury
ARDS	acute respiratory distress syndrome
ARF	acute respiratory failure
BAL	bronchial alveolar lavage
BALF	bronchial alveolar lavage fluid
cGMP	cyclic guanosine monophosphate
CPAP	continuous positive airway pressure
CT	X-ray computer tomography
DAD	diffuse alveolar damage
EE	extraction efficiency
ELF	epithelial lining fluid
ENaC	epithelial sodium channel
FD-4	fluorescein isothiocyanate dextran 4000Da
GFR	glomerular filtration ratio
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte macrophage colony-stimulating factor
HSP	heat shock protein
IFN	interferon

IL	interleukin
IR	ischemia-reperfusion
MCP	monocyte chemotactic protein
MIP	macrophage inflammatory protein
MOF	multiple organ failure
MPAP	mean pulmonary artery pressure
Na+-K+ ATPase	sodium-potassium pump
NF-κB	nuclear factor-kappa B
NO	nitric oxide
N-PCP-III	N-terminal procollagen peptide-III
NAD^+/NADH	nicotinamide adenine dinucleotide
AAA	abdominal aortic aneurysm
PAI-1	plasminogen activator inhibitor-1
PEEP	positive end-expiratory pressure
PMN	polymorphonuclear neutrophils
RCT	randomized controlled trials
SMA	superior mesenteric artery
TGF	transforming growth factor
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor

Introduction

The living organisms general response to ischemia-reperfusion (IR), trauma, bleeding, infection, transfusion, transplantation, heat and cold is a host defense response. The host defense response comes from the innate immune system and is characterized by a non-specific, immediate maximal, humoral, cell-mediated and neuronal response[1, 2].
This thesis is dedicated to improve the method of monitoring of the bronchial epithelial lining fluid (ELF) and thereby better describe the immediate inflammatory response in the lung as a response to distant IR injury as measured by cytokines. This piece in the large puzzle of the innate immune response has intrigued researchers and clinicians for decades.

Lung injury in response to distant trauma

The lung responds to distant trauma with a variety of presentations. Generally the severity of the lung failure varies with the severity of the trauma, but to a large degree the response also varies between individuals exposed to similar trauma. The clinical presentation of lung failure can be mild and transient resolving within 24 hours or with severe dysfunction in both the oxygenation and ventilation function. These presentations are stratified in the scientific literature as acute respiratory failure (ARF), acute lung injury (ALI) or acute respiratory distress syndrome (ARDS).

Definitions

One of the major problems with research on lung injury in response to distant trauma is the lack of unique and accurate definitions.
ARF is a problematic denomination since it is not uniquely defined. The most common criteria used are defining oxygenation failure, ventilatory failure or the need for endotracheal intubation and mechanical ventilation:

1. PaO_2/FiO_2-ratio less than 26.7 kPa[3].
2. Hypoxia <8kPa and/or hypercarbia >6.5 kPa[4].
3. Endotracheal intubation and mechanical ventilation ≥ 24 hours[5].

ARDS was first defined by Ashbough in 1967[6]. A new and expanded consensus definition of ALI and ARDS came in 1994 and has been used of clinicians and researchers until this year, table A[7].

Table A. American European Consensus Conference criteria for ALI and ARDS (1994)[7].

Timing	Acute onset
Oxygenation	ALI: Pao2/FiO2 <300 mm Hg (<40kPa) ARDS: Pao2/Fio2 <200 mm Hg (<26.7kPa) Regardless of positive end-expiratory pressure (PEEP)
Chest radiograph	Bilateral infiltrates seen on frontal chest radiograph
Pulmonary artery wedge pressure	<18 mmHg when measured or no clinical evidence of left atrial hypertension

"However, after 18 years of applied research, a number of issues regarding various criteria of the AECC definition have emerged, including a lack of explicit criteria for defining acute, sensitivity of PaO_2/FIO_2 to different ventilator settings, poor reliability of the chest radiograph criterion, and difficulties distinguishing hydrostatic edema", and to address the previous limitations this year the new Berlin criteria for ARDS was presented[8], table B.

Table B. The Berlin definition of ARDS (2012)[8].

Timing	Within 1 week of a known clinical insult or new or worsening respiratory symptoms.
Chest imaging[a]	Bilateral opacities-not fully explained by effusions, lobar/lung collapse, or nodules.
Origin of edema	Respiratory failure not fully explained by cardiac failure or fluid overload. Need objective assessment (eg, echocardiography) to exclude hydrostatic edema if no risk factor is present.
Oxygenation[b]	
Mild	200 mmHg < PaO_2/FiO_2 ≤ 300 mmHg with PEEP or CPAP ≥5 cm H_2O[c]
Moderate	100 mmHg < PaO_2/FiO_2 ≤ 200 mmHg with PEEP ≥5 cm H_2O
Severe	PaO_2/FiO_2 ≤ 100 mmHg with PEEP ≥5 cm H_2O

Abbreviations: CPAP, continuous positive airway pressure; FiO_2, fraction of inspired oxygen; PaO_2, partial pressure of arterial oxygen; PEEP, positive end-expiratory pressure.

[a]Chest radiograph or computed tomography scan.

[b]If altitude is higher than 1 000 m, the correction factor should be calculated as follows: (PaO_2/FiO_2 x (barometric pressure/760)).

[c]This may be delivered noninvasively in the mild acute respiratory distress syndrome group.

Incidence

Luhr et al found the incidence in Scandinavian countries to be for ARF 77.6/100 000, ALI 17.9/100 000 ARDS 13.5/100 000 which is in accordance to findings in similar studies from Europe and Northern America[5, 9].

Mortality and morbidity

Luhr et al found mortality of ARF (41%) to be similar to the mortality of ALI and ARDS[10–13]. According to two meta-analysis papers from 2008-2009 mortality of ARDS was 43-44%[5]. Phua et al points out that the mortality was lower in randomized controlled trials (RCTs) in fact 36% and that there were no reduction in mortality after 1994[14, 15]. Zambon and Vincent found a reduction in mortality of 1.1% per year in the period 1994-2006[15]. In a review published in 2012, Matthay et al refers to trials where the ARDS network has tested new interventions as low tidal volume ventilation, conservative fluid therapy and enteral additives (omega-3, gammalinolenic acid and antioxidants) and found reduced mortality from 36 to 22% in the period from 1996 until the most recent presented trials[14].
Morbidity and mortality in ARF, ALI and ARDS are mainly caused by multiple organ failure (MOF)[16]. In a recent 5 year follow up study Herridge et al found pulmonary function to be normal or near normal, but physical capacity were reduced and recovery was slower in older versus younger patients[17]. Bienvenu et al found an accumulated incidence of depression of 40% and physical impairment of 66 % the first two years after ALI[18].
Hopkins et al found that neurocognitive function was reduced in 70% at hospital discharge and in about 50 % in 1 and 2 year follow up. Quality of life was still reduced at 2-year follow up[19].

Etiology, pathogenesis and pathophysiology

There is no uniformly accepted description for ARF. ALI and ARDS have been extensively described and this section will deal with these conditions.
Causes are divided into direct (pulmonary) and indirect (extrapulmonary). Direct causes are amongst others pneumonia, aspiration of gastric contents, inhalational injury, fat embolism, near drowning and reperfusion injury after lung transplantation and lung embolectomy. Examples of indirect causes are sepsis, severe trauma, transfusion of blood products, cardiopulmonary bypass, vascular surgery with IR, drug overdose and pancreatitis[10–13, 20].

In the acute phase (circa day 1 to 6), the exudative phase, there is alveolar and interstitial edema with accumulation of polymorphonuclear neutrophils (PMN), macrophages and red blood cells (RBC) in the alveoli. Lung endovascular injury and lung epithelial injury causes hyper-permeability. Hyper-permeability allows protein-rich fluid to flow into the lung interstitium and the alveoli, and establish interstitial and alveolar edema. The lung is affected with disturbed gas-exchange, reduced lung compliance, increased work of breathing and mismatch of ventilation and perfusion[21]. The lung tissue present with diffuse alveolar damage (DAD), accumulation of PMNs, disrupted epithelial integrity, denuded basal membrane, pulmonary edema, fibrin depositions, endothelial injury and micro-thrombi[16].
In the sub-acute phase (circa day 7 to 14) there are signs of repair with proliferation of lung epithelial type II cells, occurrence of lung fibroblasts and start of collagen deposition.
In the chronic phase, the fibro-proliferative phase, there is resolution of the acute PMN infiltrate and edema. There are increased numbers of mononuclear cells and alveolar macrophages. In some patients there are fibrosis, but in many patients resolution progresses without fibrosis[16, 22].

Atelectasis

The edematous lung parenchyma and airways filled with secretions result in non-ventilated alveoli. The gas in these areas is resorbed with resulting consolidated lung. Atelectasis cause ventilation-perfusion mismatch with shunting of deoxygenated blood[21, 23].

Coagulation

Coagulation and inflammation interacts during secondary ARF, ALI and ARDS. The initial trauma initiates an imbalance between pro- versus anti-coagulants and fibrin building versus fibrinolytic activity. Deposition of intra-alveolar fibrin inactivates surfactant and acts as and chemo-attractant of PMNs. Pro-coagulant activity is characterized with increased activity of tissue factor and factor VIIa. Decreased fibrinolytic activities are due to inhibition of plasminogen activators (urokinase and tissue type) by plasminogen activator inhibitor -1 and blockage of plasmin by α2-plasmin inhibitor[24, 25].
The outcomes are microthrombosis and diffuse endothelial dysfunction/damage.

Polymorphonuclear cells and platelets

The PMN pathway is the best described in secondary lung failure. The activation of PMNs is a major factor in the development, persistence and outcome of ARDS. PMNs in conjunction

with platelets cause lung endothelial damage. In experimental studies with PMN depletion and inhibition of chemo-attractants the severity of lung injury was reduced[26].
In the blood stream activated PMNs release pro-inflammatory and pro-coagulant mediators, reactive oxygen species and proteases. Leukotrienes, thromboxanes and prostaglandins, metabolites from arachidonic acid are released. The result is aggregation of platelets and leukocytes and vascular constriction. Obliteration of pulmonary vessels cause dead space ventilation and subsequent ventilation-perfusion mismatch[27].
In response to distant trauma endothelial cells express increased activity of the transcription factor nuclear factor-κB (NF- κB) and this causes increased expression of adhesion molecules on the endothelial surface (intercellular adhesion molecule-1, vascular adhesion molecule-1). β2-Integrins are expressed on endothelial cells and interacts with adhesion molecules in the migration of PMNs from blood to the interstitium[27].
In patients undergoing open abdominal aortic aneurysm (AAA) repair the concentration of PMNs in vena cava are found to higher than on the arterial side[28]. This was interpreted as an indication of removal of activated PMNs in the pulmonary circulation.

Volume and pressure trauma of ventilation

The affection of the lung parenchyma is heterogeneous with regional atelectasis and regional thrombosis. The ventilation-perfusion mismatch leads up to reduced oxygenation of arterial blood. The choice of ventilator strategy to overcome this challenge is still under debate. There is general agreement upon a strategy with low tidal volumes (6(-8)ml)[29]. Still the question of optimal positive end expiratory pressure (PEEP) question is unsettled. Some studies support that moderate PEEP prevent over-distension of alveoli that are open. Other studies suggest more aggressive recruitment maneuvers and higher PEEP to open the lung and keep it open to avoid “atelectrauma” with shear stress forces on airways during cyclic closing and reopening[30–32].

Pulmonal hypertension

Pulmonary hypertension appear early in the course of ALI, ARDS and ARF. The pulmonary hypertension is a result of several factors.
The lung endothelium produce mediators that regulates vascular tone (endothelins, prostanoids and nitric oxide) and metabolize vasoactive mediators as angiotensinogen-I by angiotensinogen converting enzyme. In ALI there is a shift towards increased vascular tone.

With edema formation and atelectasis some lung regions are poorly ventilated. In hypoxic lung-regions the compensatory mechanism of pulmonary hypoxic vasoconstriction shifts blood to ventilated regions leading to reduced ventilation-perfusion mismatch[33].
The pro-coagulant state as discussed earlier results in diffuse micro-thrombi and infarctions. In addition cellular sequestration contributes to capillary occlusion.
Interstitial edema compresses capillaries. Positive pressure ventilation with high PEEP and plateau pressure may add to this effect.

The role of biomarkers in secondary lung failure

The cascades of cytokines, coagulation factors and growth factors are closely related and interconnected. The mediators of inflammation interact with lung tissue and alter function. Lung endothelium and epithelium are activated. With progress of inflammation dysfunction and disruption evolves. In the early phase there is an increased amount of fluids in the lung interstitium and alveoli. This is caused by increased leakage through the lung endothelium and epithelium. There is also decreased transport of salt and water from the alveoli and into the lung interstitium.

Bronchial Cytokines

Altered concentrations of cytokines have been found to correlate to the risk for development of ALI and ARDS.
Donnelly found bronchial alveolar lavage fluid (BALF) IL-8 > 200 pg/ml to be predictive of for the development of ARDS in intensive care unit patients at risk[34]. In a recent study Raymondos et al found that patients with BALF IL-8 > 200 pg/ml also had increased BALF concentrations of IL-6, TNF-α, IL-1β, IL-10 and IL-1ra levels. Plasma pro-inflammatory cytokines were also increased[35]. In a study of ARDS and non-ARDS patients admitted to the intensive care unit Lee at al found a similar inflammatory profile in both groups except for HSP 47[36]

Systemic cytokines

Pro-inflammatory cytokines as IL-1β and IL-8, pleiotropic cytokines as TNF-α, IL-6 and IL-13 and anti-inflammatory cytokines as IL-10 are all correlated to outcome in patients with ALI/ARDS[22, 37, 38].

Coagulation and fibrinolysis

In patients with ALI/ARDS studies have identified abnormal levels of tissue factor, factor VIIa, vonWillebrand factor, protein C, plasminogen activator inhibitor (PAI)-1, α2-plasmin inhibitor, thrombomodulin and fibrin[25, 39]. Increased plasma Protein C, elevated BALF PAI-1 and thrombomodulin are associated with worse outcome[24, 40].

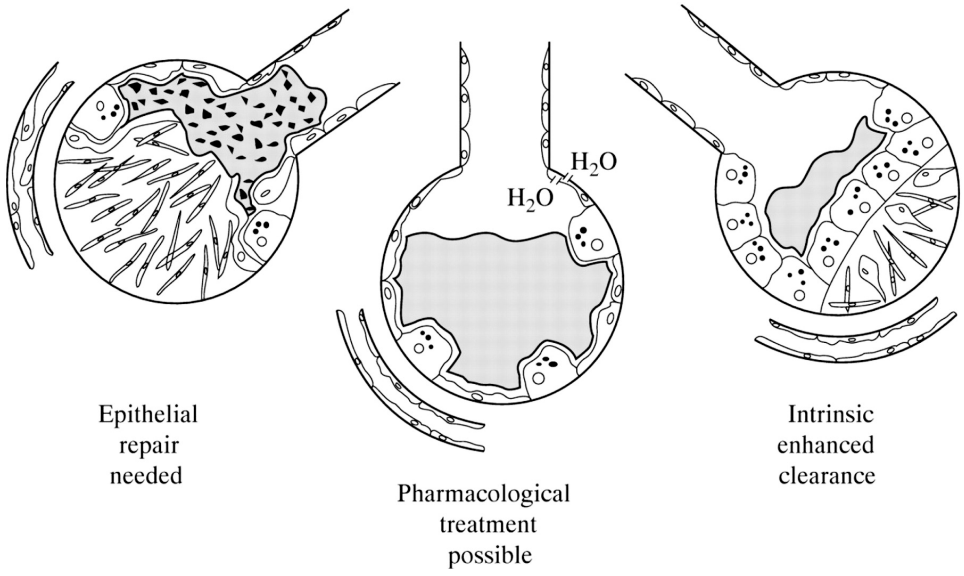
Growth factors

In the classic separation between time periods in ALI/ARDS proliferation occurs after 7 to 14 days. But in ARDS/ALI patients N-terminal pro-collagen peptide-III (N-PCP-III) a mediator of fibro-proliferative activity have been found already within 24 hours of the diagnosis, and increased N-PCP-III was associated with increased mortality[41–43].
Vascular endothelial growth factor (VEGF) has a role both to increase vascular permeability and as a mitogen for endothelial proliferation. Measurements of VEGF in BALF have been conflicting and the role of VEGF as a biomarker remains unclear. Low concentrations in BALF of ARDS patients may be caused by alveolar flooding of fluid[44].
Keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) are potent mitogens for type II alveolar epithelial cells and they are found to enhance motility, increase resistance to injury, surfactant production, decrease apoptosis and release of autocrine factors[45].

The formation and resolution of alveolar edema

In the intact lung epithelia separates compartments and facilitates transport. One important mechanism is salt and water transport. Active Na^+ transport and secondary active Cl^- transport with consequent osmotic transport of water. There are two stages of damage to the blood-alveolar barrier. In secondary acute mild to moderate lung injury there is dysfunction in the tissue, but in severe states of acute secondary lung injury the disruption of endothelium and epithelium requires repair with cellular growth to cover the basement membrane before regulation of ion transport can be restored, figure 1[46].
The endothelium is a semi-permeable barrier that lines the vasculature and regulates fluid and solute exchange between the blood and interstitial space. The surface of the endothelium is anti-thrombotic and anti-inflammatory. With the exposure to activated PMNs, platelets, chemokines and cytokines the endothelium shifts to a state of dysfunction. The endothelium expresses adhesion molecules and pro-coagulant factors (tissue factor, vonWillebrand Factor). IL-1β increases permeability by inducing TGFβ, increasing actin-stress fibers and disruption of gap junctions[47].

Figure 1. Schematic diagram illustrating 3 potential alveolar environments that can be encountered in an injured lung. In regions where there is significant injury to the epithelium, epithelial repair will be needed before stimulation of ion transport can be achieved. In regions where normal epithelial function is preserved, pharmacological stimulation is possible. Finally, in regions where a proliferative response is present, there could be an intrinsic enhanced clearance[46]. Used with permission.



TNFα is proposed to contribute to endothelial apoptosis and edema formation by inducing reactive oxygen species production, destabilizing microtubules and thereby changes in the cytoskeleton with paracellular gap formations[48–52]. But Börjesson et al found TNFα to be essential to protect the alveoli from flooding in a rodent intestinal IR model[53].
Although there are early and profound changes in the lung vascular endothelium it seems that the prerequisite for alveolar edema is changes in function in the alveolar epithelium. In the normal lung the alveolar epithelium is impermeable to proteins. Fluid transport is regulated through apical epithelial sodium channels (ENaC). The fluid transport is driven by the basal adenosine triphosphate Sodium-Potassium pump (Na^+-K^+-ATPase) and up-regulation of the basal Na^+-K^+-ATPase can itself be sufficient to increase alveolar fluid clearance[54, 55]. The lung epithelial is resistant to injury and in models of hypoxemia, intestinal IR and intravenous or intra-alveolar infusion of endotoxin (LPS) the alveolar fluid clearance is increased by induction of apical ENaCs and basal Na^+-K^+-ATPase activity[54–56].

The apical ENaCs are sensitive to many stimuli. ENaC activity is reduced by amiloride, β-blockers, IL-1β, TGFβ and NO via soluble guanylyl cyclase and increased cGMP production. On the other hand β-agonists, dopamine, glucocorticoids and reactive oxygen species are all shown to increase ENaC activity. TNFα show conflicting results. Some studies concludes that TNFα decrease ENaC activity and others have concluded that TNFα increases alveolar fluid clearance when comparing to groups receiving TNFα antibody[53, 57–59].
The lung epithelium maintains the ability to clear alveolar fluids in the absence of circulation. Even in the presence of moderate interstitial edema and moderately elevated left atrial pressure alveolar fluids are cleared by compensatory increased clearance. The lymphatic system and in some instances the pleural space can clear interstitial fluids. Nevertheless the lung circulation is the most important system for the clearance of excess fluids in the lung interstitium. When the pressure of the lung circulation and/or the lung interstitium increases further the clearance of alveolar fluids will be limited and insufficient[55]. Nevertheless in early secondary lung involvement, the inflammatory process is fully activated even prior to alveolar flooding.

Inflammatory response to open abdominal aortic aneurysm surgery

The overall trauma of open AAA repair is a combination of general anesthesia with positive pressure ventilation, surgery (surgical dissection, bowel manipulation, intestinal hypothermia, mesenteric traction, aneurysm manipulation) and IR-injury. The procedure of elective open AAA repair is a surgical two hit model with an increased risk for development of remote injury in lungs, failure of kidneys and MOF. In addition patients with ruptured aneurysms suffer an initial period of variable hypotension and hypovolemic shock. In this group a more severe and sustained inflammatory response occurs and MOF is more frequent. The inflammatory response is characterized by an early activation of the cascades of coagulation, cytokines, eicosanoids and immune cells (PMNs)[60, 61].

Cytokine response to surgery

The local release of cytokines at the site of surgery elicits a guided inflammatory response that in the end results in wound healing. Research on the quantity and the quality of the cytokine response has resulted in cytokine measurements being a parameter on prognosis. Roumen et al studied plasma cytokines in blunt trauma, elective and acute open aortic surgery, and found that IL-1β, TNF-α and IL-6 were increased in non survivors[62]. This underlines the importance of cytokines as markers of prognosis.

Cytokines in ischemia-reperfusion injury by infrarenal aortic cross clamp

Open AAA repair induces a systemic inflammatory response with substantial release of cytokines in blood[28, 61, 63–71]. Our group has previously studied this response in the evaluation of open versus endovascular repair of abdominal aortic aneurysms[60]. IL-6 correlates to the duration of aortic crossclamp and the development of SIRS and late complications[61, 70]. In addition to find overall outcome parameters a substantial effort has been done to find the main source of the lung response after aortic crossclamp.

Gut-lung hypothesis

During the procedure of open AAA repair the gastrointestinal tract are exposed to manipulation, hypothermia and hypoperfusion/ischemia. The hypoperfusion/ischemia may be facilitated by traction, bend on the mesenterium and absent blood flow of the inferior mesenteric artery.

Mediators from the gut can be transported by blood or lymph to the systemic circulation. Blood samples from the portal or the inferior mesenteric vein have shown increased IL-6 concentrations when compared to systemic values[63, 68]. Norwood et al compared IL-6 from the portal vein with the femoral vein and systemic samples, and they found that IL-6 values were highest in the portal vein[61]. But in experimental animal models with isolated gut IR-injury repeated experiments have shown that ligation of the thoracic duct lymph vessel to a great extent blunt the PMN recruitment in lungs and reduce vascular leak both in lung and intestine. The lymph in reperfusion contained high concentrations of TNFα, IL-1β and IL-10[72, 73]. It was further shown that lung tissue from animals exposed to IR-injury with ligated thoracic duct had lower levels of IL-1β, IL-10 and VEGF[74].

Limb-lung hypothesis

In patients undergoing open AAA repair there are infiltration of PMNs in muscle tissue, endothelial activation and release of cytokines from the lower limb[75–77]. Concentrations from the femoral vein of IL-6 and IL-8 are found to be higher than the systemic concentration[78]. Norwood et al compared the release of activated PMNs, as measured by increased expression of integrins (CD11b), in the femoral and portal vein compared to the systemic circulation and found that activated PMNs mainly came from the lower limb[79]. In experimental animal models lymph collected from ischemic hind legs activates human pulmonary microvascular cells and human PMNs[80, 81].

Summing up the trauma of open AAA repair is sufficient to activate PMNs, lung alveolar endothelium inflammatory and coagulation cascades. The gut and the lower limbs send out their mediating signals via the lymph and thereby play up to the concert of organ crosstalk.

Bronchial measurements

Hemodynamic and respiratory parameters available in the clinical setting fail to identify the inflammatory changes proceeding to clinical manifest ARF, ALI and ARDS.

Both systemic and respiratory markers of inflammation have been studied to predict ALI/ARDS[34, 82, 83]. Donnely et al found BALF IL-8 to predict the development of ALI/ARDS[34]. In a clinical study on BALF from patients 2 to 6 hours after trauma Raymondos et al used IL-8 >200pg/ml to assign patients to a high-risk for ARDS group. Five out of eight patients in the high-risk group and only two out of sixteen in the low risk group developed ARDS. In addition the high-risk group had increase levels of alveolar IL-1β, IL-1ra, IL-6, IL-10 and TNFα[35].

In a recent review of the biomarkers of ALI/ARDS the conclusion still have similarities with the state of previous decades. There are limited findings and no consensus. New markers are still not thoroughly evaluated. Proteomics and genomics are new fields that remain to be explored. Combination of biomarkers improves the predictive value, but there is still no "gold standard"[38]. A main problem not addressed by the review is the content of the samples and the methods used to validate existing biomarkers.

ELF of the bronchi has been examined by BAL, direct aspiration, microsampling, and exhaled breath condensates. BAL and other bronchoscopic techniques have in common that they are invasive, may create lung injury[84–86], are based on single or intermittent samples and therefore have limited value as continuous and dynamic monitors of the ELF of the lung. The exhaled breath condensates technique is non-invasive and continuous, but is indirect and best suited for collection of non-volatile hydrophilic solutes and measurements do not correlate to BAL measurements[87–89].

The natural course from a sufficient stimulus to an inflammatory response, early tissue dysfunction and to manifest clinical lung symptoms is a dynamic process. Each time period of minutes, hours and days have their own characteristic presentation. The dynamics of inflammatory markers in the period before and in the beginning of the microscopic changes may reveal new knowledge with continuous sampling and greater time spatiality.

Microdialysis can continuously monitor metabolism and protein concentration with a potential of good time spatiality and may be a tool to improve the monitoring and prognostication of ARF, ALI and ARDS.

Aims of the study

The overall aim of this thesis was to explore and evaluate a new method, bronchial microdialysis, for continuous sampling of the molecule composition, especially cytokines, in ELF in early lung involvement after distant IR-injury in both an experimental model and in the clinical setting. The specific aims were:

- Evaluate bronchial microdialysis for continuous and dynamic monitoring of the time-to-time changes in the composition of the ELF by lactate and FD-4 in a pig model. Evaluate arteriobronchial urea gradient as a correction factor.

- To determine if bronchial microdialysis can identify the immediate inflammatory response in the bronchi as measured by IL-1β, IL-8, and TNFα and monitor the time-to-time changes of these cytokines during the first 4 h of reperfusion after intestinal ischemia in a pig model.

- To investigate a broad spectrum of the bronchial and systemic inflammatory response in a time relation to the phases of open AAA repair and the immediate postoperative period by measuring 18 cytokines by microdialysis.

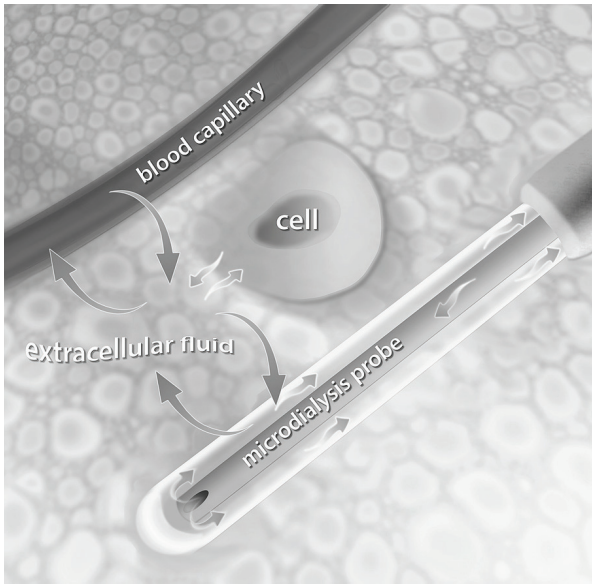
Methodological considerations

The methods have been discussed in detail in each manuscript. Only general comments of the methods will be given.

Microdialysis

The microdialysis technique as we know it today developed over decades[90]. It all started with dialysis sacs, push-pull cannulas and dialytrodes[91–93]. In 1974 Ungerstedt and Pycock established the first setup with "hollow fibers" in the brain[94]. The "hollow fiber" was intended to mimic a capillary blood vessel, figure 2.

Figure 2. The Microdialysis Catheter mimics a blood capillary. Substances from the extracellular fluid of the tissue diffuse across the membrane of the catheter into the perfusion fluid inside the catheter. Used with permission.

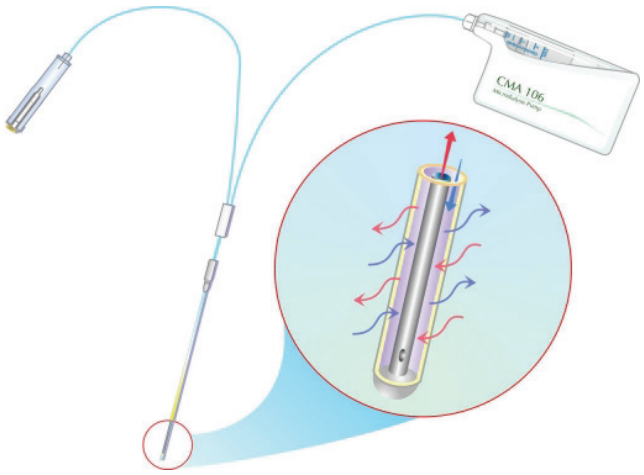


The method was refined and used in experimental animal models throughout the early eighties[95–98]. In the late eighties early nineties microdialysis was applied in humans[99–101]. In the beginning microdialysis in humans was clinically used as a monitor of aerobe versus anaerobe metabolism (lactate-pyruvate ratio), cell injury (glycerol) in brain tissue after neurotrauma, neurosurgery and skin flaps after reconstructive surgery. New tissues have been

targeted. Examples are measurements of larger molecules as cytokines and concentration of medication in tissues of interest. A search on “microdialysis” on PubMed October 2012 returns a number of 14425 publications (2709 on humans).

Microdialysis probes were designed to monitor the extracellular compartment and to mimic capillaries, figure 2. The probe consists of two plastic tubes with one inside the other and an inlet and outlet part. At the end the microdialysis probe is formed as a concentric tube. The perfusion fluid enters the space between the inner tube and the outer dialysis membrane. This is where the “dialysis” takes place, i.e. the diffusion of molecules between the extra cellular fluid and the perfusion fluid. The diffusion passively follows the concentration gradient (Fick’s first law of diffusion). Perfusion fluid is pumped into the inlet and is collected at the end of the outlet line, figure 3. Though microdialysis is most often used to measure molecule concentration in different tissues the method can also be used a substance delivery system with reversed concentration gradients.

Figure 3. Microdialysis catheter with pump and collection vial connected. Schematic presentation of the semipermeable membrane. Used with permission.



Today there are a wide range of probes for research and also clinical application. The physical appearance is adjusted to the application with difference in length of the probe and the semipermeable membrane (1 to 30 mm). The semi-permeable membrane material characteristics and pore-size (6-100kDa) vary to determine which molecules that can be collected.

Microdialysis was traditionally used in brain and skin flaps after reconstructive surgery. Today microdialysis is used in most solid organs, glands, in the intestinal lumen and in tumors. Microdialysis is applied as a monitor of metabolism, sampling of proteins, pharmacodynamic/pharmacokinetic studies and as a drug delivery unit.
When a probe is implanted into solid tissue bleeding and inflammation ensue and a washout period is necessary. A probe can function for a long time, up to 30 days, and the membrane is resistant to bio-fouling[102]. But in solid tissue a foreign body reaction can lead up to the build of a capsule that alter the local milieu shown to decrease the recovery of IL-6 already after 3 days[103]. To my knowledge this challenge is not applicable to luminal microdialysis.

Extraction efficiency, relative recovery and mass transport

In two fluid compartments with different composition separated by a semipermeable membrane the molecules that can diffuse over the membrane will equilibrate over time. With microdialysis fluid on the inside of the membrane is exchanged regularly. The speed of exchange is dependent on the microdialysis pump flow, usually 0.1 to 5 µl/min.
The ability to measure the molecule of interest is called the extraction efficiency, formula 1.

Formula 1. Extraction efficiency (EE).
C_{out} and C_{in} are the concentrations of the molecule in the dialysate and perfusate. C_{medium} is the absolute concentration in the surroundings of the membrane (tissue/fluid).

$$EE = \frac{C_{out} - C_{in}}{C_{medium} - C_{in}}$$

If C_{in} is zero the formula can be simplified to C_{out}/C_{medium}. This factor is called the relative recovery, which is used to communicate the overall performance of the microdialysis method.
The absorption of the molecule is a total description of the mass transport of the molecule in and out of the microdialysis catheter. This is used to calibrate the system. The mass transport is composed of sequential properties by the molecule and the external medium (fluid/tissue). The components are mass transport of the molecule through the external medium (fluid/tissue), the semipermeable membrane of the probe and properties of the perfusate/dialysate.
The resistance (R) is related to the EE as shown in formula 2[104].
The resistance of the perfusate/dialysate (Rd) is dependent upon the length of the lumen and the diffusion constant in the perfusate/dialysate. The resistance of the membrane (Rm) is dependent of the geometry with inner and outer diameter, outer diameter of the inner cannula, length of the membrane, volume fractions of these three transport regions and the molecule

diffusion constant. The resistance in the external medium (tissue/fluid) (Rv) is a function of diffusion and kinetic processes in the external medium (tissue/fluid)[104].

Formula 2. Mass transport.
Q_d is the perfusate flowrate. The mass transport in the perfusate/dialysate (Rd), the microdialysis membrane (Rm) and the external medium (tissue/fluid) (Rv) is to a great extent dependent on the molecules diffusional capacity.

$$EE = \frac{C_{out} - C_{in}}{C_{medium} - C_{in}} = 1 - \exp\left(\frac{-1}{Q_d(R_d + R_m + R_v)}\right)$$

Calibration

The microdialysis catheter must be calibrated before one can conclude about the absolute concentrations of a molecule in the surroundings of the microdialysis membrane. There are several techniques to find EE of the microdialysis catheter.

With the no-net-flux method the microdialysis catheter is perfused with several known concentrations of the molecule of interest and a regression line is created between the increase/decrease in concentration of the molecule in the dialysate and the concentration of the molecule in the perfusate. At the no-net-flux point the EE is similar to the constant in the regression equation. The method assume steady state during the calibration[99].

The zero-flow technique measures the recovered concentration of the molecule at different flow rates and extrapolates the results to zero-flow, and thereby the EE of the microdialysis catheter[105].

Internal reference is also used. A known concentration of the molecule is added to the perfusate. The microdialysis membrane is immersed in a medium without the molecule of interest. The decrease in concentration of the molecule in the dialysate is used to calculate the EE.

In vitro calibration, both of quiescent and stirred media have been repeatedly validated. The limitation of the method is that the resistance of the medium (tissue/fluid) in vivo is not included. In vitro calibration will generally overestimated the EE.

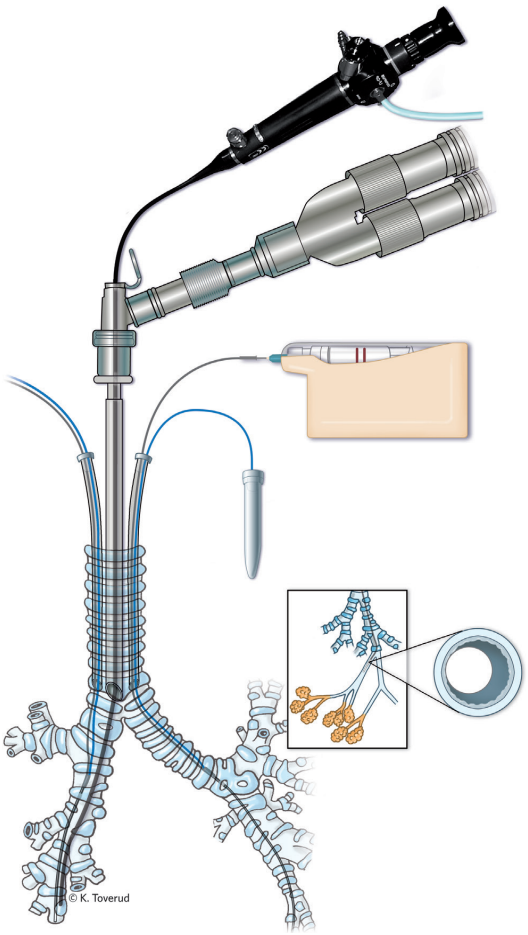
The above-mentioned methods are time-consuming. Endogenous compounds have been evaluated to find a calibration method that can be carried out during the experiment. Urea is a small molecule that moves fast and freely between compartments and immediately equilibrates in the tissue water space. Different applications of the medium/dialysate ratio of

urea have been proposed. Strindberg and Lönnroth found urea as an endogenous reference to correlate significantly with internal reference method and no-net-flux calibration[106].
The "real" concentration is not easy to find and researchers regularly experience the fact that results from different studies are not directly comparable even if the method used was the same. Even though a great effort has been done to find good calibration methods this lead up to that analysis of data relates to the relative relation between data values, and that conclusions relate to change more than the "real" concentrations.

Choice of microdialysis catheters

The application of bronchial microdialysis in pigs and humans were not previously described. The feasibility of the method is an important part of this thesis contribution to new knowledge on possible methods to monitor the composition of the ELF.

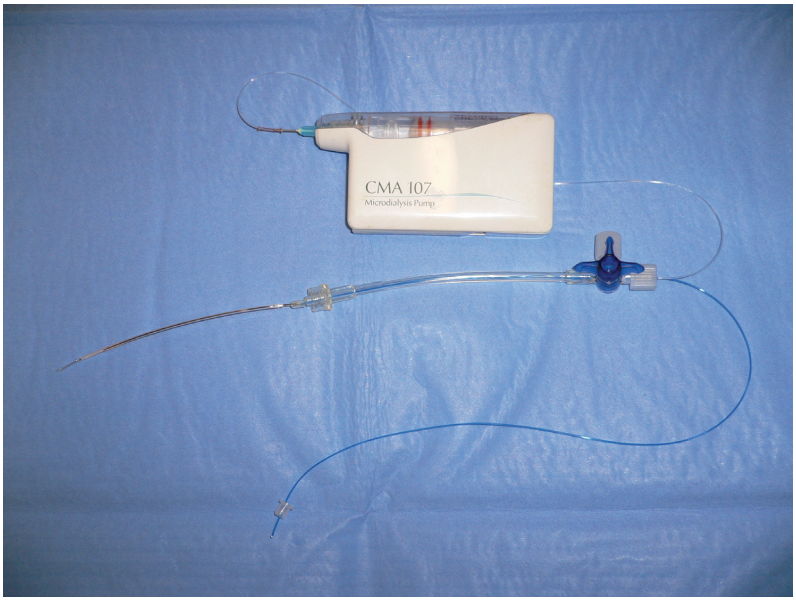
Figure 4. The application of bronchial microdialysis.



In the animal studies (Paper I and II) we used custom made probes based on the CMA20. The membrane was standard with a diameter of 0.5 mm and a length off 10 mm with 100 kDa cut-off. The shaft was lengthened to 90 mm and inlet and outlet were 435 mm. The catheter was placed under guidance of fiberoptic bronchoscopy, figure 4. We also created our own design for intra-arterial placement of these custom made catheters through a venous catheter with a luer-lock connection, figure 5.

In the human study (Paper III) a catheter that was approved for use in humans had to be used to avoid material compatibility issues. CMA used their CMA71 catheter for human application as a template. The membrane was standard with a diameter of 0.5 mm and a length of 10 mm with 100 kDa cut-off. The shaft was lengthened to 90 mm and inlet and outlet were 600 mm. The catheter was placed under direct laryngoscopy and wedged. The position was confirmed by fiberoptic bronchoscopy.

Figure 5. Custom made microdialysis catheter modified with luer-lock connector for intravenous application



Biochemical analysis

CMA 600

The analysis of urea, lactate and glycerol was done on a miniature clinical analyzer (CMA 600) “bed-side” with the results within 5 to 10 minutes after each sampling period. Lactate and glycerol reacts with the reagent and produces hydrogen peroxide in amounts reflecting lactate and glycerol concentrations. Hydrogen peroxide catalyzes the reaction forming a red-violet quinoneimine. The rate of formation is measured photometrical at 546 nm and is proportional to the concentration of lactate and glycerol.

Urea is hydrolyzed by urease to ammonium and carbon dioxide. The ammonium ions react with 2-oxygoutarate in the presence of glutamate dehydrogenase and nicotinamide adenine dinucleotide (NADH) (reducing agent - electron donor) to form glutamate and NAD^+ (oxidizing agent – accepts electrons). The utilization of NADH is measured photometrical at 365 nm and is proportional to the urea concentration.

Luminex with xMAP technology and Bio-Plex manager software

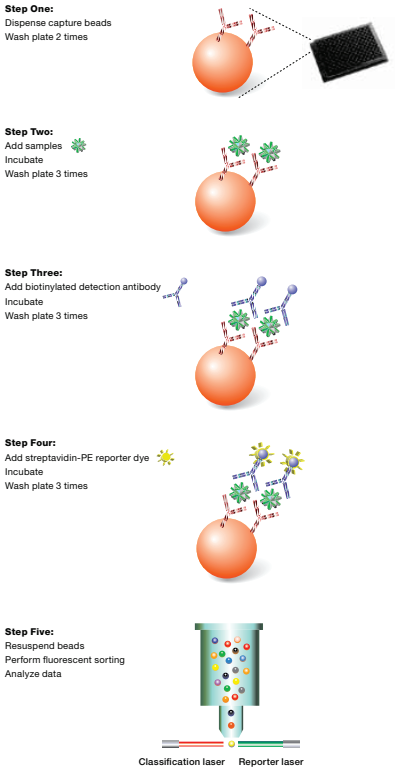


Figure 6. Schematic presentation of a sandwich-based Bio-Plex assay workflow. Used with permission.

The Luminex XY platform with xMAP technology is based on flow cytometry technology. Microplates with 96 wells are used. With this technology sample volumes down to 15 µl can be used to quantify several cytokines of interest[107]. In short the sample is added to a solution of a defined selection of colored capture beads. Thereafter the mix is incubated with biotinylated detection antibody. In the end streptavidin-PE reporter dye is incubated. The beads are re-suspended and each microsphere is analyzed separately in a tube by a classification laser that reads the color of the bead and simultaneously a report laser read the intensity of reporter dye.

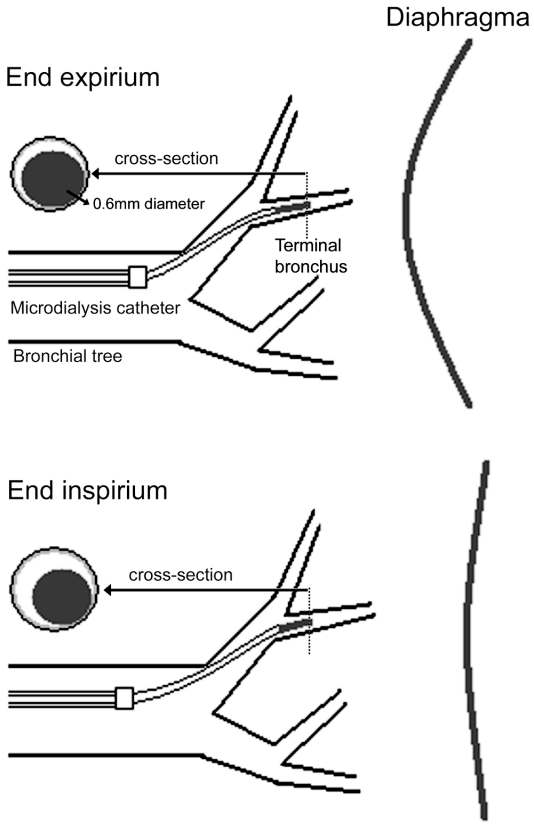
Urea as a correction factor

Urea (60 Da) is a small molecule which rapidly equilibrates in extracellular compartments included the ELF of the lung[108]. Urea has been repeatedly used as an endogenous marker in equilibrium to calculate the ELF amount in BAL and to calibrate microdialysis samples from various tissues[99, 109–112].

In bronchial microdialysis there is constant movement within the respiration-cycle. The microdialysis catheter was fixed proximally in the trachea and we postulated that the part of the microdialysis membrane in contact with the ELF would vary during inspiration and expiration, figure 7. The arteriobronchial urea-gradient as presented by Rennard was used to

estimate the part of the microdialysis membrane that was in contact with the ELF in Paper I and II[109].

Figure 7. Schematic presentation of bronchial microdialysis during the respiratory cycle. The microdialysis membrane will move within the bronchi caused by the inflation and deflation of the lung.



Permeability

Generally permeability experiments in hollow organs can either be performed by administration of the marker molecule to the lumen of the hollow organ with measurements in plasma or urine, or the marker molecule can be administered to blood with a standardized sampling procedure within the hollow organ. Tight junctions mainly regulate permeability by the paracellular route. Hydrophilic marker molecules with a cross sectional diameter of 1-3 nanometer most likely use the paracellular route caused by breaks in the tight junctions

induced amongst other molecules by cytokines[113–116]. Fluorescein isothiocyanate dextran 4000 Da (FD-4) matches these criteria.

In Paper I and II, we estimated the permeability from blood to the terminal bronchi as measured by bronchial microdialysis. We used an intravenous infusion of FD-4 and clearance was measured with a fluorescence spectrophotometer. In Paper II the concentration of FD-4 was measured in baseline and during 4 hours of intestinal reperfusion after 2 hours crossclamp of the superior mesenteric artery.

In Paper II we measured intestinal permeability changes. A microdialysis catheter for measuring FD-4 was introduced into a jejunal loop 60 to 90 cm distal of the ligament of Treitz. The loop was closed at each end with ligatures and flushed with 50 mL Ringer acetate until the fluid was clear by visual control. Detection of FD-4 in the microdialysate was used as a marker of alterations in intestinal permeability.

Histology

In Paper II a lung specimen was collected from the right diaphragmatic lobe through an anterior transdiaphragmatic access from the abdominal cavity.

Hematoxylin-eosin stained sections were examined for alveolar hemorrhage, fibrin deposition, thickening of alveolar septa, vascular congestion, and neutrophil granulocytes in 10 random areas at 10X magnification in each section. Neutrophil granulocytes were counted, and the other findings were scored on a semi-quantitative scale (0, no injury; 1, mild injury; 2, moderate injury; and 4, severe injury), modified from Douzinas et al. [117]. The M30 Cytodeath (Roche, Basel, Switzerland) antibody recognizes a specific caspase cleavage site within cytokeratin 18 present in early apoptosis. Apoptotic cells were counted in 10 random areas at 10X magnification in each section. Areas with bronchial tissue were excluded in histological examinations.

Study models

Young pigs, weighing 22-31 kg, were used in Paper I and II. Pigs are widely used in experimental circulatory research. Pigs like humans have relatively low concentrations of xanthine oxidase with the consequence that reperfusion injury and no-reflow phenomenon may be less pronounced than in rodents and cats[118, 119].

Intestinal ischemia-reperfusion is a well-known model of secondary lung injury. The PubMed search, (("Lung"[Mesh]) AND "Intestines"[Mesh]) AND ("Ischemia"[Mesh] OR "Reperfusion Injury"[Mesh]), returned 179 publications October 2012.

Inhalational anesthesia with volatile anesthetics increase concentration of some bronchial inflammatory mediators and decrease others[120–123]. To avoid lung effects of volatile anesthetics, fentanyl and ketamine were used.

To avoid ventilation induced lung injury the ventilator strategy was harmonized with the current research on optimal ventilator strategy in humans[30, 31]. To avoid ventilation-induced lung injury, peak inspiratory pressures was less than 20 cm H2O, positive end-expiratory pressure was set at 7 cm H_2O, and tidal volumes were 6 to 8 mL/kg. Fluid balance was obtained by an intravenous infusion of heated (37°C) crystalloid fluids throughout the experiments. Rectal temperature was maintained within the normal range (37°C-39.6°C) by a heating mattress and wrappings[124].

The trauma of surgery was minimized by the assistance of experienced surgeons within our own group.

In Paper III the method of bronchial microdialysis was evaluated in open AAA repair patients. Both the feasibility and the ability to monitor the inflammatory response were controlled. open AAA repair induce a systematic inflammatory response bi IR. The IR-injury of both the gut and the lower limbs is sufficient to induce remote organ failure of the lung and other organs[28, 61, 63–71].

Statistics

In Paper I parametric test-statistics was used. Evaluation of bronchial microdialysis and urea-corrected bronchial microdialysis as an approximation of arterial microdialysis were presented as accuracy. To evaluate improvement in precision by urea-correction, the coefficient of variation was used. *Coefficient of variation = Standard deviation/Mean·100.* A significant reduction in the coefficient of variation by use of urea-correction was used to confirm the justification of the method.

In our papers there was a limited number of observations for each parameter and it was open to discussion whether the criteria for parametric testing always was present. Therefore we used non-parametric testing in Paper II and III. To assess changes within the groups over time, the Friedman test was used. Wilcoxon signed rank test was used to compare different time points within groups, and in Paper III the Bonferroni correction for multiple testing was used to avoid false positive results. Mann-Whitney U test was used to compare groups. Fisher exact test was performed on binomial data. The Spearman's ρ was used to identify correlations. Spearman's ρ uses ranks to calculate the correlation coefficient.

Summary of results

Paper I. Continuous monitoring of the bronchial epithelial lining fluid by microdialysis

The aim of the first paper was to evaluate bronchial microdialysis as a possible method for continuous and dynamic monitoring of the time-to-time changes in the composition of the ELF. Lactate and FD-4 were administered intravenously by continuous infusion to maintain two levels of steady state. Arteriobronchial urea-gradient was used as a correction factor to calculate the absolute concentration of lactate and FD-4 in the bronchial ELF. The arteriobronchial lactate gradient was 1.2 ± 0.1 and FD-4 gradient was 4.0 ± 1.2.
Arterial and bronchial concentrations of lactate and FD-4 showed similar relative concentration changes, figure 8 and 9. Correction of the bronchial lactate concentrations with the arteriobronchial urea-gradient improved the accuracy and the precision of the measurements. For FD-4 there were a trend to improvement in accuracy and precision.
We concluded that bronchial microdialysis was an applicable continuous monitor of the dynamic changes in the composition of the ELF. The absolute concentrations of the measured molecules could be estimated with acceptable precision using a correction by the arteriobronchial urea gradient.

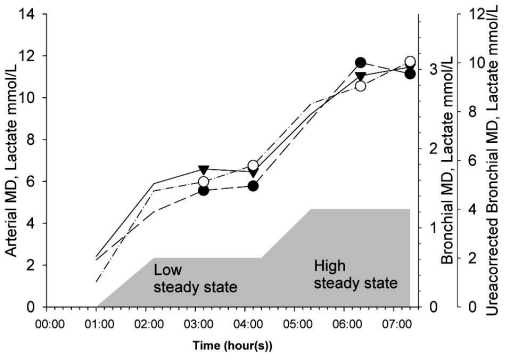


Figure 8. Overview of the lactate infusion with two steady states. Data are presented as mean lactate values by arterial microdialysis (solid line) with mean values in steady state (filled triangles), bronchial microdialysis (long dash line) with mean values in steady state (filled circles) and ureacorrected bronchial microdialysis (dash-dot line) with mean values in steady state (open circles).
The circles and the triangles represent the time where the microdialysis vials were exchanged for the steady state samples. The gray area is a graphical presentation of the intravenous lactate infusion. During the low steady state the infusion of sodium lactate was gradually increased to maintain an arterial blood lactate of ~5 mmol/L. During the high steady state the infusion of sodium lactate was increased to maintain an arterial blood lactate of ~10 mmol/L.

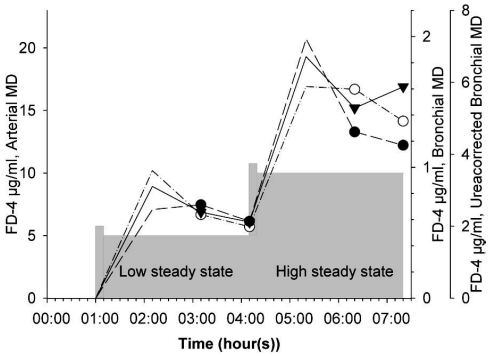


Figure 9. Overview of the fluorescein isothiocyanate dextran 4000 Da (FD-4) infusion with two steady states. Data are presented as mean FD-4 values by arterial microdialysis (solid line) with mean values in steady state (filled triangles), bronchial microdialysis (long dash line) with mean values in steady state (filled circles) and ureacorrected bronchial microdialysis (dash-dot line) with mean values in steady state (open circles). The circles and the triangles represent the time where the microdialysis vials were exchanged for the steady state samples. The gray area is a graphical presentation of the intravenous FD-4 infusion. The infusion was started with an intravenous bolus of 10 µg/kg over 10 minutes. In the low steady state an infusion of FD-4 at 5 µg/kg/hour was maintained. After the low steady state a new intravenous FD-4 bolus of 10 µg/kg over 10 minutes was infused. In the high steady state an infusion of FD-4 at 10 µg/kg/hour was maintained.

Paper II. Bronchial microdialysis of cytokines in the epithelial lining fluid in experimental intestinal ischemia and reperfusion before onset of manifest lung injury

At the time there was no continuous monitor to measure cytokines in the bronchial ELF or the intestine. The aim of the study was to evaluate bronchial microdialysis as a method to continuous monitor early lung cytokine response (IL-1β, TNFα and IL-8) and altered blood-alveolar permeability (FD-4) secondary to intestinal IR in pigs. We also measured the cytokine response and permeability changes in the intestine by luminal microdialysis. Intestinal IR-injury was induced by cross-clamp of the superior mesenteric artery for 120 min followed by 240 min of reperfusion. We hypothesized that microdialysis could identify the immediate inflammatory response and permeability changes in the bronchi and the intestines as measured by IL-1β, TNFα, IL-8 and FD-4, and monitor the time-to-time changes of these cytokines during the first 4 hours of reperfusion after intestinal ischemia. Sham-operated pigs served as controls.

In the ischemia group during reperfusion, mean pulmonary artery pressure and pulmonary vascular resistance increased to values higher than reference values for conscious pigs.

During reperfusion the ischemia group presented individual differences in the concentrations of bronchial IL-8 and IL-1β. Bronchial IL-8 and IL-1β in the ischemia group increased during the reperfusion phase as compared with baseline, and there were increase between different time points and between the ischemia group and the control group. Already within 1 hour of reperfusion bronchial epithelial lining fluid levels of both IL-8 and IL-1β increased. Most bronchial TNFα samples were below the limit of detection. Luminal intestinal IL-8 increased in the ischemia group. The measured levels of intestinal IL-1β were low, and there were no changes during reperfusion compared with baseline. Intestinal TNF-α was below the limit of detection in all samples in both groups. Cytokine levels obtained by arterial microdialysis were mostly below the limit of detection and were not suitable for statistical analysis. All plasma samples on IL-1β, TNFα and IL-8 in all animals were below the limit of detection. During reperfusion, the concentration of IL-6 in pulmonary serum increased in the ischemia group compared to the control group. In this study we had a high limit of detection for TNFα and this must be considered when interpreting the data.

Intestinal luminal lactate and FD-4 was increased during reperfusion. Bronchial lactate and FD-4 were the same in both groups. The baseline FD-4 concentration in the bronchi was approximately one half of the arterial concentration; and in the intestinal lumen, the concentration was less than one tenth of the arterial concentration.

There was no histological pathology as sign of diffuse alveolar damage in hematoxylin-eosin/stained sections or number of apoptotic cells as indicated by immunohistochemistry.

Paper III. Bronchial microdialysis monitoring the inflammatory response in open abdominal aortic aneurysm repair

The aim of this study was to investigate a broad spectrum of the bronchial and systemic inflammatory response in a time relation to the phases of open AAA repair and the immediate postoperative period by measuring 18 cytokines by microdialysis. Sixteen consecutive patients scheduled for open AAA repair were included. The patients served as their own controls. Cytokines were measured in bronchial ELF and venous blood by microdialysis during open AAA repair and the first 120 minutes of reperfusion. Serum samples were collected the day of surgery and the three following days. Three patients were admitted to the critical care unit directly after surgery and had organ failure(s) that persisted more than 24 hours (Organ Failure-group).

Eight out of eighteen bronchial cytokines measured by microdialysis presented an overall change in relation to the trauma. IL-5, IL-6, IL-13, and GM-CSF had their peak during

reperfusion, IL-2, IL-4 and TNF-α showed an overall change with their highest concentration during reperfusion and IL-7 was reduced during ischemia and reperfusion. Bronchial cytokine concentrations by microdialysis in the Organ Failure-group were statistically different from the Non Organ Failure-group in seventeen out of eighteen cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, MCP-1, MIP-1α, MIP-1β and TNF-α). Bronchial cytokines correlated to aortic aneurysm diameter, s-creatinine, GFR, previous coronary intervention, kidney disease, statin use, duration of cross-clamping, duration of surgery, duration of anesthesia, critical care days, volume of erythrocyte transfusion and MPAP.

Venous IL-2, IL-5, IL-7, IL-13, G-CSF, IFN-γ measured by microdialysis were below limit of quantification in more than half of the samples. Further analysis was omitted for these six cytokines. Eleven out of the remaining twelve cytokines presented an overall change in relation to the trauma. IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12 (p70), IL-17, MCP-1, MIP-1α, MIP-1β and TNF-α had a peak during reperfusion and in addition TNF-α, MIP-1α and MIP-1β increased already during ischemia. Venous cytokine concentrations by microdialysis in the Organ Failure-group were statistically different from the Non Organ Failure-group in four out of twelve cytokines (IL-4, IL-17, MCP-1 and MIP-1). Venous cytokines as measured by microdialysis correlated to age, aortic aneurysm diameter, kidney disease, acetylsalicylic acid use, smoking status, duration of cross-clamping, duration of surgery, duration of anesthesia, volume of erythrocyte transfusion, volume of other fluids, MPAP, lung compliance, temperature, duration of stay, and number of organ failures.

Serum IL-2, IL-17 and GM-CSF were below limit of quantification in more than half of the samples. Further analysis was omitted for these three cytokines. Fifteen out of the remaining fifteen cytokines presented an overall change in relation to the trauma. IL-1β, IL-4, IL-5, IL-7, IL-12 (p70), IL-13, IFN-γ and TNF-α had their highest concentration in the sample collected immediately after induction of anesthesia, IL-8, IL-10, G-CSF, MCP-1, MIP-1β had their highest concentration during reperfusion, IL-6 peaked on the morning of the 1st postoperative day and MIP-1α had the lowest concentration on the first postoperative day. Serum cytokine concentrations in the Organ Failure-group were statistically different from the Non Organ Failure-group in seven out of fifteen cytokines (IL-1β, IL-5, IL-8, GM-CSF, MIP-1α, MIP-1βand TNF-α). Serum cytokines correlated to acetylsalicylic acid use and MAP.

General discussion

How to assess the composition of the bronchial epithelial lining fluid?

Existing methods and "gold standards" of measurement

Studies on bronchial epithelial lining fluid have been limited by the available methods. BAL has been judged being the "gold standard". But the method has defined weaknesses. BAL allows only a few intermittent samples in each individual caused by the invasiveness and the side effects of the procedure. The method itself elicits pulmonary hypertension with right ventricle distension, arterial deoxygenation and an acute phase response[85, 86, 125]. Although regarded as a safe procedure there is a considerable frequency of complications of 3 to 10 %[126, 127]. The one major challenge with BAL is to determine the exact concentration of the molecules of interest. The method is based on repeated instillation of fluids within a bronchus, washout of bronchial fluids and thereafter collection of the alveolar fluids. To approximate the absolute concentration in the ELF a correction factor is needed to estimate the proportion between instilled fluid and ELF within the collected sample. Urea is frequently used as a correction factor, but there is still a controversy on the reliability. Instillation volume, dwell time, injured versus normal lung and other individual applications of the technique influence the composition of the collected sample[128–131].

Other techniques for collecting ELF existed at the time for start of this project. Exhaled breath condensates are noninvasive, but compromised by uncertainties concerning the sources of the droplets and by the extreme and variable dilution of ELF droplets with condensed water vapor (approximately 20,000-fold) and exhaled breath condensates measurement results are not comparable to BAL[87–89]. Bronchial microsampling and direct aspiration are invasive and only allows intermittent sampling. The diameter of the equipment limits the possibility to get samples from the smallest airways[132, 133].

The overall aim of this thesis was to explore and evaluate the molecular composition, especially cytokines, in ELF in early lung involvement after distant IR-injury in both an experimental model and in the clinical setting. None of the existing techniques were suitable for this and we concluded that we were in need of a new method for continuous sampling from bronchial ELF.

Bronchial microdialysis, the new technique

Microdialysis was originally designed to be a capillary within cellular organs and tissues and thereby collect molecules from the immediate surroundings[101]. The technique was later applied to the lumen of intestines. In our group we had experience with microdialysis applied in the intestinal lumen[134–136]. After a Pubmed search we found that bronchial microdialysis had been used in one rodent study[137]. New challenges presented with the idea of using microdialysis in the lungs within the bronchi of large animals and humans. As opposed to soft intestines the bronchi are stiff lumens held open by cartilage rings and connective tissue.

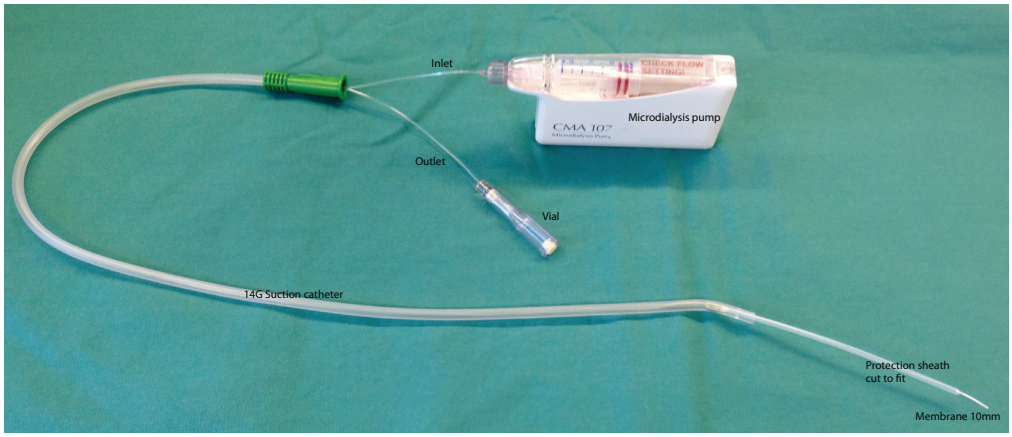
The bronchial tree has been well characterized through morphometrical studies and terminal bronchi and bronchioles have a diameter of 0.6-1.5 mm[138, 139]. The diameter of the microdialysis membrane of 0.5-0.6 mm allows positioning within a terminal bronchus or a bronchiole in humans.

We started pilot studies on bronchial application of microdialysis in pigs and the method as presented in Paper I and II was designed, figure 4. In the pig model we succeeded with a fiberoptic guided technique with visual control during insertion of the microdialysis catheters. With a tracheostomy the angle and distance to the distal airway was kept to a minimum. We tried to verify the position of a bronchial microdialysis catheter perfused with a radio-contrast agent by DynaCT, but we were not able to detect the tip of the catheter in vivo probably caused by the minimal diameter of the microdialysis catheter. We also tested perfusion of the microdialysis catheter with methylene blue and post mortem dissection, but the exact position of the bronchial microdialysis catheter was not determinable. This is a limitation of the method at present. An in vivo technique to confirm the exact position within the bronchial tree would improve the reliability of the method.

In Paper III, the human study, we had to use microdialysis catheters that were approved for use in humans with a different material in the tubing. Patients were endotracheally intubated via the oral route. In pilot studies in pigs we were able to insert the catheter through the bronchial blocker port in the Arndt Multiport adapter (an adapter placed on the endotracheal tube with ports for fiberoptic bronchoscopy, bronchial blocker and connection to ventilator) and wedged in a terminal bronchus. But after attempts in patients we concluded that this was not possible and the method was totally redesigned. The bronchial microdialysis catheter was prepared before the experiment by placement within a 14 G suction catheter and the biocompatible sterile outer sheath were manually cut to fit this application, see figure 10. Our

bronchial microdialysis catheter was introduced under direct laryngoscopy until it wedged and thereafter the patient was endotracheally intubated. The position of the catheter was confirmed by fiberoptic bronchoscopy with a thin bronchoscope (outer diameter 2.1 to 2.3 mm) without working channel. The ideal image was to see the microdialysis catheter disappear within a terminal bronchus.

Figure 10. The bronchial microdialysis catheter prepared for insertion in patients.



In pilot studies and Paper I, II and III about 60 bronchial microdialysis catheters have been placed in pigs and humans. The method is feasible and appears safe with no observed major complications as visible bleeding, deoxygenation or other respiratory complications.

Bronchial microdialysis, need for a correction factor?

The bronchi move distally during inspiration and proximally during expiration. With a microdialysis catheter fixated proximally in the trachea the membranous area in contact with the epithelial lining fluid will vary, figure 7. Rennard et al and Eisenberg and Eckoff had previously presented urea as an endogenous factor with equal concentration and equilibration between body compartments[109, 110]. Strindberg evaluated urea applicable and as good as other calibration techniques for in vivo calibration of microdialysis catheters[106]. In Paper I we addressed the issue of variable area of microdialysis membrane in contact with the thin film of bronchial ELF. To produce standardized conditions a lactate and FD-4 clamp at two levels of concentration in blood were administered by continuous intravenous infusion. Urea was measured and used as a correction factor. Urea-correction increased accuracy and reduced coefficient of variation for lactate and a trend to improvement for FD-4 that did not

reach significance. The FD-4 results must be judged in light of technical challenges as described in Paper I[1]. Based on the improved accuracy and reduced coefficient of variation we chose urea as an in vivo correction factor of the microdialysis membrane function within the bronchi in Paper I and II. One weakness in our application in Paper I and II was that we did not add urea to the perfusion fluid in microdialysis catheters. This may have influenced results by a depletion of urea in the immediate surroundings of the microdialysis catheter within the bronchus. In circulating blood it is reasonable to assume that the issue with urea depletion could be neglected. As a consequence the corrected bronchial concentrations of molecules could be overestimated. In Paper III urea-correction was omitted because a number of measured readings on the Luminex were above the standard curves and the exact concentrations measured were not possible to estimate. This was further complicated since the measurements had to be separated on two microplates with separate standard curves. With the uncertainty of the real concentrations of the high readings we decided to omit the urea-correction in Paper III. Regardless of this Paper III showed an increase in bronchial cytokines in accordance with our findings in Paper II.

Bronchial cytokines and the blood-bronchial barrier in inflammation

Inflammation increases the permeability of the endothelium both in quantity and in quality. With severe inflammation the disruption of gap junctions allows fluids, ions and proteins increase access to lung interstitium. Inflammation also increases the permeability of alveolar epithelium. But the alveolar epithelium has a great capacity for compensatory increase in transport of fluids and ions by increased activity in apical ENaCs driven by the basal Na+-K+-ATPase. When it comes to the alveolar permeability to larger molecules the knowledge is more limited. Caused by the complexity of the blood-bronchial barrier, with three different functional units (endothelium, interstitium with lymph and epithelium) all sensitive to external stimuli, most studies on molecular transport have been conducted in cell-cultures and isolated organs. Molecule-size dependent paracellular and transcellular transport have been verified and FD-4 is found to be a marker of paracellular transport[113, 114]. In Paper I we

[1] Pilot in vitro studies of low volume FD-4 samples were conducted with wells containing 15 µl, 25 µl and 40 µl. With wells containing 15 µl there was a great variance and by visual inspection we saw that this small volume did not cover the bottom of the well. Wells containing 25 µl and 40 µl gave similar results and variance. During analysis of the last fluorescein isothiocyanate results from the in vivo experiment, we registered that wells with higher concentrations of FD-4 increased the registered result of neighboring wells with lower concentration of FD-4. We concluded that this maybe was caused by the use of microplates with translucent wells and in paper II we used microplates with white opaque wells.

found that there was a blood-bronchial gradient of about 4:1 for FD-4 in anesthetized positive pressure ventilated pigs. In Paper II we found no difference in the blood-bronchial FD-4 gradient in pigs after intestinal IR-injury and sham-operated controls. This finding indicated that in our pig-model of intestinal IR-injury there were no disruptions of the tight junctions in alveolar epithelium. This was in accordance with the histological findings in Paper II with no sign of diffuse alveolar damage[2].

Challenges in validating bronchial microdialysis

In Paper I we examined bronchial microdialysis under standardized conditions with a lactate and a FD-4 clamp by intravenous infusion. We evaluated the methods accuracy and precision. We concluded that bronchial microdialysis could be an applicable method to continuously measure the concentration of molecules in the bronchial ELF.

The term accuracy demands knowledge of a true value. The term is well accepted in the validating literature, but a more suitable term would have been agreement. Even though the ABL700 (Radiometer, Copenhagen, Denmark) is considered the best analyzer available (the gold standard) we have no evidence that we measure the true value with certainty. In addition lactate concentrations in plasma is higher than in erythrocytes and this introduce uncertainty when comparing blood to microdialysate[140].

Blood lactate concentrations were measured on the ABL700 (Radiometer, Copenhagen, Denmark). The concentration of the microdialysate in arterial microdialysis and bronchial microdialysis was measured on the CMA600 (CMA Microdialysis AB, Stockholm, Sweden). We found the CMA600:ABL700 ratio for lactate concentrations to be 1.13:1.

In Paper I we present our findings as accuracy and coefficient of variation (precision). In Paper II and III we have based our conclusions on the precision more than the accuracy or agreement. The ability to measure molecules continuously and detect substantial changes between several time-intervals with good time-resolution is the strength of the method and the status of the methods validity at present.

Bronchial cytokines by microdialysis

In Paper II and III we found increased concentrations of bronchial cytokines in response to IR-injury. The cytokine concentrations were higher in bronchi than in blood. Our data support

[2] We had also performed biopsies for electron microscopy of the alveolar cells and their tight junctions, but caused by capacity problems at the electron microscopy laboratory the sections were not produced.

that cytokine production is compartmentalized, with local production (alveolar epithelial cells and alveolar macrophages) of the majority of cytokines found in the bronchi.
IR-injury in both an animal model of intestinal IR and in open AAA repair with crossclamping of the abdominal aorta gave an immediate response. In Paper II we found an increased concentration of IL-1β and IL-8 already within the first 60 minutes of reperfusion. In Paper III we found decreased IL-7 during ischemia, increased IL-6, IL-13 and GM-CSF within the first 60 minutes of reperfusion and an overall change in TNFα. In Paper III we additionally found a difference in the concentration of 17 out of 18 cytokines in patients with perioperative organ failure group when compared to patients without perioperative organ failure. None of the included individuals fulfilled criteria of ALI or ARDS. We found that production of bronchial cytokines came early. We found a measureable inflammatory response within minutes and up to an hour after the distant IR-injury. In summary Paper II and III found concentration changes in well-defined and important cytokines in the early inflammatory response (IL-1β, IL-6, IL-8 and TNFα). Bronchial cytokines seem to have interest beyond the scope of lung organ failure as defined by ARF, ALI and ARDS. Our findings open up a possible research area of lung inflammatory response in immediate organ crosstalk when the organism is exposed to trauma; in our project the trauma was IR-injury.

Cytokines by microdialysis and intermittent samples

A special quality with microdialysis is continuous sampling. With continuous sampling, the measured concentration is the mean area under the curve for the time-interval when the sample was collected. The time-resolution is determined by the time between the exchanges of the collection vials. In studies on cytokines a volume of 15 µl is sufficient allowing a time resolution of about 15 minutes[107]. The concentration measured in each individual sample is the actual area under the curve for the measurement period. In Paper III we had designed the study with microdialysis and intermittent serum samples with sample time-point and time-intervals according to previously conducted studies on open AAA repair patients[28, 62]. The first serum sample was drawn immediately after anesthesia induction. When comparing cytokines in samples from venous microdialysis and serum samples we were surprised with the difference in concentration-profile of the cytokines measured within the same compartment. To our knowledge the timing of the first serum sample have not been previously addressed. With a literature-search we found that some studies used samples from the day before surgery and some after anesthesia induction[28, 62, 64, 65]. Venous microdialysis was established immediately after anesthesia induction with a median sample-

period of 2 hours and 5 minutes and the cytokine concentrations were low when compared to ischemia and reperfusion periods. In serum samples we found high cytokine concentrations in our after-anesthesia-induction-samples when compared to ischemia- and reperfusion-samples. In Paper II the concentrations in all intermittent samples from blood were generally below limit of quantification, maybe caused by lower sensitivity of the analysis method. The finding in Paper III is not conclusive, but the occurrence of the phenomenon puts a question mark behind previously conducted projects and calls for studies to clarify the issue.

Conclusion - answer to the aims

Aim 1: *Evaluate bronchial microdialysis for continuous and dynamic monitoring of the time-to-time changes in the composition of the ELF by lactate and FD-4 in a pig model. Evaluate arteriobronchial urea gradient as a correction factor.*

In Paper I we evaluated Bronchial microdialysis in a pig model with standardized conditions. Lactate and FD-4 clamp were established at two levels. A method for standardized application of bronchial microdialysis was developed, see figure 4. Lactate and FD-4 could be measured in the bronchial microdialysate. However the precision of the measurements could have been better. The coefficients of variation were 60-65 percent without correction. With correction by the arteriobronchial urea-gradient the precision for lactate improved significantly with a coefficient of variation of 17 percent. For FD-4 the improvement in precision was not significant.

Paper I led up to the conclusion that bronchial microdialysis could be a method to measure substantial concentration changes of molecules in the bronchial ELF, and that correction by the arteriobronchial urea-gradient improved the precision of the method.

Aim 2: *To determine if bronchial microdialysis can identify the immediate inflammatory response in the bronchi as measured by IL-1β, IL-8, and TNFα and monitor the time-to-time changes of these cytokines during the first 4 h of reperfusion after intestinal ischemia in a pig model.*

In Paper II we found increased IL-1β and IL-8 during the first 60 minutes of reperfusion in the bronchial ELF as a response to intestinal IR-injury in our pig-model. We could not reliably measure TNFα. This could be caused by a high limit of detection of 100 pg/ml. In Paper III we measured TNFα in both blood and bronchi with concentrations below 30 pg/ml. In Paper III the limit of quantification was below 1 pg/ml. Microdialysis of proteins such as cytokines have limitations caused by a relatively low relative recovery and this limits the possibility to measure low concentrations of large proteins. The improving sensitivity of multiplexed ELISAs reduce this issue, but still it requires attention.

In Paper II lung biopsies did not show signs of diffuse alveolar injury, and FD-4 measurements were the same in the IR-injury and he control group. We concluded that

bronchial cytokines occurred as an immediate response to intestinal IR before manifest organ failure and that bronchial microdialysis could be an early continuous monitor of the inflammatory response. A future issue would be to see if this method could expedite clinical judgment and intervention with improved outcome as a consequence.

Aim 3: *To investigate a broad spectrum of the bronchial and systemic inflammatory response in a time relation to the phases of open AAA repair and the immediate postoperative period by measuring 18 cytokines by microdialysis.*

In Paper III a broad spectrum of eighteen cytokines were measured in blood and bronchi in sixteen open AAA repair patients. With the application of bronchial microdialysis in a human model new methodological challenges came along. The method had to be totally revised and a modified device was developed, see figure 10. In Paper III we found that venous microdialysis reliably could detect the systemic inflammatory response as measured by cytokines. Cytokines measured by bronchial microdialysis detected the inflammatory response. All but one bronchial cytokine in our material were increased in patients suffering organ failure in direct time-relation to open AAA repair. Paper III, the human study, partially addresses the issue of expedited clinical judgement. Paper III indicated that early continuous monitoring of cytokines could be of value if the results were presented close to real time.

Issues for future research

The method of bronchial microdialysis has proven useful in this thesis. Still the method needs further validation. The most critical issue is to verify a good method to correct for the amount of microdialysis membrane in contact with the bronchial ELF. Urea-correction is validated in microdialysis earlier up against the no net flux method for measurements in muscle and fat[106]. The validity of urea-correction as a parameter of membrane in contact with the bronchial ELF in Paper I indicate that it is useful, but it is not yet finally verified.

The microdialysis method allows continuous follow up of inflammatory markers in controlled trials on treatments in intubated patients. The inflammatory process is important both in the diagnosis, but also in the prognosis. Patients with similar premorbid status have different outcome when exposed to standardized trauma. Our present research indicated that microdialysis could improve the methodology with continuous sampling and better spatiality. Our new research application of microdialysis in bronchi has a potential to add new knowledge. Patient groups of special interest are patients undergoing major surgery, trauma patients and medical and surgical intensive care patients.

For application as a clinical diagnostic tool one technical obstacle is the time from sample collection to laboratory-result. The potential of close to real time monitoring of cytokines must be brought to attention for the developers of analysis equipment. This is a necessary step for clinical application.

Our research models did not alter lung permeability according to the recorded data, but in a model of altered permeability in the bronchial/alveolar epithelium it may be that bronchial microdialysis can prove useful in experimental settings. The ability to measure continuously with good time spatiality in vivo can prove useful.

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Paper I

Respiratory Research

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Continuous monitoring of the bronchial epithelial lining fluid by microdialysis

Stig S Tyvold*[1,2], Erik Solligård[1,2], Oddveig Lyng[3], Sigurd L Steinshamn[2,4], Sigurd Gunnes[2,5] and Petter Aadahl[2,5]

Address: [1]Department of Anesthesia and Intensive Care, St. Olavs Hospital, Trondheim, Norway, [2]Department of Circulation and Medical Imaging, Norwegian University of Science and Technology, Trondheim, Norway, [3]Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway, [4]Department of Lung Medicine, St. Olavs Hospital, Trondheim, Norway and [5]Department of Heart and Lung Surgery, St. Olavs Hospital, Trondheim, Norway

Email: Stig S Tyvold* - stig.s.tyvold@ntnu.no; Erik Solligård - erik.solligard@ntnu.no; Oddveig Lyng - oddveig.lyng@ntnu.no; Sigurd L Steinshamn - sigurd.steinshamn@ntnu.no; Sigurd Gunnes - sigurd.gunnes@ntnu.no; Petter Aadahl - petter.aadahl@ntnu.no

* Corresponding author

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Abstract

Background: Contents of the epithelial lining fluid (ELF) of the bronchi are of central interest in lung diseases, acute lung injury and pharmacology. The most commonly used technique broncheoalveolar lavage is invasive and may cause lung injury. Microdialysis (MD) is a method for continuous sampling of extracellular molecules in the immediate surroundings of the catheter. Urea is used as an endogenous marker of dilution in samples collected from the ELF. The aim of this study was to evaluate bronchial MD as a continuous monitor of the ELF.

Methods: Microdialysis catheters were introduced into the right main stem bronchus and into the right subclavian artery of five anesthetized and normoventilated pigs. The flowrate was 2 μl/min and the sampling interval was 60 minutes. Lactate and fluorescein-isothiocyanate-dextran 4 kDa (FD-4) infusions were performed to obtain two levels of steady-state concentrations in blood. Accuracy was defined as [bronchial-MD] divided by [arterial-MD] in percent. Data presented as mean ± 95 percent confidence interval.

Results: The accuracy of bronchial MD was calculated with and without correction by the arteriobronchial urea gradient. The arteriobronchial lactate gradient was 1.2 ± 0.1 and FD-4 gradient was 4.0 ± 1.2. Accuracy of bronchial MD with a continuous lactate infusion was mean 25.5% (range 5.7–59.6%) with a coefficient of variation (CV) of 62.6%. With correction by the arteriobronchial urea gradient accuracy was mean 79.0% (57.3–108.1%) with a CV of 17.0%.

Conclusion: Urea as a marker of catheter functioning enhances bronchial MD and makes it useful for monitoring substantial changes in the composition of the ELF.

Background

The epithelial lining fluid of the lung is important in the understanding mechanisms in acute lung injury, inflammatory lung diseases, cardiac failure, and in pharmacokinetic studies.

Today there are no established methods of direct continuous monitoring of the epithelial lining fluid of the lower respiratory tract. The epithelial lining fluid of the bronchi has been examined by bronchioalveolar lavage (BAL), direct aspiration, microsampling, and exhaled breath condensates. BAL and other bronchoscopic techniques have in common that they are invasive, may create lung injury, are based on single or intermittent samples and therefore have limited value as continuous and dynamic monitors of the epithelial lining fluid of the lung. The exhaled breath condensates technique is non-invasive and continuous, but is indirect and best suited for collection of non-volatile hydrophilic solutes[1].

Microdialysis is a method for continuous sampling of extracellular molecules in the immediate surroundings of the catheter. The technique is based on the principle of diffusion of substances along a concentration gradient through a semipermeable membrane on a thin catheter with an outer diameter of 0.6 mm. Microdialysis has routinely been used in parenchymatous organs and tissues, but has also gained widespread acceptance in hollow organs. Intestinal endoluminal microdialysis has been used as a continuous monitor of intestinal dysfunction both in experimental [2-4] and in clinical settings[5]. As far as we know there is only one published article on bronchial microdialysis in rats for pharmacokinetic measurements of aminoglycosides[6]. The value of microdialysis as a continuous and dynamic monitor of the time to time changes in the epithelial lining fluid has not previously been evaluated.

The recovered amount of epithelial lining fluid by various techniques, especially BAL, has been estimated by endogenous and exogenous markers of dilution. Urea (60 Da) is a small molecule in equilibrium in all body compartments. The recovered concentration of urea has been used as an indicator of the recovered concentration of epithelial lining fluid in the dialysate [7-10]. The arteriobronchial urea gradient has been used to calculate the absolute concentration of the molecules measured in the epithelial lining fluid by BAL and the ureagradient has also been used to calculate the absolute concentration of molecules measured in the extracellular fluid[9,11].

Lactate (90 Da) is a small molecule which has previously been studied as a marker of intestinal barrier dysfunction[2]. In the intestines lactate only passes the blood-luminal barrier of the intestines when the intestines suffer an ischemia-reperfusion injury[4]. The question is if lactate freely passes the blood-bronchial barrier.

Molecular transport across rat epithelial monolayer by the paracellular route has previously been investigated with the macromolecule fluorescein isothiocyanate dextran 4000 Da (FD-4)[12,13]. According to the present literature an increased leakage of FD-4 is believed to be via the paracellular route by changes in the composition and confirmation of the tight junctions of the epithelium and the endothelium in response to stimuli[14,15]. In this study, we used FD-4 to see if larger molecular size influences the gradient across the paracellular blood-bronchial barrier.

The aim of our study was to evaluate bronchial microdialysis as a possible method for continuous and dynamic monitoring of the time to time changes in the composition of the epithelial lining fluid. Lactate and FD-4 were measured during two levels of steady-state concentrations in anesthetized pigs under positive pressure ventilation. Arteriobronchial urea gradient was used as a correction factor to calculate the absolute concentration of lactate and FD-4 in the bronchial epithelial lining fluid.

Methods

Anesthesia and surgical preparation

Five outbred pigs (Norwegian Landrace 50%, Duroc 25%, Yorkshire 25%) (range 22–31 kg) were acclimatized and treated in accordance with the "European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes", Strasbourg, 18.III.1986. The pigs fasted overnight with free access to water prior to the experiment. They were premedicated with intramuscular diazepam 10 mg (Stesolid, Dumex-Alpharma, Copenhagen, Denmark) and azaperon 400 mg (Stresnil, Janssen-Cilag, Vienna, Austria), and anesthesia was induced with atropine 1 mg (Nycomed Pharma AS, Oslo, Norway), ketamin HCl 10 mg/kg (Parke-Davis, Solna, Sweden) and thiopenthal sodium 10 mg/kg (Pentothal, Abbot Scandiavia AB, Solna, Sweden).

Tracheostomy was performed with the pig in the supine position, and an endotracheal tube 8.0 was inserted 5 cm.

The pigs received total intravenous anesthesia with a continuous infusion of fentanyl 20 to 30 μg/kg/hour (Fentanyl, Pharmalink, Spanga, Sweden) and ketamin HCl 8 to 12 mg/kg/hour. Pancuronbromide 2 mg/ml (Pavulon, Organon Teknika, Baxtel, The Netherlands) was used as needed to eliminate spontaneous ventilation.

The pigs were ventilated in pressure control mode with a positive end expiratory pressure of 7 cmH2O, a peak inspiratory pressure less than or equal to 20 cmH2O and a tidal volume of 6 to 8 ml/kg (Servo Ventilator 900C, Siemens-Elema, Sweden)[16,17].

After a recruitment maneuver of 3 breaths at 40 cmH2O, respiratory rate was adjusted to an arterial partial pressure of carbon dioxide ($PaCO_2$) of 4.5 to 5.5 kPa in baseline. Fraction of inspired oxygen (FiO_2) was maintained at 25%

during the experiment. Similar recruitment maneuvers were performed when the bronchial microdialysis catheter was in position. In 2 out of 5 pigs a recruitment maneuver was necessary during the experimental protocol due to spontaneous ventilation in conflict with the ventilator and derecruitment. This was solved with pancuronium bromide, increased infusion of the anesthetetic agents and a recruitment maneuver to reestablish the lung compliance as measured by lung tidal volume/peak inspiratory measure.

The pigs received an intravenous infusion of heated (37°C) Ringers acetate at 14 to 18 ml/kg/hour throughout the experiment. Rectal temperature was maintained within the normal range (37 to 39.6°C) by a heating mattress[18].

The right femoral artery was cannulated with a triple lumen catheter (Certofix-Trio S715, Braun, Melsungen, Germany) for sampling of arterial blood gases and invasive blood pressure monitoring (Tram-rac 4A/Tram-scope 12C, Marquette Electronics, USA). A single lumen catheter (Sekalon-T 16G, Becton Dickinson, Singapore) was inserted in the femoral vein for blood sampling. The right carotid artery and the right jugular vein were surgically prepared. A pulmonary artery catheter (Swan Ganz CCOmbo 7.5 Fr, Edwards Lifescience, USA) was inserted, and positioned using the pulmonary arterial blood pressure curve and the pulmonary artery wedge pressure curve. Continuous cardiac output and central venous saturation were monitored (Vigilance, Edwards Lifescience, USA).

Microdialysis

Alongside the 8.0 endotracheal tube a 4.0 endotracheal tube was inserted into the trachea and advanced to a position 1 cm proximal to the carina confirmed by fiberoptic bronchoscopy. A thread was used to secure the two tracheal tubes and to avoid air leakage. The microdialysis catheter (CMA Custom made, 10 mm membrane length, 100 kDa cut-off, outer diameter 0.6 mm, CMA Microdialysis, Stockholm, Sweden) was introduced through the 4.0 endotracheal tube and guided fiberoptically into the left main stem bronchus. The microdialysis catheter was gently guided forward until wedged and then retracted 0.5 to 1 cm to avoid atelectasis. The proximal opening of the 4.0 endotracheal tube was sealed with wax to avoid air leakage.

Another microdialysis catheter (CMA Custom made, 10 mm membrane length, 100 kDa cut-off, outer diameter 0.6 mm, CMA Microdialysis, Stockholm, Sweden) was inserted into the right subclavian artery through a venous catheter (Optiva2 18G, Medex, Great Britain) in the right brachial artery.

Both microdialysis catheters were connected to microdialysis pumps (CMA 107, CMA Microdialysis AB, Stockholm, Sweden) and perfused by sterile phosphate buffered saline (PBS) at a flow rate of 2 μl/min.

The microdialysis catheters were perfused with a flow of 2 μl/min. The volume on the inside of the membranous part of the microdialysis catheters used in this study was less than 1.4 μl. Thus the time period of membrane contact of the microdialysis perfusion fluid was short.

The catheters were perfused in situ for ≥60 minutes before the experiment was started. Samples from each microdialysis catheter were collected in microvials that were exchanged every 60 minutes, and analyzed online for lactate and urea on the CMA 600 (CMA Microdialysis AB, Stockholm, Sweden), using the peroxidase methodology.

To verify the position of the catheter at the end of the experiment, the microdialysis catheter either was perfused with methylene blue and the bronchial tree dissected or the catheter was left in situ and the bronchial tree dissected (figure 1).

Experimental protocol

After surgical preparations the animals were allowed to stabilize for 60 minutes.

Lactate

An infusion of sodium lactate ~50% (Merck KGaA, Darmstadt, Germany) was adjusted to an arterial blood lactate

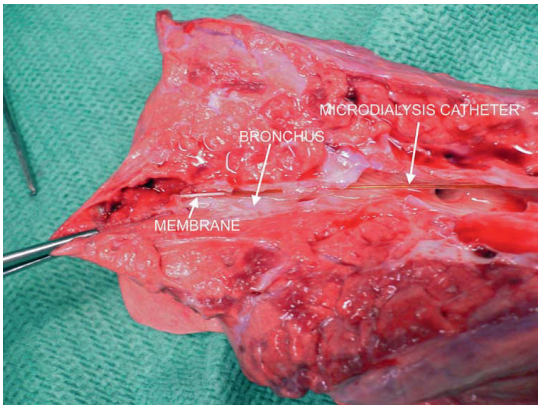

Figure 1
The microdialysis catheter in situ. The picture shows the microdialysis catheter in the distal bronchus. The distal white part is the microdialysis membrane. It is this part of the catheter that is in contact with the epithelial lining fluid and collects molecules by diffusion.

concentration of ~5 mmol/l for 120 minutes. Thereafter, the infusion rate was increased to an arterial blood lactate concentration of ~10 mmol/l (figure 2). Arterial blood was collected every 15 minutes throughout the experiment to establish a steady-state of lactate and $PaCO_2$ (ABL700, Radiometer Copenhagen, Denmark).

Fluorescein isothiocyanate dextran 4000 Da infusion

A solution of FD-4 (Sigma Chemical, St. Louis, MO, USA) in PBS was prepared fresh each day at a concentration of 10 mg/ml. The FD-4 infusion started with an intravenous loading dose of 10 mg/kg over 10 min followed by an infusion of 5 mg/kg/hour for 180 minutes. Then parallel to the FD-4 infusion at 5 mg/kg/hour a new bolus of 10 mg/kg was given. After the bolus the infusion was increased to 10 mg/kg/hour for the last 180 minutes of the experiment (figure 3).

Beginning during in the stabilizing period, 0.5 ml of arterial blood was sampled every 60 minutes throughout the experiment. Fluorescence of plasma and microdialysate

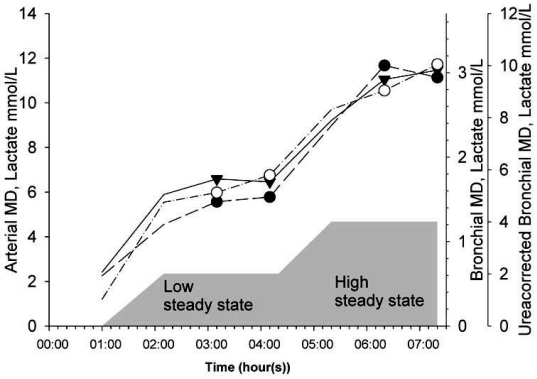

Figure 2
Overview of the lactate infusion with two steady states. Data are presented as mean lactate values by arterial microdialysis (solid line) with mean values in steady state (filled triangles), bronchial microdialysis (long dash line) with mean values in steady state (filled circles) and ureacorrected bronchial microdialysis (dash-dot line) with mean values in steady state (open circles). The circles and the triangles represent the time where the microdialysis vials were exchanged for the steady state samples. The gray area is a graphical presentation of the intravenous lactate infusion. During the low steady state the infusion of sodium lactate was gradually increased to maintain an arterial blood lactate of ~5 mmol/L. During the high steady state the infusion of sodium lactate was increased to maintain an arterial blood lactate of ~10 mmol/L.

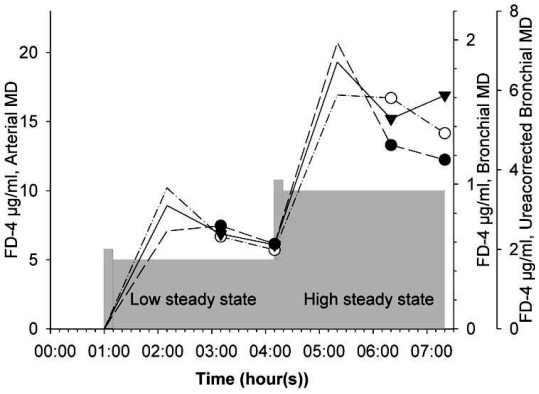

Figure 3
Overview of the fluorescein isothiocyanate dextran 4000 Da (FD-4) infusion with two steady states. Data are presented as mean FD-4 values by arterial microdialysis (solid line) with mean values in steady state (filled triangles), bronchial microdialysis (long dash line) with mean values in steady state (filled circles) and ureacorrected bronchial microdialysis (dash-dot line) with mean values in steady state (open circles). The circles and the triangles represent the time where the microdialysis vials were exchanged for the steady state samples. The gray area is a graphical presentation of the intravenous FD-4 infusion. The infusion was started with an intravenous bolus of 10 µg/kg over 10 minutes. In the low steady state an infusion of FD-4 at 5 µg/kg/hour was maintained. After the low steady state a new intravenous FD-4 bolus of 10 µg/kg over 10 minutes was infused. In the high steady state an infusion of FD-4 at 10 µg/kg/hour was maintained.

samples was measured on a fluorescence spectrophotometer (Fluoroskan II, Labsystem).

Arteriobronchial urea gradient

Urea concentrations are the same throughout various body compartments. The arteriobronchial urea gradient in each sampling period is a measure of the microdialysis catheter functioning in the bronchial as opposed to the arterial compartment.

In vivo recovery

Blood lactate was measured every 30 minutes. Mean arterial blood concentration of lactate during a 60-minutes period was defined as the mean of the value at 0, 30 and 60 minutes for the period.

Arterial blood FD-4 was measured every 60 minutes. Mean arterial blood concentration of FD-4 during a 60-minutes period was defined as the mean of the value at 0 and 60 minutes for the period

Arterial microdialysis was sampled over the same 60-minute period.

In vivo recovery was calculated as $[Molecule]_{arterial\ microdialysis}/[Molecule]_{arterial\ blood}$.

In vitro microdialysis

Lactate wells were prepared in PBS at lactate concentrations of ~1, ~5 and ~10 mmol/L. Three microdialysis catheters (CMA20, 10 mm membrane length, 100 kDa cut-off, outer diameter 6 mm, CMA microdialysis, Stockholm, Sweden) were perfused with PBS at 2 μl/min and put in the first well, lactate ~1 mmol/L, for 60 minutes to stabilize. Thereafter vials were exchanged every 60 minutes for 180 minutes. Samples from the wells were collected and analyzed at the beginning and at the end of the experiment. The procedure was then repeated for lactate ~5 and ~10 mmol/L. All lactate samples were analyzed on the CMA 600 (CMA Microdialysis AB, Stockholm, Sweden).

The procedure was repeated for FD-4 wells prepared with PBS at FD-4 concentrations of ~10, ~50 and ~150 μg/ml. This setup gave good and stable volume recovery with FD-4 samples being analyzed in triplicate or quadruplicate, each well containing 25 μl (Fluoroskan II, Labsystem).

In vitro recovery was calculated as $[Molecule]_{well\ microdialysis}/[Molecule]_{well}$.

Statistics and calculations

Arterial microdialysis values were considered to be the real arterial concentration during each sample period. Bronchial values are presented with and without correction by the arteriobronchial urea gradient. Ureacorrection was done in accordance to the formula in Rennard's article[9]. $([Urea]_{arterial}/[Urea]_{bronchial})/([Molecule]_{arterial}/[Molecule]_{bronchial})$.

Evaluation of bronchial microdialysis and urea-corrected bronchial microdialysis as an approximation of arterial microdialysis are presented as accuracy. The bronchial microdialysis values, corrected and uncorrected, are presented as fractions of the arterial microdialysis values. Accuracy = $[Molecule]_{bronchial}/[Molecule]_{arterial}$. To evaluate the benefit of the urea correction, the coefficient of variation was used. Coefficient of variation = Standard deviation/Mean · 100.

Calibrations of the microdialysis catheters were done by in vitro experiments and in vivo calculations. In vitro relative recovery = $[Molecule]_{well\ microdialysis}/[Molecule]_{well}$. In vivo relative recovery = $[Molecule]_{arterial\ microdialysis}/[Molecule]_{arterial\ blood}$.

The data are presented as values and mean ± 95% confidence interval. Paired t-test was used to compare means between arterial, bronchial and urea-corrected bronchial microdialysis with significance defined as $p < 0.05$.

Results

Circulatory and respiratory measurements

Heart rate, mean artery pressure, cardiac output, central venous oxygen saturation and partial pressure of carbondioxide remained stable throughout the entire experiment (table 1).

Recovery measurements

The in vivo lactate relative recovery was 105.9 ± 7.2%. The in vivo FD-4 relative recovery was 28.8 ± 4.4%.

The in vitro lactate relative recovery was 54.9 ± 3.2%. The in vitro FD-4 relative recovery was 14.2 ± 1.0%.

Arteriobronchial urea gradient

Arterial urea by microdialysis was 3.69 ± 0.59 mmol/L. Bronchial urea by microdialysis was 1.19 ± 0.21 mmol/L. Arteriobronchial urea gradient was 3.78 ± 0.85.

Lactate

During the steady-state periods lactate measured by arterial microdialysis was 6.5 ± 0.9 and 11.8 ± 1.4 mmol/L, by bronchial microdialysis 1.5 ± 0.8 and 3.0 ± 0.9 mmol/L and by urea-corrected bronchial microdialysis 5.5 ± 0.8 and 9.6 ± 1.3 mmol/L respectively. There was an arteriobronchial gradient of 1.2 ± 0.1 for lactate as measured by arterial microdialysis and bronchial microdialysis, corrected by the arteriobronchial urea gradient. Steady-state values and mean values for arterial lactate, bronchial lac-

Table 1: Central hemodynamic and respiratory variables.

Parameter	Stabilizing	Low steady state	High steady state
Mean arterial pressure (mmHg)	65 ± 3	64 ± 2	60 ± 1
Heart rate (beats/min)	81 ± 15	88 ± 6	89 ± 3
Cardiac output (l/min)	4.2 ± 0,9	4.8 ± 0.4	4.9 ± 0.4
Central venous oxygen saturation (percent)	59 ± 5	62 ± 3	60 ± 3
Arterial partial pressure of carbon dioxide (kPa)	4.81 ± 0.23	4.78 ± 0.09	4.94 ± 0.11

Values are mean ± 95% confidence interval.

tate and urea-corrected bronchial lactate, as measured by microdialysis are presented in figure 4. Paired t-test show a significant difference ($p < 0.05$) between lactate by arterial microdialysis and urea-corrected bronchial microdialysis at low steady state (blood lactate ~5 mmol/L).

The accuracy of bronchial microdialysis with a continuous lactate infusion was mean 0.26 ± 0.08 with a coefficient of variation of 62.6%. The accuracy of bronchial microdialysis with a continuous lactate infusion and a correction by the arteriobronchial urea gradient was mean 0.81 ± 0.06 with a coefficient of variation of 17.0% (figure 5). The reduction in the coefficient of variation is in accordance with urea being a molecule in almost immediate equilibrium within all body compartments and thereby is a correction factor of bronchial microdialysis catheter functioning.

Non-microdialysis data are presented in table 2.

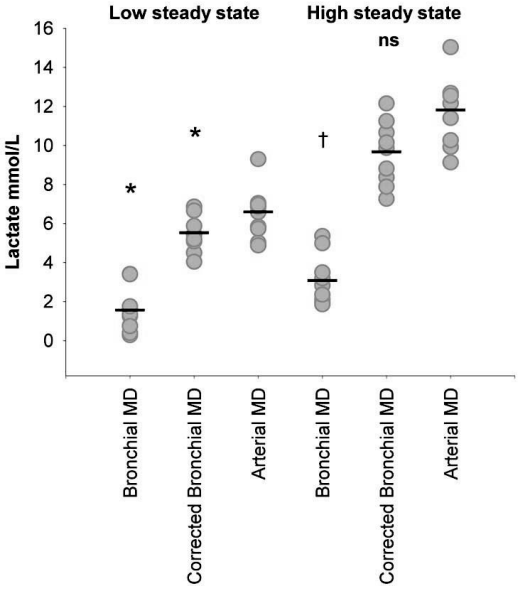

Figure 4
Microdialysate lactate. All values (gray circles) and mean (-) are presented. Lactate values by corrected bronchial microdialysis are corrected by the arteriobronchial urea gradient. * Paired T-test showed significant difference from arterial microdialysis at low steady state (arterial blood lactate ~5 mmol/L) ($p < 0.05$). † Paired t-test showed significant difference from arterial microdialysis at high steady state (arterial blood lactate ~10 mmol/L) ($p < 0.05$). ns Non significant from arterial microdialysis at high steady state (arterial blood lactate ~10 mmol/L).

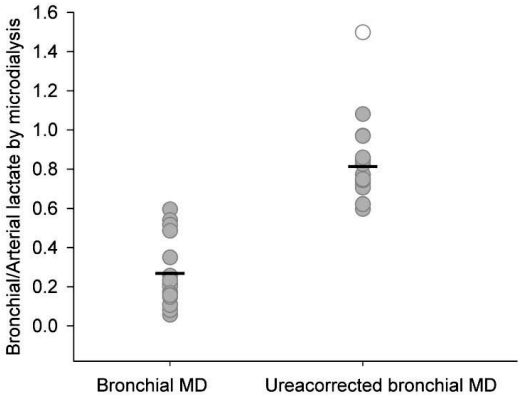

Figure 5
Accuracy of bronchial microdialysis of lactate. All values (gray circles) and mean (-) are presented. One value (open circle) is excluded as an outlier due to extreme deviation in the arterial microdialysis value. The accuracy of bronchial microdialysis with a continuous lactate infusion was mean 0.26 ± 0.08 with a coefficient of variation of 62.6%. The accuracy of bronchial microdialysis with a continuous lactate infusion and a correction by the arteriobronchial urea gradient was mean 0.81 ± 0.06 with a coefficient of variation of 17.0%. The reduced coefficient of variation after correction by the arteriobronchial ureagradient sustains the ureacorrection as useful correction factor to estimate the absolute concentrations of molecules in the epithelial lining fluid as measured by microdialysis.

Fluorescein isothiocyanate dextran 4000 Da

During the steady-state periods FD-4 measured by arterial microdialysis was 6.5 ± 1.9 and 16.0 ± 4.0 μg/ml, by bronchial microdialysis 0.7 ± 0.3 and 1.2 ± 0.5 μg/ml and by urea-corrected bronchial microdialysis 2.2 ± 0.6 and 5.3 ± 2.5 μg/ml respectively. There was an arteriobronchial gradient of 4.0 ± 1.2 for FD-4 as measured by arterial microdialysis and bronchial microdialysis, corrected by the arteriobronchial urea gradient. Steady-state values and means for arterial FD-4, bronchial FD-4 and ureacorrected bronchial FD-4, as measured by microdialysis, are presented in figure 6. There was a defined barrier between blood and bronchi for the diffusion of FD-4.

The accuracy of bronchial microdialysis with a continuous FD-4 infusion was mean 0.09 ± 0.03 with a coefficient of variation of 65.2%. The accuracy of bronchial microdialysis with a continuous FD-4 infusion and a correction by the arteriobronchial urea gradient was mean 0.35 ± 0.10 with a coefficient of variation of 54.8%. The reduction in the coefficient of variation was less than expected. Previous knowledge of FD-4 analyses on the Fluoroskan II is

Table 2: Non-microdialysis data.

Parameter	Low steady state	High steady state
Lactate (mmol/L)	5.9 ± 0.4	11.2 ± 0.3
Fluorescein isothiocyanate dextran 4000 Da (μg/ml)	25.2 ± 3.8	49.5 ± 6.7

Lactate was measured in blood and fluorescein isothiocyanate dextran 4000 Da was measured in plasma. Non-microdialysis data are intermittent samples and not continuous measurements. Values are mean ± 95% confidence interval.

from experiments with wells containing 150 μl. Due to small volumes sampled by microdialysis, pilot in vitro studies were conducted with wells containing 15 μl, 25 μl and 40 μl. With wells containing 15 μl there was a great variance and by visual inspection we saw that this small volume did not cover the bottom of the well. Wells containing 25 μl and 40 μl gave similar results and variance (data not shown). During analysis of the last fluorescein isothiocyanate results from the in vivo experiment, we registered that wells with higher concentrations of FD-4 increased the registered result of neighboring wells with lower concentration of FD-4. Due to lack of material the analysis could not be repeated and controlled.

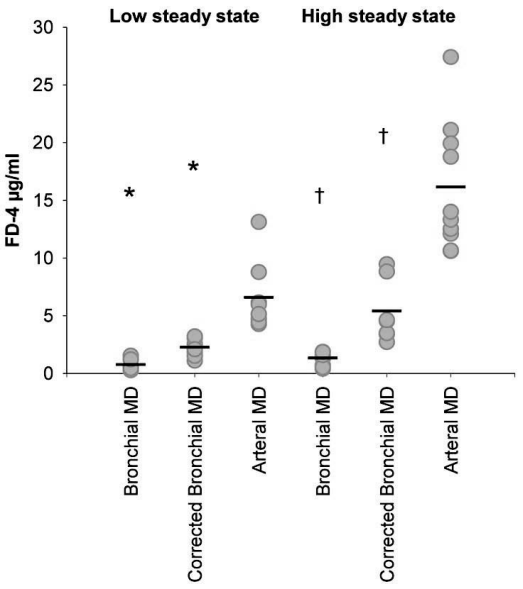

Figure 6
Microdialysate fluorescein isothiocyanate dextran 4000 Da. All values (gray circles) and mean (-) are presented. Fluorescein isothiocyanate dextran 4000 Da (FD-4) values by corrected bronchial microdialysis are corrected by the arteriobronchial urea gradient. * Paired t-test showed significant difference from arterial microdialysis at low steady state (FD-4 5 μg/kg/hour) ($p < 0.05$). † Paired t-test showed significant difference from arterial microdialysis at high steady state (FD-4 10 μg/kg/hour) ($p < 0.05$).

Non-microdialysis data are presented in table 2.

Individual correlation

To evaluate bronchial microdialysis as a tool of continuous monitoring all pigs are presented in XY-plots with individual values and correlation lines (figure 7). There was a significant improvement in the correlation between urea-corrected bronchial and arterial lactate values versus bronchial and arterial lactate values by paired t-test. For fluorescein isothiocyanate, the improvement was only a tendency and did not reach significance.

Discussion

Extensive research on the bronchial epithelial lining fluid has been done to understand mechanisms of permeability, pharmacokinetics and pathophysiology. The lack of techniques for continuous monitoring and the possibility of causing lung injury have limited previous work to describe the dynamics of physiological phenomena. Lung microdialysis as previously described, with an open surgical technique and "interstitial" introduction of the microdialysis catheter by visual control, is only suitable for patients undergoing thoracic surgery [19-21].

This study indicates that bronchial microdialysis can be used as a continuous monitor of the epithelial lining fluid. Correction of bronchial microdialysis by the arteriobronchial urea gradient enhances bronchial microdialysis, as measured by lactate, by reducing the coefficient of variation. Correction by the arteriobronchial urea gradient makes bronchial microdialysis suitable as a monitor of substantial concentration changes in the epithelial lining fluid.

There is an ongoing controversy on how to estimate the amount of epithelial lining fluid recovered. Urea rapidly diffuses and equilibrates into the total volume sampled with BAL technique. Therefore recovered concentration of urea as a marker of recovered concentration of epithelial lining fluid has a tendency to overestimate the recovered concentration of epithelial lining fluid and hence underestimate the bronchial concentration of the measured molecule. Total BAL volumes in humans vary from 100 to 300 ml[9,22]. The tendency of overestimating the recovered volume of epithelial lining fluid also increases with increasing dwell time with BAL technique. The dwell time of BAL varies from about 3 to more than 10 minutes. Technetium-99 m diethylenetriaminepenta-acetic acid has been used as an alternative marker of recovered concentration of epithelial lining fluid without the problem

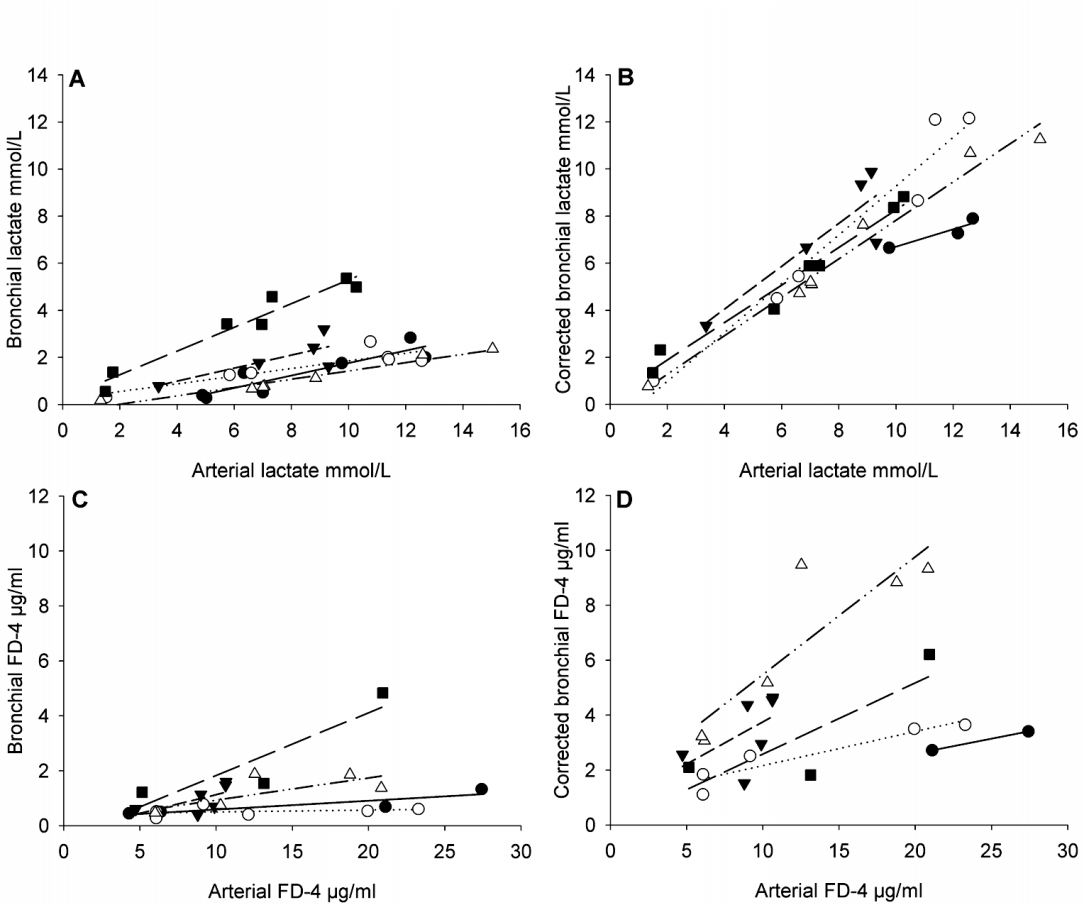

Figure 7

XY-plot of bronchial and corrected bronchial microdialysis data against arterial microdialysis data of lactate and fluorescein isothiocyanate dextran 4000 Da. All five pigs are represented; number one closed circles and line, number two open circles and dotted line, number three closed triangles and medium dashed line, number four open triangles and dash-dot-dot line, and number five closed squares and long dashed line. Plot A and B show arterial lactate along the X-axis and bronchial (R^2 0.82 ± 0.18) and ureacorrected bronchial lactate (R^2 0.91 ± 0.11) along the Y-axis respectively. Paired t-test of R^2 for the individual bronchial and ureacorrected bronchial lactate XY-plots showed significant difference ($p < 0.05$). Plot C and D show arterial fluorescein isothiocyanate dextran 4000 Da (FD-4) along the X-axis and bronchial (R^2 0.53 ± 0.38) and ureacorrected bronchial FD-4 (R^2 0.72 ± 0.34) along the Y-axis. Paired t-test of R^2 for the individual bronchial and ureacorrected bronchial FD-4 XY-plots was not significant.

of additional influx in the instilled lavage volume[23]. However the method is time consuming and this limits its applicability in both clinical and experimental settings. With small volume lavage (3 aliquots of 1 ml) with short dwell time (less than 1 minute) urea is a valid marker of dilution in BAL fluids in both the normal, diseased and recovering lung in infants, according to Dargaville et al[7]. With microdialysis, the additional volume to equilibrate was minimal (less than 1.4 µl), limited to the volume on the inside of the semi-permeable membrane of the catheter. With a perfusion flow of 2 µl/minute and the short time period of contact between the microdialysis perfusion fluid and the epithelial lining fluid, the arteriobronchial urea gradient was a reasonable correction factor

for the recovered concentration of epithelial lining fluid. Thus it could be a correction factor to calculate the absolute concentrations of lactate and FD-4 in the epithelial lining fluid[7,10,11,23].

In vivo studies of transport of molecules are complicated by the metabolic activity of the organism. Molecules will to some extent be depositioned both extra- and intracelluarly. Metabolism in all tissues and renal clearance of the molecule one measures are factors that must be corrected and/or considered when the conclusions are drawn.

Lactate is depositioned in all body compartments, especially red blood cells and muscle[24]. The lung itself is a possible producer of lactic acid[25]. Urea (60 Da) and lactate (90 Da) differ in size and shape and may have different diffusion properties over the blood-bronchial barrier. Lactate passed the blood-bronchial barrier rather freely with a gradient of 1.2:1. Whether the gradient is a true blood-bronchial gradient is not known at present.

FD-4 crossed the blood-bronchial barrier with a gradient of about 4:1. As described previously in monolayer cell cultures of rat alveolar epithelial cells, FD-4 is transported through paracellular pores in human cultured alveolar epithelial cell monolayer[26]. FD-4 was chosen as a macromolecule with restricted transport through paracellular pores. Matsukawa showed this in rat alveolar epithelial cell monolayer[12]. As far as we know there is no previous study that describes FD-4 transport in vivo across the blood-bronchial barrier. In our study FD-4 had to cross intact endothelium, lung interstitium and lung epithelium. Due to methodological limitations with small volume samples, our FD-4 results varied. The blood-bronchial gradient was 4.0:1 ± 1.2. FD-4 was transported across the blood-bronchial barrier with restriction, in accordance with previous findings, but this requires further research to be confirmed.

The arteriobronchial urea gradient as a marker of bronchial catheter function reduced the coefficient of variation from 62.6% to 17.0% of the bronchial microdialysis as measured for lactate. The arteriobronchial urea gradient significantly improved correlation of arterial and bronchial lactate sampled over the same time interval in the same pig. A coefficient of variation of 17% still limits the use of bronchial microdialysis in the measurement of minor changes in lactate concentration of the bronchial epithelial fluid.

Bronchial microdialysis has some limitations. Bronchi are relatively stiff lumens with continuous movements during the respiratory cycle. The epithelial lining fluid is only a thin film. Microdialysis is developed for analyzing the composition of extracellular fluids in compact tissues with little movement. In the bronchi, one must assume that only varying parts of the microdialysis membrane are in contact with the epithelial lining fluid during the respiratory cycle. Thus, a marker of catheter functioning is necessary for characterizing each individual microdialysis catheter, measuring the recovered amount of epithelial lining fluid and the absolute concentrations of the molecules in the epithelial lining fluid.

With correction of bronchial microdialysis by the arteriobronchial urea gradient, the coefficient of variation of FD-4 decreased only slightly, from 65.2% to 54.8%. There was only a tendency toward improved correlation between arterial and bronchial FD-4 sampled over the same time interval in the same pig. The pilot studies on concentration and variation of different volumes on fluorescence spectrophotometry concluded that 25 μl in each well was sufficient to get precise numbers with a small degree of variation. But the FD-4 results from our study were not optimal with a high degree of variation. One of the problems was that wells with high concentration of FD-4 increased the measured FD-4 concentration in neighboring wells with low concentration of FD-4 according to our fluorescence spectrophotometer. This problem could be a contributing factor to the lack of precision with our method. The lack of reduction in the coefficient of variation of FD-4 by urea correction is probably due to methodological errors in analyzing FD-4 in small volumes by fluorescence spectrophotometry. Thus FD-4 measurement in small volumes still has to be refined to increase the precision of the method.

In vivo lactate recovery was more than 100% (105.9 ± 7.2%). The non-microdialysis samples were analyzed in whole blood. The microdialysis probe was placed in plasma in the subclavian artery. The plasma lactate is higher than the lactate value in erythrocytes[27].

In our laboratory we have shown that the CMA 600 (CMA Microdialysis AB, Stockholm, Sweden) and the ABL700 (Radiometer Copenhagen, Denmark) return different results from the same sample with a known lactate concentration (data not shown). The CMA600:ABL700 ratio for lactate in our laboratory is mean 1.13:1. The in vivo recovery percent for lactate must be evaluated with these factors in mind.

There was a major difference, about 2:1, between in vivo and in vitro recovery. In vitro the microdialysis catheter was placed in a well, collecting molecules from the immediate surroundings. In general, this means that there will always be a concentration gradient within the well around the membrane of the microdialysis catheter. Accordingly, there was a local concentration gradient and a smaller concentration of the measured molecule in the immediate

surroundings of the microdialysis catheter. This effect increased with increasing perfusion volume per time unit. 2 μl/min is a relatively high perfusion rate with the microdialysis technique. In vivo, the catheter was in the arterial bloodflow with a continuous replacement of the fluid surrounding the microdialysis membrane so that the concentration of the measured molecule was the same in the immediate surroundings of the microdialysis membrane as in the rest of the fluid compartment.

Based on good volume recovery in the in vitro experiments, an isotonic crystalloid perfusion fluid was chosen. A microdialysis membrane with 100 kDa cut-off has large pores which easily can contribute to a loss of collected volume limiting the possibility for analyzing triplicates. The literature indicates that use of a colloid perfusion fluid increases the recovered volume, but has no significant influence on the concentration of the sampled molecules at a perfusion rate of 2 μl/min[28]. Thus a colloid perfusion fluid would probably increase the recovered volume and more frequently allow triplicate analyses of each sample and larger volumes in each analysis.

The method as presented in this study is well suited for monitoring substantial changes in the composition of the epithelial lining fluid as seen with major physiological trauma such as sepsis and ischemia-reperfusion injury, and may even be used in the clinical setting. Combining the application of microdialysis in the bronchi with present knowledge of the microdialysis technique can provide better knowledge of the dynamics of the epithelial lining fluid in normal (pharmacokinetics) and pathological processes (altered permeability and measurement of markers of inflammation).

Conclusion

Bronchial microdialysis is an applicable continuous monitor of the dynamic changes in the composition of the epithelial lining fluid. The absolute concentrations of the measured molecules can be estimated with acceptable precision using a correction by the arteriobronchial urea gradient.

Further research on bronchial microdialysis has to be done.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

SST, ES and PA have made major contributions to conception and design of the study. OL, SLS and SG have made major contributions to conception and design on laboratory methods and surgical preparations. All have contributed in the acquisition of data. SST, OL and ES have made major contributions in analysis and interpretation of the data. All have been involved in drafting the manuscript or revising it critically for important intellectual content. All have given final approval of the version to be published.

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