Role of NO-cGMP signalling pathway in mediation of ischemia-reperfusion lung injury

Inaugural Dissertation
submitted to the
Faculty of Medicine
in partial fulfilment of the requirements
for the PhD-Degree
of the Faculty of Medicine
of the Justus Liebig University Giessen

by

Bakytbek Egemnazarov
of
Bishkek, Kyrgyz Republic

Giessen 2008

From the Medical Clinic II, University of Giessen Lung Centre

Chairman: Werner Seeger, Prof., M.D.

of the Faculty of Medicine of the Justus Liebig University Giessen

First Supervisor and Committee Member:

Second Supervisor and Committee Member:

Committee Members:

Date of Doctoral Defence:

Role of NO-cGMP signalling pathway in mediation of ischemia-reperfusion lung injury

Inaugural Dissertation
submitted to the
Faculty of Medicine
in partial fulfilment of the requirements
for the PhD-Degree
of the Faculty of Medicine
of the Justus Liebig University Giessen

by

Bakytbek Egemnazarov
of
Bishkek, Kyrgyz Republic

Giessen 2008

INDEX OF CONTENTS

1 INTRODUCTION

1.1 Ischemia/reperfusion injury. Definition.

Ischemia/reperfusion injury (I/R) is a tissue damage, which occurs during organ transplantation, thrombendarterectomy, myocardial ischemia and revascularisation (1). Lung I/R results in endothelial damage and dysfunction leading to the development of high permeability pulmonary edema. Clinical picture of lung I/R may vary from interstitial edema to multiple organ dysfunction syndrome. The pathogenesis of I/R is complex and involves both local and systemic inflammatory response characterized by oxidant production, complement activation, leukocyte–endothelial cell adhesion, transendothelial leukocyte migration, platelet–leukocyte aggregation, increased microvascular permeability and decreased endothelium dependent relaxation (1, 2). Over the past two decades, the role of free radicals and other inflammatory mediators in IR injury have been extensively investigated. However, these responses and mediators appear to contribute only in part to lung IR injury.

1.2 Role of NO-cGMP signalling pathway in I/R injury

Many authors reported an impairment of nitric oxide (NO) production under conditions of I/R to contribute significantly to endothelial dysfunction (3, 4). NO-cGMP signalling pathway in the lung is involved in control of many biological functions involving vascular tone, microvascular permeability, and inhibition of neutrophil-endothelium interactions. NO is generated via oxidation of L-arginine by a family of three NO synthases (NOS): neuronal NOS (nNOS, type 1), inducible NOS (iNOS, type 2), and endothelial NOS (eNOS, type 3). All of the three NOS isoforms are expressed in the lung (5). In addition, in the lung a wide variety of cell types including epithelial cells, inflammatory cells, and endothelial cells express NOS (6).

However, there is little knowledge about their interaction and pathophysiologic relevance in I/R-associated inflammatory processes.

Blockage of NOS has been shown to be both beneficial (7, 8) and deleterious (9) in the condition of I/R. Moreover, administration of NO, the product of NO synthase, produced ambiguous results. NO inhalation has been demonstrated to attenuate I/R lung injury in experimental (10) and clinical settings (11). However, other authors (12) reported that nitric oxide application after bilateral lung transplantation did not improve early graft dysfunction. In the randomized clinical trial (13) prophylactic NO administration had no effect on pulmonary edema formation and resolution following lung transplantation. Possible explanation for these differing results could be that different NO synthase isoforms have different functions and influence differently inflammatory reactions in the course of I/R. Indeed, whereas eNOS has been demonstrated to reduce inflammation (14), iNOS has been demonstrated to be involved in mediation of inflammation (15).

1.2.1Role of iNOS in I/R injury

Induction of high-output iNOS during I/R might lead to overproduction of NO and contribute to vascular damage. NO overproduction in an oxidant-rich environment is suggested to cause tissue injury by formation of toxic peroxynitrite (16). Activation of iNOS and excessive peroxynitrite formation have been demonstrated to contribute to I/R induced vascular dysfunction and selective iNOS inhibition prevented lungs from the dysfunction (17).

1.2.2Role of eNOS in I/R injury

eNOS has been demonstrated to be protective against ischemia reperfusion injury. Activation eNOS has been shown to attenuate post-ischemic inflammatory injury (18). Moreover, over-expression of eNOS in mice led to reduced infarct size after myocardial I/R (19). On the other hand, oxidative stress, as a result of I/R (20), can induce oxidation of cofactors like tetrahydrobiopterin (21) and therefore lead to eNOS uncoupling and switch from NO production to superoxide production (22). Indeed, eNOS uncoupling in diabetic transgenic mice has been demonstrated to exacerbate I/R induced liver injury (23). In contrast, other authors reported that eNOS is involved in development of vascular leakage in acute inflammation, and eNOS knock out (KO) mice are protected against the injury (24).

1.2.3Role of nNOS in I/R injury

Role of nNOS in the development of lung I/R injury has not been studied intensively. However, this isoform has been shown to contribute to brain injury. Increased expression of nNOS has been demonstrated in the brain after I/R (25). The increased expression of nNOS led to increased cell death after reoxygenation in the model of cerebellar I/R (26). Moreover, selective nNOS inhibition reduced cerebral injury in preventive and curative therapeutic approaches (27). In model of myocardial I/R injury nNOS lacking mice developed infarct of the same size as their wild type counterparts did (28).

1.2.4Role of sGC in I/R injury

Soluble guanylyl cyclase (sGC) is a downstream molecule in NO-cGMP signalling pathway. This enzyme is responsible for conversion of GTP to cGMP. The latter

messenger affects cGMP dependent phosphodiesterases, protein kinases, ion channels, thus exerting its effect. Enhancing the effect of sCG has been demonstrated to protect against I/R lung injury. Schütte et al. have revealed that inhibition of PDE5, which is responsible for degradation of cGMP, protects against I/R lung injury (29). Application of cGMP analogue 8-BrcGMP to organ preservation solution (30) has been shown to reduce lung damage.

1.3 Role of NADPH oxidase in I/R injury

In many studies the role of reactive oxygen species (ROS) in development of I/R injury has been demonstrated (31). There are many enzyme systems capable of ROS production in the cell, including mitochondrial respiratory chain, xanthine oxidoreductase, uncoupled NO synthase, and NADPH oxidase. Among them, NADPH oxidases are expressed in many cell types in the lung and produce large amounts of ROS. Indeed, I/R lung injury was associated with activation of NADPH oxidase (32). Moreover, inhibition of NADPH oxidase has been reported to be protective against vascular leakage in reperfused lungs (33). Mice lacking functional NADPH oxidase were protected against development of stroke (34).

1.4 Interaction between NO-cGMP signalling pathway and NADPH oxidase

Reactive oxygen species can not only induce tissue damage directly, by oxidizing variety of biomolecules, but also interfere with function of other signalling pathways. ROS could influence NO-cGMP signalling pathway via binding with NO, thereby limiting its bioavailability. This is underlying mechanism of endothelial dysfunction. Importance of this mechanism has been demonstrated for development of atherosclerosis (35), and stroke (36). In presence of large amounts of NO, reaction with superoxide leads to peroxynitrite formation, which in turn contributes to

oxidative damage. Less is known about the influence of NO-cGMP pathway on NADPH oxidases. It has been shown that NOS activation can inhibit NADPH oxidase via interfering with its assembly (37, 38) and changing its expression (39, 40).

1.5 Introduction into NO-cGMP pathway

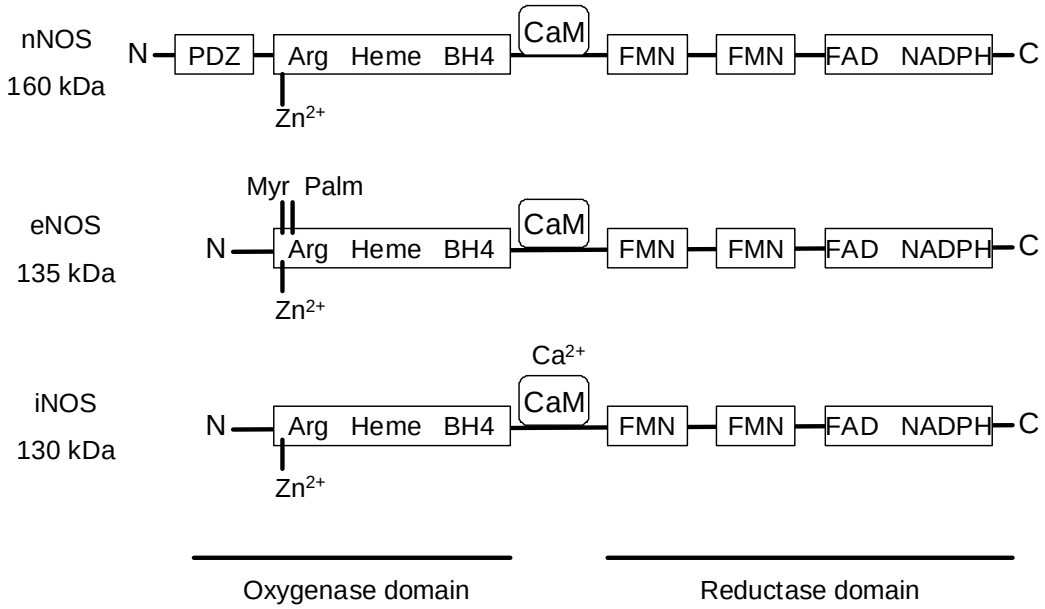
NO synthases consist of two catalytic domains: N-terminal oxygenase domain and C-terminal reductase domain (Scheme 1). Oxygenase domain contains binding site for L-arginine, heme and tetrahydrobiopterin (BH4), while reductase domain has binding sites for FAD, FMN and NADPH. These two catalytic domains are connected by a calmodulin binding site. Flavins mediate transfer of electrons from NADPH to the heme iron, bringing about NO production via oxidation of L-arginine to L-citrulline. BH4 is important for the electron shuttling process and effects the homodimeric conformation of NOS. NOS isoforms share approximately 50% of primary sequence homology, suggesting that they may differ from each other in regulatory aspects.

1.5.1Endothelial NO synthase

eNOS is expressed primarily in endothelial cells but also in cardiac myocytes and platelets. In the lung eNOS expression was demonstrated in endothelial cells and bronchial epithelium (6). Regulation of eNOS activity is complex. Large variety of factors are known to regulate eNOS activity including shear stress, acetylcholine, bradykinin, histamine, VEGF, thrombin, estrogen. eNOS activity is regulated on the transcriptional level or by post-translational modifications (41). The latter include interactions like calcium-dependent calmodulin binding, post-translational modifications (e.g. phosphorylation, acylation) as well as sub-cellular compartmentalization (42).

1.5.2Neuronal NO synthase

Neuronal NOS has been shown to be expressed in neurons. Additionally, other non-neuronal cell types including: cardiac myocytes, skeletal myocytes, and the adventitia of a subset of neuronal blood vessels have been demonstrated to express nNOS (43). In the lung nNOS expression has been demonstrated in bodies and termini of non-adrenergic non cholinergic nerves innervating airway and vascular smooth muscle cells (44) as well as in capillary endothelial cells (45). Regulation of nNOS is



Scheme 1. Structural domains of NOS enzymes: alignment of conserved regions of the NOS enzymes. Calmodulin (CaM), cofactor and substrate binding regions. Isoform specific features including membrane-localizing PDZ (nNOSa and nNOSl), myristoylation (Myr) and palmitoylation (Palm) sites (eNOS) are also shown. Arg (l-arginine), BH4 (tetrahydrobiopterin), FAD (flavin adenine dinucleotide), FMN (flavin mononuclotide), NADPH (nicotinamide adenine dinucleotide phosphate), Zn2+ (zinc).

Adopted with modifications from N. Mungrue, Acta Physiol Scand 2003, 179, 123–135

complex. It is regulated acutely through reversible calcium–calmodulin binding. Brain derived neurotrophic factor (BDNF) and glial derived neurotrophic factor (GDNF) were demonstrated to control expression of nNOS. Interesting and specific feature of nNOS is presence of variety spicing variants, which are expressed in different tissues (46).

1.5.3Inducible NO synthase

Inflammation mediators have been demonstrated to induce expression of inducible NOS (iNOS, NOSII, NOS2) in many cell types including endothelial cells, smooth muscle cells, macrophages (47).

Regulation of iNOS is calcium-insensitive. iNOS is predominantly regulated at expressional level. The expression of iNOS is upregulated by LPS, oxidative stress and variety of cytokines such as interleukin-1β (IL-1β), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). Different inducers of iNOS activate different signaling pathways, leading either to enhanced transcription via nuclear factor kappa B (NF-kB), interferon regulatory factor-1 (IRF-1) or other transcription factors. Regulation of iNOS occurs also via modulation of mRNA stability. Kleinert et al. summarized in his recent review the latest advances in this field of research (48).

1.5.4Soluble guanylyl cyclase (sGC)

There are two isoforms of guanylyl cyclase: particulate (pGC) and soluble (sGC). sGC is a main down stream target of nitric oxide. Therefore, in our work we will discuss only this isoform. Its expression has been demonstrated in the cytoplasm of almost all mammalian cells (49).

NO activates sGC via binding to heme group and changing its conformation. Conformational changes result in activation of the enzyme. Recently a novel NO-independent sGC stimulation mechanism has been described. YC-1 and BAY 582667 have been demonstrated to bind to α-subunit of sGC leading to its activation. This activation takes place even in absence of NO. In presence of the stimulators effect of NO is enhanced (50).

1.6 Aim of the study

Despite intensive research exact pathomechanisms of I/R syndrome remain unclear. Previous studies produced equivocal results about the role of NO synthases in mediation of I/R injury. NO synthase inhibition has been shown to reduce lung injury (7, 8, 51). However, reduced iNOS expression and reduced activity of NO-cGMP signalling pathway in the course of I/R have been described (52). On the other hand, upregulation of eNOS attenuated I/R induced lung injury (18).

Although activation of sGC seems to be protective against I/R injury (30), exact mechanisms of the protective effect remain unclear.

Aim of this study was to evaluate: 1) the role of different NO synthases in the mediation of I/R induced lung injury and 2) the role of NO-cGMP signalling pathway in the control of NADPH oxidase activity in the course of I/R lung injury.

To answer these questions we applied the model of isolated perfused rabbit and mouse lung, because this model enables us to study processes taking place in the lung without regulatory influence of the whole body. We applied inhibitors of different NOS isoforms to determine contribution of different isoforms to development of I/R injury. Since inhibitors can display unspecific effects, mice with genetic depletion of eNOS and iNOS were employed. To reveal effects of NADPH oxidase, its stimulator and inhibitor were applied.

2 MATERIALS AND METHODS

2.1 Applied substances

All chemicals used were of analytical grade purity. Alphabetical list of used chemicals, reagents and consumables is provided in tables 1 and 2.

Table 1. Alphabetical list of used substances.

Substance	*Chemical name*	*Company*	*Catalogue number*
1400W	N-(3-(Aminomethyl)benzyl) acetamidine . 2HCl	Alexis	ALX-270-073-M005
Apocynin	4′-Hydroxy-3′-methoxyacetophenone	Sigma-Aldrich-Aldrich	W508454
BAY 41-2272		kindly provided by Dr. Stasch	
BYK 191023		Kindly provided by Altana Pharma	
Calcium chloride dehydrate	CaCl2 x 2H2O	Fluka Biochemika	21097
Chelex 100		BioRad Laboratories	142-2842
CPH	1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine	Alexis	ALX-430-078-M250

Substance	*Chemical name*	*Company*	*Catalogue number*
D-(+)-glucose anhydrous		Fluka Biochemika	49139
Deferoxamine mesylate		Sigma-Aldrich-Aldrich	D-9533
Elektrolytlösung II/N (Krebs-Henseleit buffer)		Serag-Wiessner KG	218978
Heparin		Ratiopharm GmbH	3029843
Ketavet	Ketamin hydrochloride	Pfizer	72201-2
L-NMMA	N^G-Monomethyl-L-arginine acetate salt	Sigma-Aldrich-Aldrich	M7033
Magnesium chloride hexahydrate	MgCl2 x 6H2O	Fluka Biochemika	63068
PMA	phorbol 12-myristrate 13-acetate	Sigma-Aldrich-Aldrich	P 8139-10MG
Potassium chloride	KCl	Fluka Biochemika	60128
Potassium dihydro-phosphate	KH2PO4	Merk	74.873.1000
Rompun 2%	Xylazine hydrochloride	Bayer Healthcare	KP043Z

Substance	*Chemical name*	*Company*	*Catalogue number*
SOD	superoxide dismutase from bovine erythrocytes	Sigma-Aldrich-Aldrich	S-2515
Sodium chloride	NaCl	Fluka Biochemika	71376

Table 2. Consumables

Consumables	*Supplier*
Single use syringes Inject Luer®, 1ml, 2ml, 5ml, 10ml	Braun, Melsungen, Germany
BD Microlance needles 21G, 26G	Becton Dickinson, Heidelberg, Germany
Single use gloves Transaflex®	Ansell, Surbiton Surrey, UK
Gauze balls size 6	Fuhrman Verbandstoffe GmbH, Munich, Germany
Napkins	Tork, Mannheim, Germany
Threads Nr. 12	Coats GmbH, Kenzingen, Germany
Surgical threads non-absorbable ETHIBOND EXCEL®, size 5-0	Ethicon GmbH, Norderstedt, Germany
Surgical instruments	Martin Medizintechnik, Tuttlingen, Germany
Disposable feather scalpel	Feather Safety Razor Co, Ltd, Osaka, Japan
Pipette tips, blue, yellow, white	EPPENDORF, Hamburg, Germany

Consumables	*Supplier*
Tygon® lab tubing 3603	Cole-Parmer Instruments Company, Vernon Hills, Illinois, USA
Tracheal cannula	Customer-made
Cannula for pulmonary artery catheterisation	Customer-made
Cannula for left heart catheterisation	Customer-made
Combi-Stopper	Intermedica GmbH Klein-Winternheim, Germany
Combitrans Monitoring-Set	B.Braun Melsungen AG, Melsungen, Germany
Haematocrit sealing compound	Blaubrand; Brand GmbH, Wertheim, Germany
Filter papers	Whatman, Schleicher & Schuell GmbH, Dassel, Germany
Steritop Filter GP Express 0.22 µm	Millipore, Eschborn, Germany

2.2 Animals

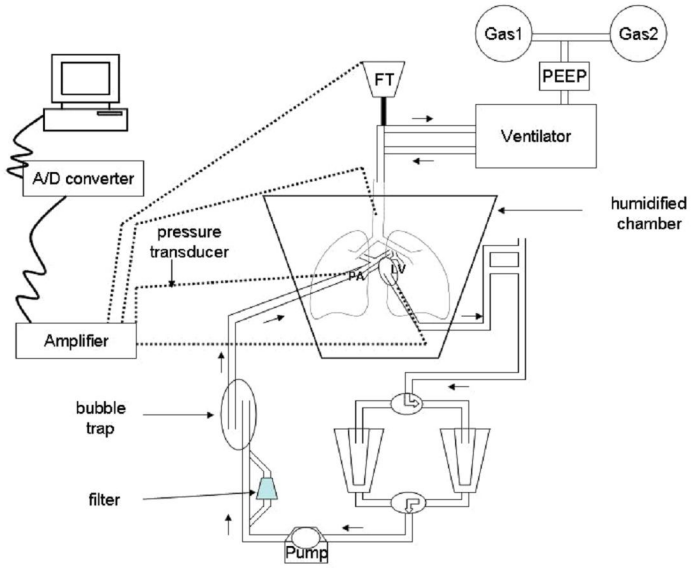
Male New Zealand Wight rabbits (body weight 2.8-3.5 kg) were used. WT C57BL/6J mice were purchased from Charles River Laboratory (Sulzfeld, Germany). iNOS and eNOS knockout (KO) mice were purchased from Jackson Laboratories (Bar Harbor, USA). All mice used in the experiments were male, 2 to 3 months of age, weighing 24 to 28 g each. Animals were provided food and water ad libitum. Animals were kept under pathogen free conditions. All animals received humane care in compliance with

the “Principles of Laboratory Animal Care” and the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health (NIH publication 86-23, revised 1985).

2.3 Isolated rabbit lung model.

2.3.1 Setup of isolated perfused rabbit lung

The model of isolated perfused and ventilated rabbit lung is depicted in the scheme 2. Devices and systems employed for the setup are listed in the table 4. The lung is canulated via pulmonary artery and left atrium and perfused in a recirculating system. Perfusion is flow-constant. Ventilation enables application of different gas mixtures. Ventilation is volume-constant. The lung is placed in warmed and humidified jacket. The model offers the opportunity to study specific pulmonary functions (53). This model possesses many advantages. It enables to determine physiological parameters, cells of the organ remain in their physiological and anatomical environment containing cell-to-cell contacts and native extracellular matrix. To the same time,



Scheme 2. Schematic presentation of the model of isolated perfused rabbit lung. Abbreviations: A/D converter – analog-to-digital converter, FT – force transducer, LV – left ventricle, PA – pulmonary artery.

controlling mechanisms of the organism are excluded. An investigator may retain control over several parameters. Limitation of this model is short duration of the experiment, since lungs deteriorate with time. Next limitation is deprivation of lymph drainage, which could contribute to fluid accumulation in the lung.

2.3.2 Animal preparation

Animals were anticoagulated with 1,000 U/kg of heparin and deeply anesthetized with ketamine and xylazine. A tracheostomy was performed, and the animals were room air ventilated with a tidal volume of 30 ml, a frequency of 30 breaths/min, and a positive end-expiratory pressure of 1 cm H_2O. After a midsternal thoracotomy, catheters were inserted into the pulmonary artery and left atrium, and perfusion with sterile Krebs-Henseleit buffer was started (content of Krebs-Henseleit buffer is provided in the table 3). Sterilized perfusion circuit tubing was used throughout. In parallel with the onset of artificial perfusion, the gas supply was changed to a mixture of 5.3% CO_2, 21% O_2, 73.7% N_2 provided by Messer (Siegen, Germany). For the washout of blood, the perfusate was initially not recirculated. The lungs were removed from the thorax without interruption of ventilation and perfusion and were freely suspended from a force transducer for the monitoring of organ weight in a temperature-equilibrated, humidified chamber at 39°C. In a recirculating system, the flow was slowly increased to 100 ml/min (total volume 250 ml). Left atrial pressure was set at 2 mmHg (referenced at the hilum), and the whole perfusion system was equilibrated at 39°C. Additionally, the inspiration loop of the ventilation system was connected to a humidifier and heated to 39°C.

Pressures in pulmonary artery and left atrium were registered with pressure transducers and transferred to computer. The capillary filtration coefficient (Kfc) was

determined gravimetrically from the slope of the lung weight gain curve induced by a 7.5-mmHg step elevation of the venous pressure for 8 min as previously described (54). Lung weight gain was calculated as the difference in organ weight measured directly before and 5 min after each of these pressure elevation manoeuvres.

Table 3. Content of Krebs-Henseleit buffer (Perfusate II/N) used for the setup of isolated perfused lung.

Chemical	*Concentration*
NaCl	120 mM
KCl	4.3mM
KH_2PO_3	1.1 mM
$NaHCO_3$	24 mM
MgCl x $6H_2O$	1.3 mM
$CaCl_2$	2.4 mM
Glucose	13.32 mM
hydroxyethylamylopectin (mol wt 200,000)	5% (wt/vol)

2.3.3Experimental protocols

After termination of the initial steady-state period and performance of a control hydrostatic challenge, the lungs were exposed to ischemia by stopping the perfusion and switching the inhaled gas mixture to anoxic 5,3% CO_2, 94,7% N_2 (Messer, Siegen, Germany). For maintenance of positive intravascular pressure, the arterial and venous

catheters were clamped after adjustment of initial value of arterial pressure to 6 mm Hg. During ischemia, lungs were continuously ventilated with a warmed and humidified anoxic gas mixture. At the end of ischemia, ventilation was changed to normoxia, and perfusion was re-established by increasing the flow stepwise over 3 min. Hydrostatic challenges were performed 30 and 60 after the onset of reperfusion. Duration of ischemic period and timings of hydrostatic challenges were designed based on previous studies performed in our laboratory (29, 55-57).

Lungs were treated according to one of following protocols:

1) I/R – lungs were exposed to ischemia with following reperfusion; no interventions were performed;

2) NIC – nonischemic control, lungs were perfused and ventilated throughout 7 h;

3) I/R + L-NMMA – unselective NOS inhibitor L-NMMA (400μM) was admixed to perfusion buffer 5 min before onset of ischemia;

4) I/R + 1400W – selective iNOS inhibitor 1400W (10 μM) was applied into perfusion system 5 min before onset of ischemia;

5) I/R + BYK 191023 – selective iNOS inhibitor (20 μM) was applied;

6) I/R + VNIO – selective nNOS inhibitor VNIO (1μM) was applied into perfusion 5 min before onset of ischemia;

7) I/R + BAY 41-2272 – the substance (3μM) was admixed to perfusion buffer 5 min before onset of ischemia. Dosage was chosen on the basis of our previous results as the most effective;

8) I/R + apocynin – the substance in end concentration 0.5 mM was admixed to perfusion buffer 5 min before onset of ischemia;

Criteria for termination of experiments were achieving 60 min after reperfusion or weight gain more than 25 g during reperfusion.

In separated set of experiments lungs were treated as described for NIC and I/R groups. After 10 min after onset of reperfusion experiment was stopped, lung tissue was snap frozen in liquid nitrogen and further used for western blotting.

Measurements of ROS release in perfusate were performed in additional experiments with the same protocol. Additional control experiments with SOD 150 U/ml were performed.

PMA stimulation experiments. Krebs-Henseleit buffer used for these experiments was incubated with 5 µM diethyldithiocarbamate overnight to allow sedimentation. Briefly before the experiments, 20 µM DFO and $NaHCO_3$ were added. Lungs were prepared as described previously. After the initial steady state period CPH (1mM) was added to the circulating buffer fluid. Five minutes later, one of three different protocols was started:

1) PMA - one hour perfusion period followed by a bolus application of PMA, end concentration 1µM, into the pulmonary artery. Every 5 min samples from the venous outflow of the isolated lung were taken in 50µl glass capillaries and measured by spectroscopy as described previously. After PMA stimulation measurements were performed every 2 min.

2) BAY 41-2272 + PMA – after 30 min of perfusion with CPH 1mM the sGC activator was applied with following PMA simulation after next 30 min. Measurements were performed as described for the PMA group.

3) SOD controls – after steady state period SOD 150/ml was admixed to the buffer, which was followed by CPH 1mM application. Subsequent interventions were executed as described for PMA and BAY 41-2272 + PMA groups.

Table 4. Devices and systems applied for the model of isolated perfused rabbit lung

Device	***Supplier***
Pump Masterflex Easy-load 7518-10	Masterflex, Gelsenkirchen, Germany
Heater Thermomix BM	B. Braun Biotech International, Melsungen, Germany
Cooler Frigomix	B. Braun Biotech International, Pfieffewiesen, Germany
Ventilator 6025 Cat/rabbit ventilator	Hugo Sachs Elektronik, March, Germany
Force Transducer Type U1A	Hottinger Baldwin Messtechnik, Fuchstal, Germany
Amplifier Transbridge 4M	World Precision Instruments, Berlin, Germany
Analog-to-digital converter PCLD-8115 wiring terminal board Rev. A2	Advantech, Feldkirchen, Germany
Flowmeter G10a6142fa2b1x0 with precision bore flowrator tube No. FP ¼-25-G-5/81	Bailey Fischer Porter, Solingen, Germany
Computer Unicorner	Unicorner, Stuttgart, Germany

2.4 Isolated perfused mouse lung technique

2.4.1 Setup of isolated perfused mouse lung

The model of isolated perfused and ventilated mouse lung is depicted in the scheme 2. Devices and systems employed for the setup are listed in the table 5.

Table 5. Devices and systems for the model of isolated perfused mouse lung

Device	***Supplier***
Peristaltic pump REGLO Digital MS-4/12	Ismatech SA, Labortechnik-Analytik, Glattbrugg, Switzerland
Piston pump Minivent Type 845	Hugo Sachs Elektronik Harvard Apparatus, GmbH, March-Hugstetten, Germany
Transbridge BM4	World Precision Instruments, Berlin, Germany
Thermomix UB	Braun Melsungen, Melsungen, Germany
Frigomix1495	Braun Melsungen, Melsungen, Germany
Research Grade Isometric Force Transducer	Harvard Apparatus, Holliston, USA
Blood analyser ABL 330	Radiometer, Copenhagen, Denmark
Magnetic stirrer Ret-Basic	IKA Labortechnik, Stauffen, Germany
MiniScope MS100	Magnettech, Berlin-Adlershof, Germany

2.4.2Isolated mouse lungs preparation

Mice were deeply anesthetized intraperitoneally with pentobarbital sodium (100mg/kg body weight) and anticoagulated with heparin (1000U/kg) by intravenous injection. Animals were then intubated via a tracheostoma and room air ventilated using a piston pump (Minivent Type 845, Hugo Sachs Elektronik, March-Hugstetten, Germany) with a tidal volume of 300µl, a frequency of 90 breaths/min and a positive end-expiratory pressure 3 cmH_2O. Midsternal thoracotomy was followed by insertion of catheters into the pulmonary artery and left atrium. Using a peristaltic pump (REGLO Digital MS-4/12, Ismatech SA, Labortechnik-Analytik, Glattbrugg, Switzerland), perfusion with sterile Krebs–Henseleit buffer (Serag-Wiessner, Naila, Germany), via the pulmonary artery was started at 4°C and a flow of 0.2 ml/min. In parallel with the onset of artificial perfusion, ventilation was changed from room air to a pre-mixed normoxic normocapnic gas with 21%O_2, 5.3%CO_2, balanced with N_2 (Air Liquide, Deutschland GmbH, Ludwigshafen, Germany). The lungs were removed from the thorax without interruption of ventilation and perfusion and were freely suspended from a force transducer for the monitoring of organ weight in a temperature-equilibrated, humidified chamber at 37.5°C. After rinsing the lungs with ≥20 ml buffer, the perfusion circuit was closed for recirculation. Meanwhile, the flow was slowly increased from 0.2 to 2 ml/min (total system volume 15 ml). Left atrial pressure was set at 2.0 mmHg, and the whole perfusion system was equilibrated at 37.5°C.

Pressures in pulmonary artery (Ppa) and left atrium were registered with pressure transducers via small diameter catheters and digitized with an analog-to-digital converter, thus allowing data sampling with a personal computer. The capillary filtration coefficient (Kfc) and total vascular compliance were determined gravimetrically from the slope of the lung weight gain curve induced by a 7.5-mmHg step elevation of the venous pressure for 8 min as previously described (54). Lung

weight gain was calculated as the difference in organ weight measured directly before and 5 min after each of these pressure elevation manoeuvres. Vascular compliance was calculated from the initial steep increase in lung weight on a step change in pressure.

Inclusion criteria for the study were *1*) a homogeneous white appearance of the lungs with no signs of edema, hemostasis, or atelectasis; *2*) initial Ppa and ventilation pressure values in the normal range; and *3*) constancy of organ weight during an initial steady-state period of at least 20 min.

2.4.3Experimental protocols.

After termination of the initial steady-state period and performance of a control hydrostatic challenge, the lungs were exposed to ischemia by stopping the perfusion. The arterial and venous catheters were both clamped for maintenance of a positive intravascular pressure, which was initially adjusted to 8 mmHg. During ischemia, the lungs were continuously ventilated with anoxic gas mixture (95% N_2, 5% CO_2). At the end of ischemia, ventilation was changed to normoxia, and perfusion was re-established by increasing the flow stepwise over 3 min. Hydrostatic challenges were performed 30, 60, and 90 min after the onset of reperfusion.

Lungs from wild type and NOS2 knockout mice were treated according to one of the following protocols:

1) I/R - the lungs were exposed to 90 min of ischemia with following reperfusion, no interventions were performed;

2) I/R + L-NMMA – 5 min before onset of ischemia L-NMMA 400 μM was admixed to the perfusion buffer;

3) I/R + BYK-191023 - 20 μM of BYK-191023 were admixed to the perfusion buffer;

4) I/R + VNIO - vinyl-L-NIO (VNIO) 1 μM was admixed to perfusion buffer.

In a separate set of experiments lungs from WT and iNOS KO mice were subjected to 120min continuous normoxic perfusion only or 30 min normoxic perfusion followed by 90 min anoxic ischemia. At the end of ischemia or respective normoxic perfusion period, one set of lungs was removed and snap frozen in liquid nitrogen for isolation of mRNA and protein. A second set was fixed for immunohistochemistry in a 3% paraformaldehyde solution and then paraffin embedded.

Each group encompassed four to eight independent experiments. All experiments were terminated 90 min after onset of reperfusion or when lung weight gain exceeded 1 g during reperfusion.

2.5 Measurement of cGMP

cGMP was determined in samples of pulmonary venous effluent before ischemia as well as 3, 30 and 60 min after the onset of reperfusion. The perfusate aliquots were immediately frozen in liquid nitrogen and stored in -20 °C until measurement. Samples were analysed with a commercially available RIA (Beckman-Coulter, Hamburg, Germany). Briefly, assay based on the competition between succinylated cGMP of the sample and a ^{125}I-labeled tracer for binding to polyclonal antibody coated onto tubes. Measurements were carried out in duplicate. Samples were treated with perchloric acid to inhibit PDE activity and extract the cyclic nucleotide. The extracts were then succinylated and assayed. Radiation was detected by gamma-counter “Cobra Auto-gamma” (Packard, Zurich, Switzerland).

2.6 Measurement of exhaled NO

Measurements were performed as described by Spriestersbach et al. (58).Aliquots of exhaled gas sampled from the open-end tube immediately at the ventilator exhaust valve were provided continuously for measurement by chemoluminescence technique to NO analysator “Sievers 280”. This device uses ozone to oxidize NO to an excited-state NO_2; light emission occurring on transition of excited-state NO_2, to ground-state NO_2 is quantified. Daily calibration was performed with certified gas of known concentration 100 parts per billion (ppb) (Messer Griesheim, Herborn, Germany). The detection limit of NO in gas was 1 ppb (vol/vol).

2.7 Measurement of NO metabolites in perfusate

NO metabolites (nitrite, nitrate, peroxynitrite) were determined in perfusate samples by NOA “Sievers 280” (Seeheim, Germany) according to manufacturer instructions. Perfusate probes were sampled from venous effluent before ischemia as well as 3, 30 and 60 min after the onset of reperfusion and frozen immediately and stored under -20 °C until measurement. NO reaction products in samples were reduced by vanadium chloride at 95 ºC. Resulting gaseous NO was provided to NO analysator, which was connected to computer for data transfer and analysis by “NoaWin 32” software (DeMeTec, Langgons, Germany).

2.8 Measurement of intravascular ROS release by electron spin resonance (ESR) spectroscopy

Electron spin resonance (or electron paramagnetic resonance; ESR) is based on absorption of microwave radiation stimulated by an electromagnetic field in molecules such as free radicals and transition metal ions with unpaired electrons (59). In our

laboratory we employ ESR spectroscopy advanced by spin trapping adapted for the model of isolated perfused lung (60).

ROS were measured by ESR spectroscopy using the spin probe 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine (CPH, Alexis Corporation, San Diego, USA). The triple-line spectrum of $CP^{\bullet}$ radical, a product of the reaction between ROS and CPH, was detected by ESR spectroscopy, using a "MS 100 spectrometer" (Magnettech, Berlin, Germany) ESR measurements were performed in field scan with the following settings: microwave frequency 9.78 GHz, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 18 mW.

Isolated rabbit and mouse lungs were perfused with Krebs–Henseleit buffer, pretreated for 4 h with 50 g/L Chelex 100, to minimize contamination with transition metals.

2.8.1ROS measurements in rabbit lungs

Spin trap CPH 1 mM was applied into perfusate 5 min before reperfusion. Tubings were flushed with perfusate containing spin trap, so that with first portions of perfusate the spin trap reached the lung upon onset of reperfusion. Samples from the venous outflow of the isolated lung were taken immediately after start of reperfusion in 50µl glass capillaries and measured at room temperature. Samples were taken every 15 seconds during 1^{st} min and then every 30 seconds after reperfusion. To decrease autooxidation of CPH due to iron, present in perfusate, DTPA in end concentration 0.1mM was applied before CPH.

2.8.2ROS measurements in mouse lungs

Stock solutions of CPH (10 mM), dissolved in 0.9% NaCl containing 1 mM diethylenetriamine-pentaacetic acid (DTPA) and purged with argon, were prepared

daily and kept under argon on ice. DTPA was used to decrease autooxidation of CPH catalyzed by trace amount of transition metals.

Lungs from WT and NOS2 KO mice were exposed to anoxic ischemia for 90 min. The spin probe CPH (0.5 mM) was given into perfusate 5 min before onset of reperfusion. Samples from the venous outflow of the isolated lung were taken in 50 µl glass capillary tubes and measured immediately at room temperature. The samples were taken every 15 seconds during the first 3 minutes of reperfusion, and then every minute or 5 minutes, as appropriate. Experiments were also carried out in time-matched nonischemic control lungs.

The quantity of trapped ROS was directly calculated from the ESR spectrum of the probe, while the contribution of superoxide radical to the formation of $CP^{\bullet}$ was determined in parallel experiments performed in the presence of SOD in the buffer fluid (150 U/ml).

2.9 Western blot assay

The lungs were removed and immediately frozen in liquid nitrogen. They were then homogenised in lysis buffer containing 50 mM tris-(hydroxymethyl)-aminomethane (Tris)/HCl (pH 7.6), 150 mM NaCl, 10 mM $CaCl_2$, 60 mM NaN_3, 0.1% wt/vol Triton X-100 and a cocktail of protease inhibitors (Complete™ Protease Inhibitor Cocktail Tablets; Boehringer, Ingelheim, Germany) using a tissue homogenizer. Homogenates were centrifuged at 13,000 rpm at 4°C for 30 minutes. The supernatants were measured for protein content using Dye Reagent Concentrate (Bio-Rad Laboratories, Munich, Germany). Extracts containing equal amounts of protein (100 µg) were denatured by boiling for 10 minutes in Laemmli's buffer containing β-mercaptoethanol and separated on 7.5% SDS-polyacrylamide gels at 120 V. The

separated proteins were transferred to nitrocellulose membranes with a semidry transfer unit at 130mA for 2 hours. The blots were blocked and developed with:

Mouse lung tissue - specific rabbit polyclonal Ig G antibodies: eNOS (BD Biosciences, Lexington, KY, USA, 1:1000), iNOS (Santa Cruz, Heidelberg, Germany, 1:1000) and bNOS (Abcam, Cambridge, UK, 1:1000) and goat anti-rabbit HRP-labelled secondary antibody (Promega, Germany, 1:30000).

The bands were visualized using an enhanced chemiluminescence ECL Plus Western blotting detection reagents (Amersham Biosciences, Freiburg, Germany) and quantified by densitometry (Biometra, Germany). Density values are expressed relative to the β-actin (Abcam, Cambridge, UK) level in rabbit lung tissues and to the GAPDH (Abcam Ltd, Cambridge, UK) control level in mouse lung tissues of each sample. All densities reported are means and SEM of four separate experiments.

Table 6. Reagents used for western blot assay.

Reagent	***Company***	***Catalogue number***
Tris base	Sigma-Aldrich-	T1503
	Aldrich-	
Tween	Sigma-Aldrich	P7949
Glycine	Sigma-Aldrich	G7126
SDS	Serva	20763
Glycerol	Sigma-Aldrich	G5150
Bromophenol blue		
Methanol	Fluka	65545

Skimmed milk powder	Roth	T145.2
Temed	Sigma-Aldrich	T-9281
Acrylamide	Fluka	0199
Ammonium per sulphate	Sigma-Aldrich	A-3678
Bis-acrylamide	Sigma-Aldrich	M-2022
Tris HCl	Sigma-Aldrich	T-2788

Reagent	***Company***	***Catalogue number***
Triton x-100	Sigma-Aldrich	T-8787
Full range Rainbow ™ recombinant molecular weight marker	Amersham	RPN800V
ECL plus western blotting detection system	Amersham	RPN2132
Gel blotting paper 580x580 mm	Schleicher & Schuell	G8005 Ref. N:10426994
Hyperfilm 18x24 cm (5-75 sheet bones)	Amersham	RPN175K
eNOS/type II pab	BD Transduction Labs	610298
Mouse monoclonal (6c5) to GAPDH-loading control	Abcam	Ab8245-10
NOS2 (N-20)	Santa Cruz	Sc-51
Rabbit polyclonal antibodies to bNOS	Abcam	Ab3511-100
Anti-iNOS polyclonal antibodies	BD Transduction	610332

	labs	
Histostain (R)SP rabbit primary (AEC)	Zymed Labs	95-6143
Rabbit serum	Zymed Labs	50-061
Biotin-conjugated anti-rabbit goat Ig G	Zymed Labs	50-232

2.10 Immunohistochemical staining

Paraffin-embedded lung tissue sectioned at a 2 µm thickness was deparaffinized in xylene and rehydrated in a graded ethanol series to phosphate-buffered saline (PBS, pH 7.2). Antigen retrieval was performed by pressure cooking in citrate buffer (pH 6.0) for 15 min. Immunohistochemical staining was performed using anti-iNOS antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) in conjunction with an avidin-biotin-peroxidase kit as per manufacturer's instructions (Zymed Laboratories, Burlingame, CA, USA). Briefly, the sections were pre-treated with 3% hydrogen peroxide to quench endogenous peroxidase activity. Then, the sections were incubated for 1h in 10% normal goat serum to block nonspecific binding sites prior to the application of primary antibodies. Sections were then incubated overnight at 4°C with the primary monoclonal antibody (1:100). Biotinylated anti rabbit immunoglobulin (Ig G) and then avidin-biotinylated enzyme complex were applied according to the manufacturer's instructions. After each incubation step, sections were washed briefly in PBS. Development of the dye was carried out with AEC substrate for 10 min. Finally, sections were counterstained with hematoxylin and coverslipped using mounting medium.

2.11 RT-PCR

Total RNA was isolated from frozen lungs using TRIzol Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). One microgram of RNA from each sample was reverse transcribed to cDNA with Improm-II Reverse Transcription System (Promega, Madison, WI, USA). Three microliters of this RT reaction were added to each 50 μl semi quantitative PCR reaction. Oligonucleotide primers used to amplify eNOS were (forward) 5'-AAGACAAGGCAGCGGTGGAA-3' and (reverse) 5'-GCAGGG GACAGGAAATAGTT-3', for nNOS were (forward) 5'-AATGGAGACCCCCAG AGAAT-3' and (reverse) 5'-TCCAGGAGAGTGTCCACTGC-3', for iNOS were (forward) 5'-ACGCTTGGGTTTGTTCACT-3' and (reverse) 5'-GTCTCTGGGTCC TCTGGTCA-3', for GAPDH were (forward) 5'-GTGATGGGTGTGAACCACGAG-3' and (reverse) 5'-CCACGATGCAAGTTGTCA-3'. PCR products were analysed by 1.5% (w/v) agarose gel electrophoresis. The expected sizes of PCR products were 292, 281, 468 and 179 bp for eNOS, nNOS, iNOS and GAPDH respectively. Identity of the product was confirmed with sequence analysis.

2.12 Data analysis

Data are expressed as means ± s.e.m. Differences between two groups were evaluated by t-test. For multiple comparisons one-way analysis of variance followed by a post hoc Student-Newman-Keuls test was used. P values< 0.05 were considered to represent a significant difference. Analysis was performed using SPSS statistic software package.

3 RESULTS

3.1 Role of different NO synthases in I/R injury

3.1.1 Isolated perfused rabbit lungs

Permeability and LWG

In rabbit lungs in preischemic period low Kfc values indicated intact endothelial barrier properties similarly in all experimental groups. Four hours of ischemic exposition induced dramatically elevated Kfc values on reperfusion with subsequent severe edema formation in control lungs (Fig. 1). Unspecific NO synthase inhibition prevented I/R induced lung injury. Specific iNOS or nNOS inhibition protected against I/R induced permeability and weight gain increase.

Ppa

No significant differences in Ppa between groups were observed. After reperfusion in I/R group a transient Ppa increase was detected (16.3 ± 2.1 mm Hg) with following decrease until baseline level (Fig. 6). Nonspecific NO synthase or specific iNOS or nNOS inhibition did not significantly influence this Ppa increase.

Nitric oxide

NO release detected in exhaled gas (NOex) mixture was 75.3±5.5 ppb under pre-ischemic control conditions in the rabbit lungs. Upon starting ischemia NOex decreased immediately and remained low during whole ischemic period (1.3 ± 1.1 ppb shortly before reperfusion). After onset of reperfusion levels of NOex returned to baseline levels (Fig. 5). NO metabolites in perfusate accumulated continuously (Fig. 5C). L-NMMA inhibited NO production completely, which was confirmed by rapid drop of NOex and diminished accumulation of NO metabolites after inhibitor

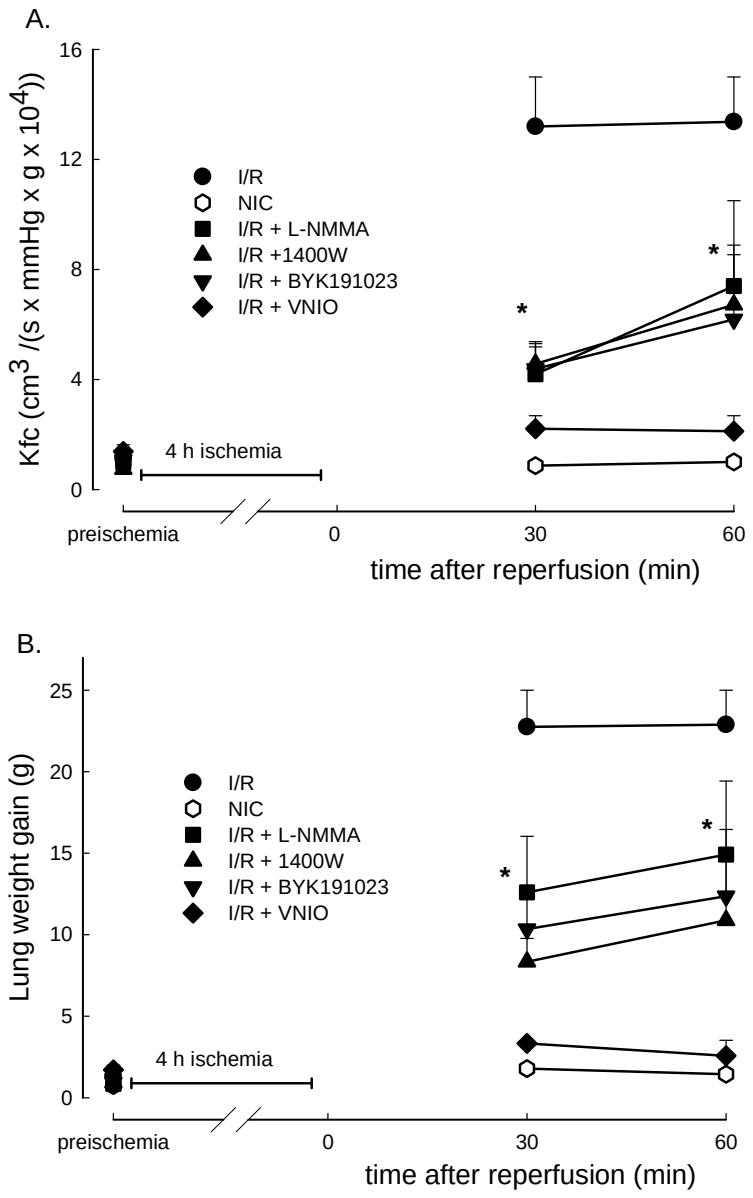


Fig. 1. Impact of NO synthase inhibition on capillary filtration coefficient (Kfc) and lung weight gain (LWG) in isolated perfused rabbit lung. I/R lungs underwent 4 hour ischemia with following reperfusion, no treatment was administered. Time matched nonischemic control (NIC) lungs were perfused and normoxically ventilated throughout 6 hrs. NOS inhibitors L-NMMA 400 μM, 1400W 10 μM, BYK 191023 20 μM, or VNIO 1 μM were applied 5 min before ischemia according to protocol. Kfc was determined gravimetrically from the slope of the lung weight gain curve induced by a 7.5-mmHg step elevation of the venous pressure for 8 min. LWG was calculated as the difference in organ weight measured directly before and 5 min after each of these pressure elevation maneuvers.

A. Thirty min after onset of reperfusion all treatment groups demonstrated significant reduction of elevated Kfc ($p<0.05$ vs. I/R). B. Thirty min after reperfusion all treatment groups showed significant reduction of elevated LWG ($p<0.05$ vs. I/R). Data presented as mean ± SEM.

application. Moreover, L-NMMA blocked the recovery of NO production after reperfusion (23.8 ± 1.9ppb, $p<0.001$ vs. I/R). Specific iNOS or nNOS inhibition did influence neither NOex nor NO metabolites accumulation in recirculating buffer.

Intravascular ROS release

In the rabbit lungs exposed to ischemia an increase in ROS release in perfusate upon onset of reperfusion was detected, whereas in nonischemic control lungs no significant

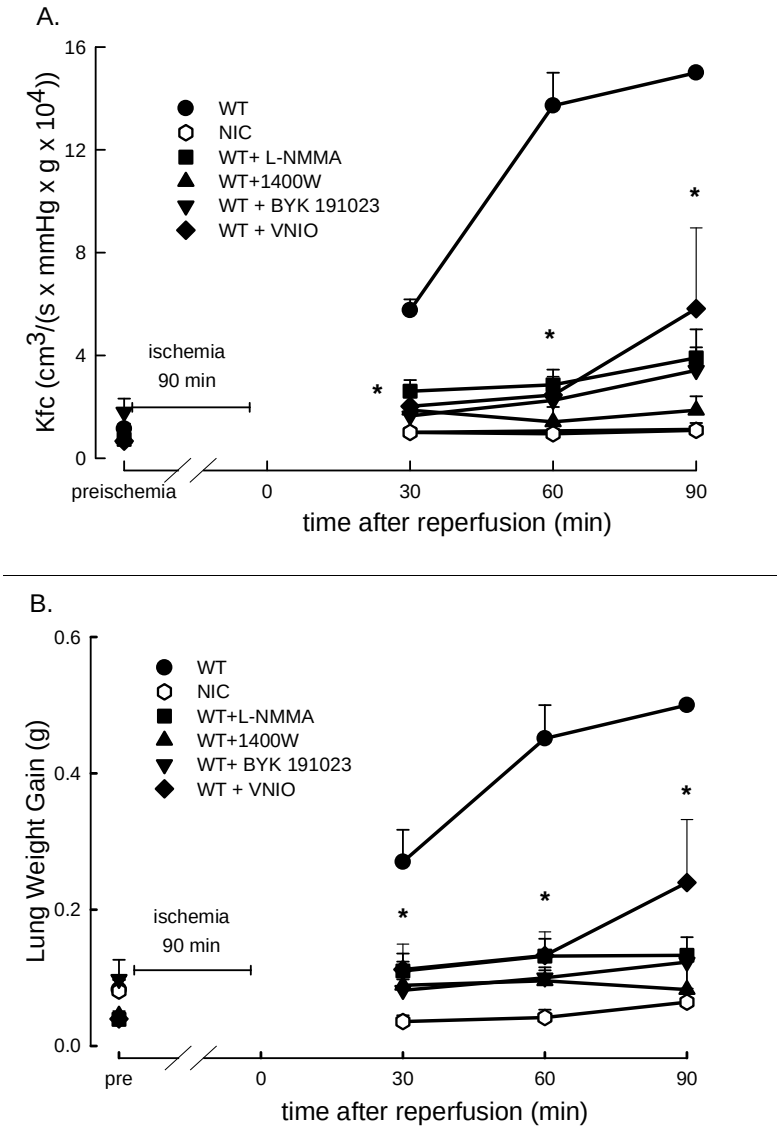


Fig. 2. Impact of NO synthase inhibition on capillary filtration coefficient (Kfc) and lung weight gain (LWG) of isolated perfused wild type mouse lungs. Lungs from wild type mice were exposed to ischemia with anoxic ventilation for 90 min with following reperfusion. Time matched nonischemic control lungs underwent 180 min perfusion with normoxic ventilation. When indicated, LNMMA 400 µM, 1400W 10 µM, BYK-191023 20 µM, or VNIO 1 µM was admixed into the buffer solution 5 min before the onset of anoxic ischemia. Data presented as mean ± SEM. A. Thirty min after onset of reperfusion all treatment groups demonstrated significant reduction of elevated Kfc ($p<0.05$ vs. I/R). B. Thirty min after reperfusion all treatment groups showed significant reduction of elevated LWG ($p<0.05$ vs. I/R). Data presented as mean ± SEM.

increase was observed (Fig. 11). Application of superoxide dismutase (SOD) reduced significantly CP^- signal suggesting that the superoxide is the main radical produced. NOS inhibition by L-NMMA diminished ROS release. Both iNOS and nNOS inhibition reduced ROS release in perfusate.

3.1.2 Wild type mouse lungs

Permeability and LWG

Mouse lungs in preischemic period demonstrated low Kfc values indicating intact endothelial barrier properties in all experimental groups. Ninety min of ischemic

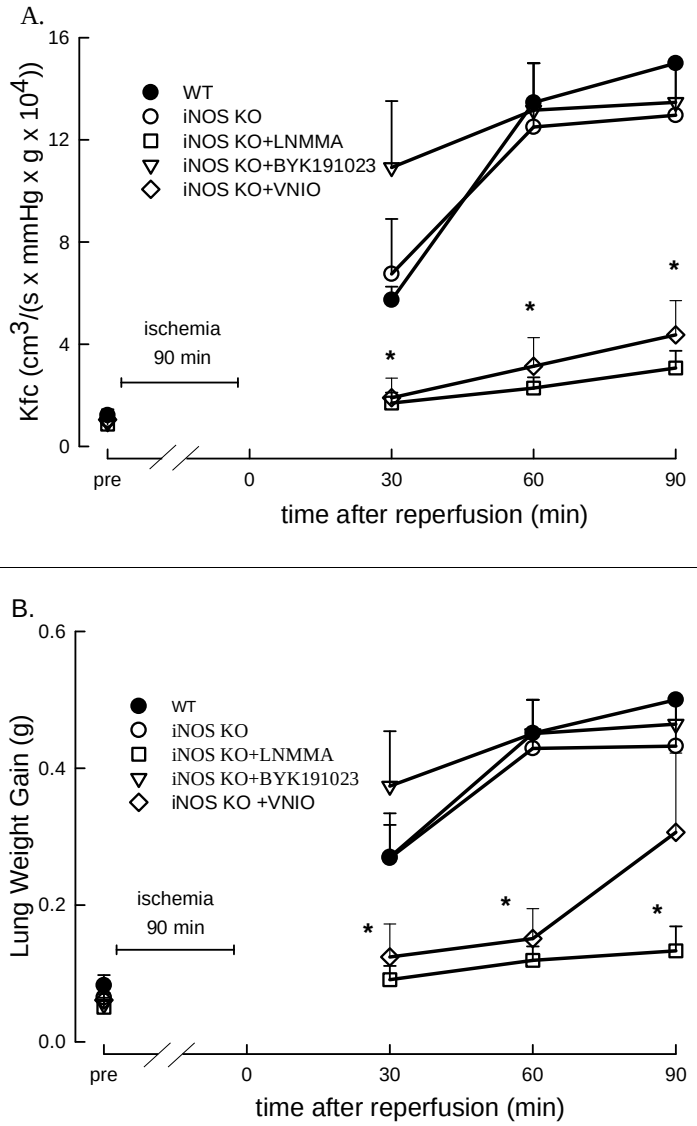


Fig. 3. Impact of NO synthase inhibition on capillary filtration coefficient (Kfc) and lung weight gain (LWG) of isolated perfused iNOS deficient mouse lungs. Lungs were exposed to ischemia with anoxic ventilation for 90 min. When indicated, L-NMMA 400 μM, BYK 191023 20 μM, or VNIO 1 μM was applied into the buffer solution 5 min before the onset of anoxic ischemia. Data presented as mean ± SEM. A. Thirty min after onset of reperfusion L-NMMA and VNIO treatment groups demonstrated significant reduction of elevated Kfc ($p<0.05$ vs. I/R). B. Thirty min after reperfusion L-NMMA and VNIO treatment group showed significant reduction of elevated LWG ($p<0.05$ vs. I/R). Data presented as mean ± SEM.

exposition induced dramatically elevated Kfc values on reperfusion with subsequent edema formation in control lungs (Fig. 2). Unspecific NO synthase inhibition prevented permeability disturbances and lung weight gain. Either specific iNOS or nNOS inhibition protected against I/R induced lung injury.

Ppa

At the baseline in all treatment groups similar values of Ppa were measured (8.1 ± 0.6 mm Hg in WT I/R group). Reperfusion induced transient Ppa increase, which was not influenced by NO synthase inhibitors (Fig. 6A).

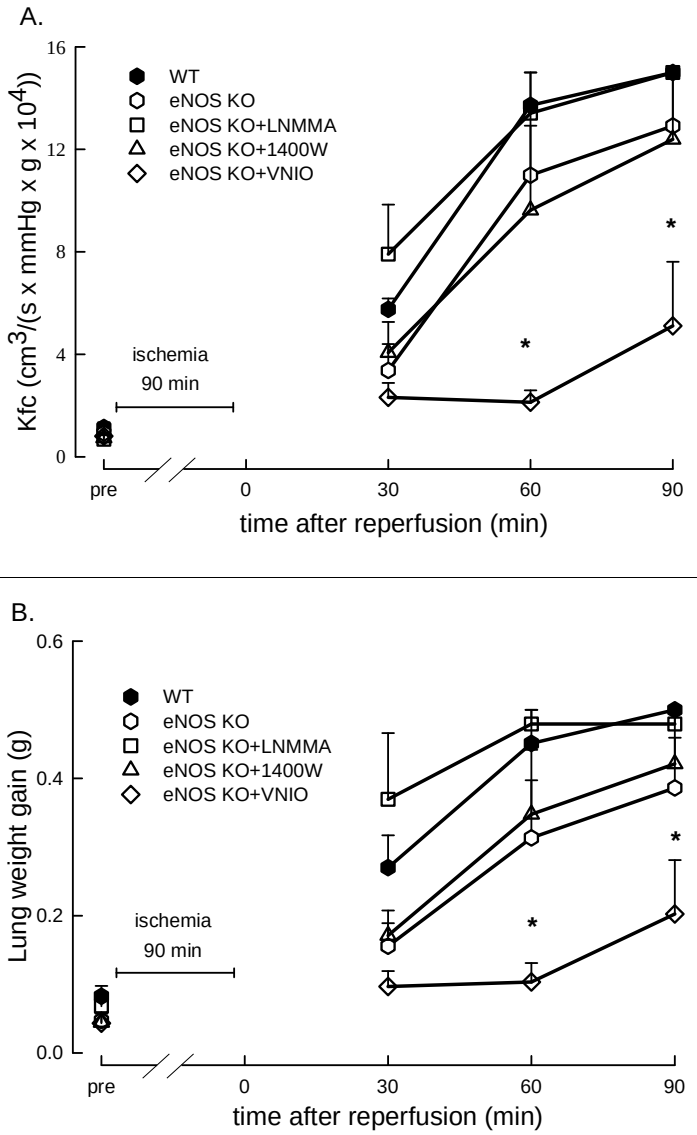


Fig. 4. Impact of NO synthase inhibition on capillary filtration coefficient (Kfc) and lung weight gain (LWG) of isolated perfused eNOS deficient mouse lungs. Lungs were exposed to ischemia with anoxic ventilation for 90 min. When indicated, LNMMA 400 µM, 1400W 10 µM, or VNIO 1 µM was admixed with the buffer solution 5 min before the onset of anoxic ischemia. Data presented as mean ± SEM. A. Sixty min after onset of reperfusion VNIO treatment group demonstrated significant reduction of elevated Kfc ($p<0.05$ vs. I/R). B. Sixty min after reperfusion VNIO treatment group showed significant reduction of elevated LWG ($p<0.05$ vs. I/R). Data presented as mean ± SEM.

NO synthase expression

In the WT mouse lungs subjected to ischemia with anoxic ventilation eNOS protein and mRNA levels were significantly reduced, while nNOS expression was moderately increased (Figs. 8 and 9). . In the WT mouse lungs subjected to ischemia with anoxic ventilation upregulation of iNOS mRNA was observed (Fig. 8). iNOS protein upregulation was noted after anoxic ischemia by immunohistochemistry. iNOS was found mainly localized in the airway epithelium as well as in smaller vessels (Fig. 10).

Intravascular ROS release

In the WT mouse lungs exposed to ischemia an increase in ROS release in perfusate upon onset of reperfusion was detected (Fig. 11). Application of superoxide dismutase (SOD) reduced significantly CP^{-} signal suggesting that the superoxide is the main radical produced. NOS inhibition by L-NMMA diminished ROS release. Both iNOS and nNOS inhibition reduced ROS release in perfusate.

3.1.3iNOS KO mouse lungs

Permeability and LWG

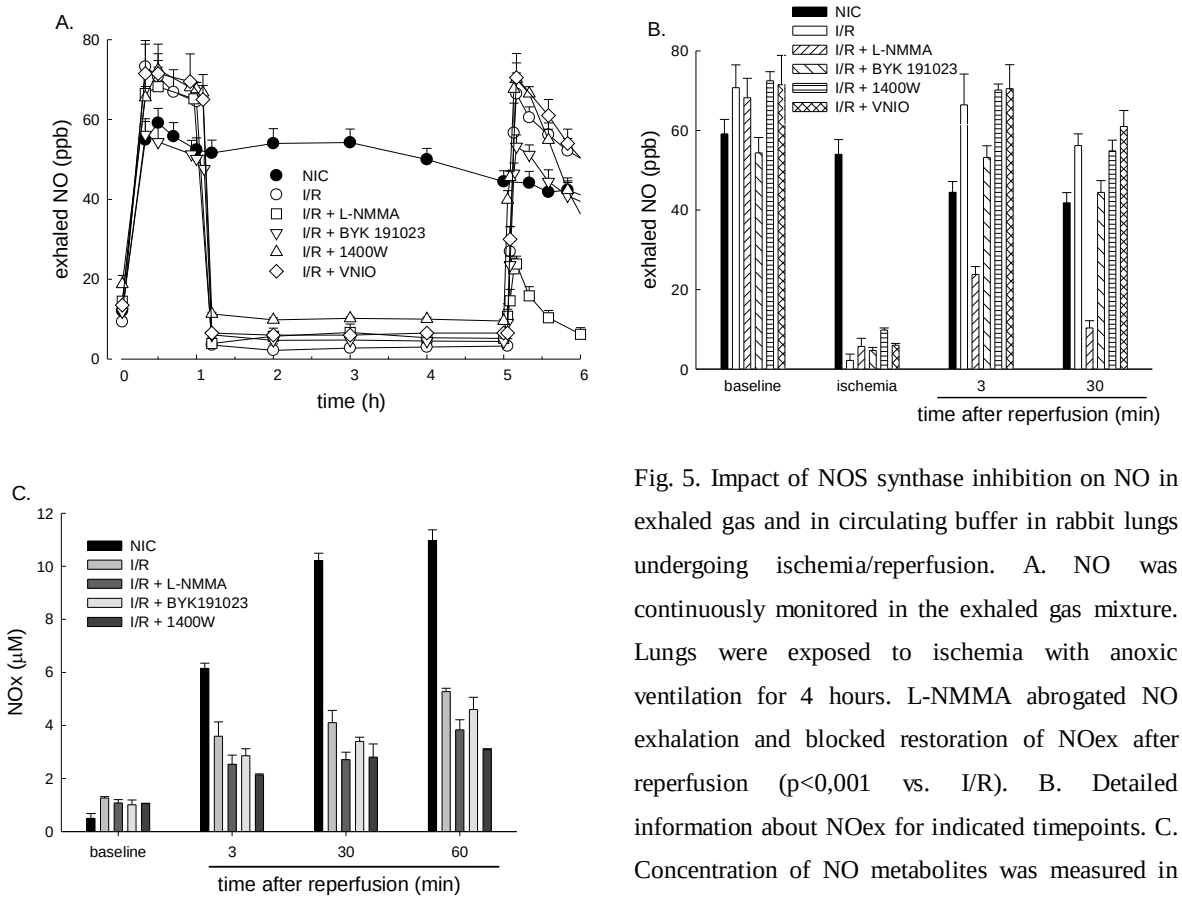


Fig. 5. Impact of NOS synthase inhibition on NO in exhaled gas and in circulating buffer in rabbit lungs undergoing ischemia/reperfusion. A. NO was continuously monitored in the exhaled gas mixture. Lungs were exposed to ischemia with anoxic ventilation for 4 hours. L-NMMA abrogated NO exhalation and blocked restoration of NOex after reperfusion ($p<0,001$ vs. I/R). B. Detailed information about NOex for indicated timepoints. C. Concentration of NO metabolites was measured in perfusate samples in preischemia, after onset of reperfusion and then every 30 min. Values are given as mean $\pm$ SEM

In preischemic iNOS KO mouse lungs demonstrated permeability not differing from that of their wild type counterparts. Interestingly, iNOS KO mouse lungs were not protected against I/R injury (Fig. 3). After exposure to ischemia and reperfusion they developed injury to the same degree as WT louse lungs did. However, when non-selective NOS inhibitor L-NMMA was administered before the ischemic period, both

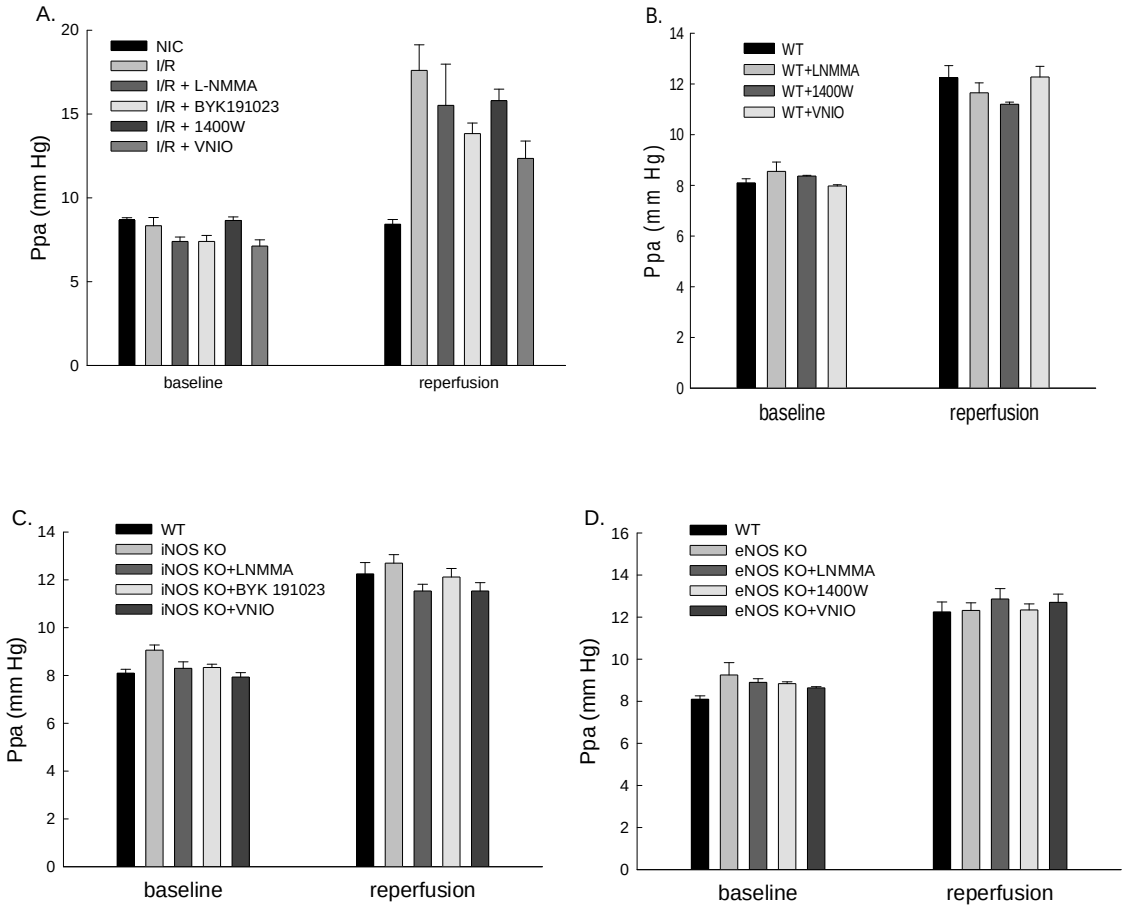


Fig. 6. Effect of NO synthase inhibition on reperfusion induced increase in pulmonary arterial pressure. Baseline Ppa represents pressure after steady state period. Reperfusion rise demonstrates Ppa on 5th min of reperfusion, when perfusion was completely re-established. A. Ppa values at baseline and after reperfusion for rabbit lungs are presented. Reperfusion induced dramatic Ppa increase after reperfusion ($p<0.05$ vs. NIC). B. Ppa values at baseline and after reperfusion of isolated perfused WT mouse lungs. C. Ppa values at baseline and after reperfusion of isolated perfused iNOS KO mouse lungs are presented. D. Ppa values at baseline and after reperfusion of isolated perfused eNOS KO mouse lungs are presented.
Data presented as mean ± SEM

the dramatic rise in Kfc and the severe edema formation were significantly attenuated, with values approaching those in WT mouse lungs pre-treated with L-NMMA. Specific nNOS inhibition prevented I/R induced permeability increase and fluid retention in lungs. Specific iNOS inhibition, as expected, did not demonstrate any effect.

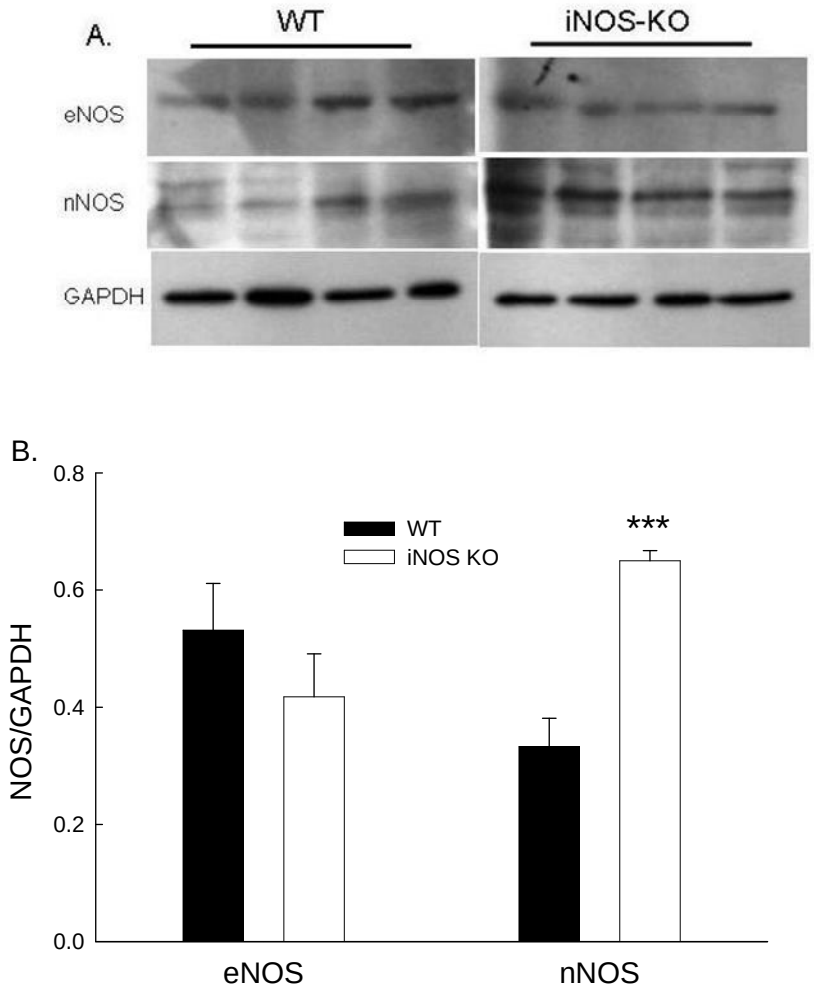


Fig. 7. Baseline expression of eNOS and nNOS in lung homogenates from wild type an iNOS-KO mice. Western blot analysis was performed by immunodetection with antibodies against eNOS and nNOS. A. The specific antibodies recognize protein at a molecular mass 140 kDa and 155 kDa respectively.
B. Quantification of the NOS signal. Values are expressed as ratio of obtained signal to GAPDH expression. iNOS KO mouse lungs demonstrate higher nNOS expression at baseline. *** $p<0.001$ vs WT mouse lungs.

Ppa

At baseline iNOS KO mouse lungs demonstrated Ppa (8.4 ± 0.1 mm Hg) not differing from that of WT mouse lungs. Extent of Ppa increase after reperfusion was similar between WT and iNOS KO mouse lungs and was not changed by NO synthase inhibitors (Fig. 6C).

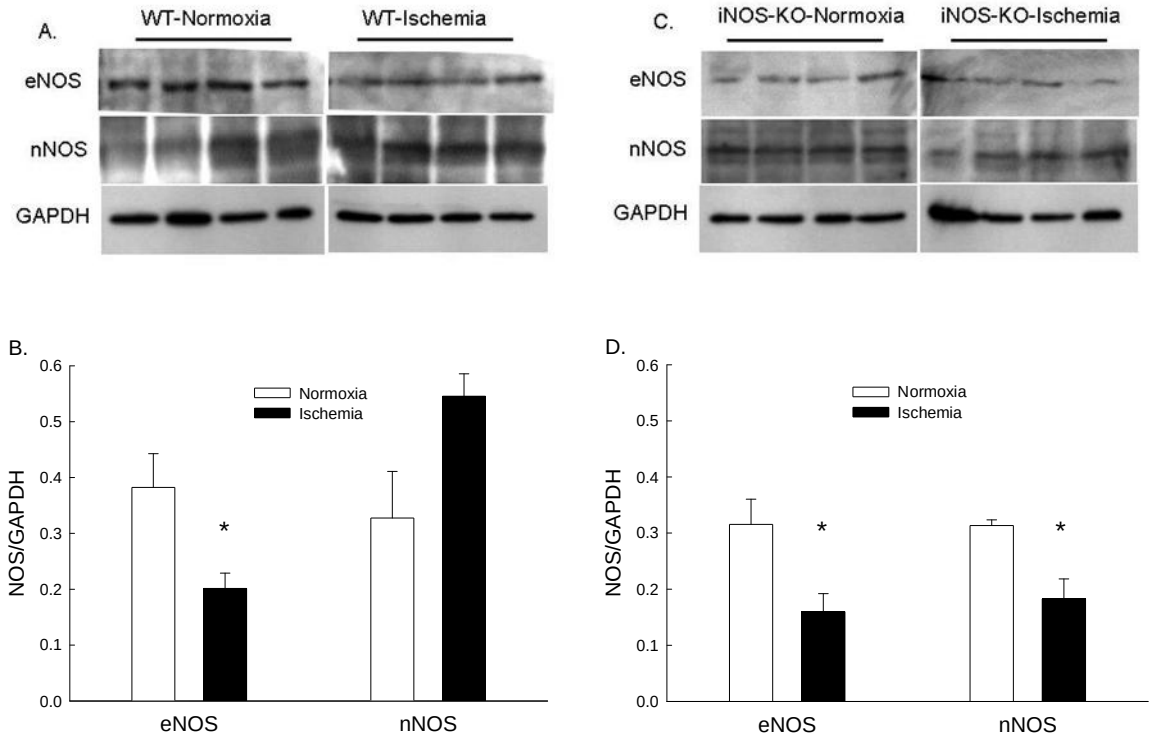


Fig. 9. Changes in the expression of NOS isoforms in lungs from WT (A) and iNOS KO (C) mice after exposure to anoxic ischemia. Mouse lungs were subjected to 120 min continuous normoxic perfusion (normoxia) or 30 min normoxic perfusion followed by 90 min anoxic ischemia (ischemia). Western blot analysis was performed from proteins obtained from the lungs. B. and D. The bar graphs illustrate quantification of eNOS or nNOS expression levels normalized to GAPDH levels. Data presented as means ± SEM. * $p < 0.05$ vs. respective normoxia group.

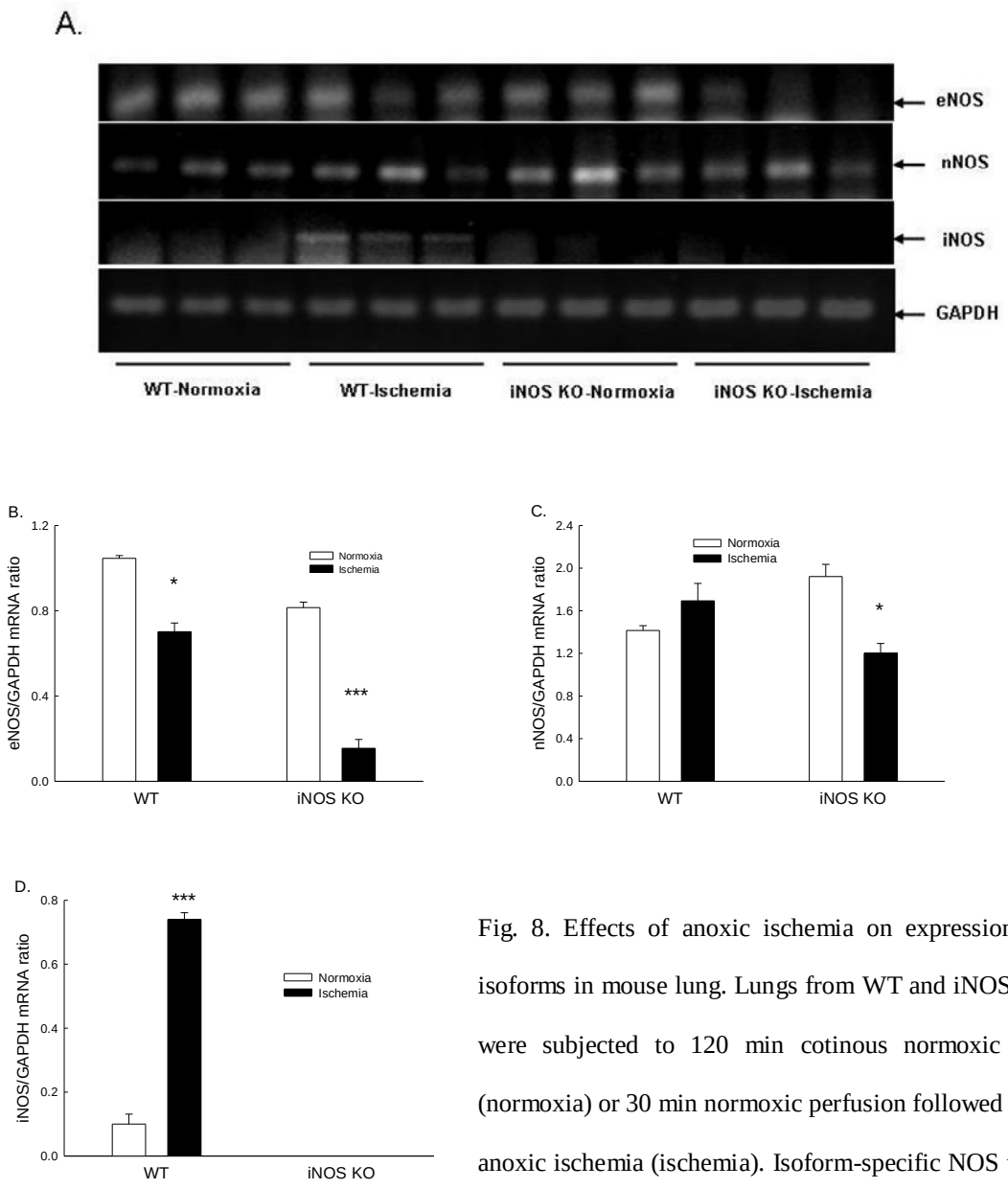


Fig. 8. Effects of anoxic ischemia on expression of NOS isoforms in mouse lung. Lungs from WT and iNOS KO mice were subjected to 120 min cotinous normoxic perfusion (normoxia) or 30 min normoxic perfusion followed by 90 min anoxic ischemia (ischemia). Isoform-specific NOS transcripts were detected by RT-PCR in RNA extracted from these lungs. A. Electrophoregram of PCR products is demonstrated. The bar graphs illustrate quantification of (B) eNOS, (C) nNOS, (D) iNOS expression levels normalized to GAPDH levels. Data presented as mean ± SEM. * $p < 0.05$, *** $p < 0.001$ compared to respective normoxia group.

NO synthase expression

At baseline in the iNOS KO mouse lungs when compared with WT mouse lungs decreased levels of eNOS and increased levels of nNOS expression were observed (Fig. 7). In that mouse lungs, ischemia with anoxic ventilation induced further decrease of eNOS protein and mRNA level as well as the decreased amount of nNOS

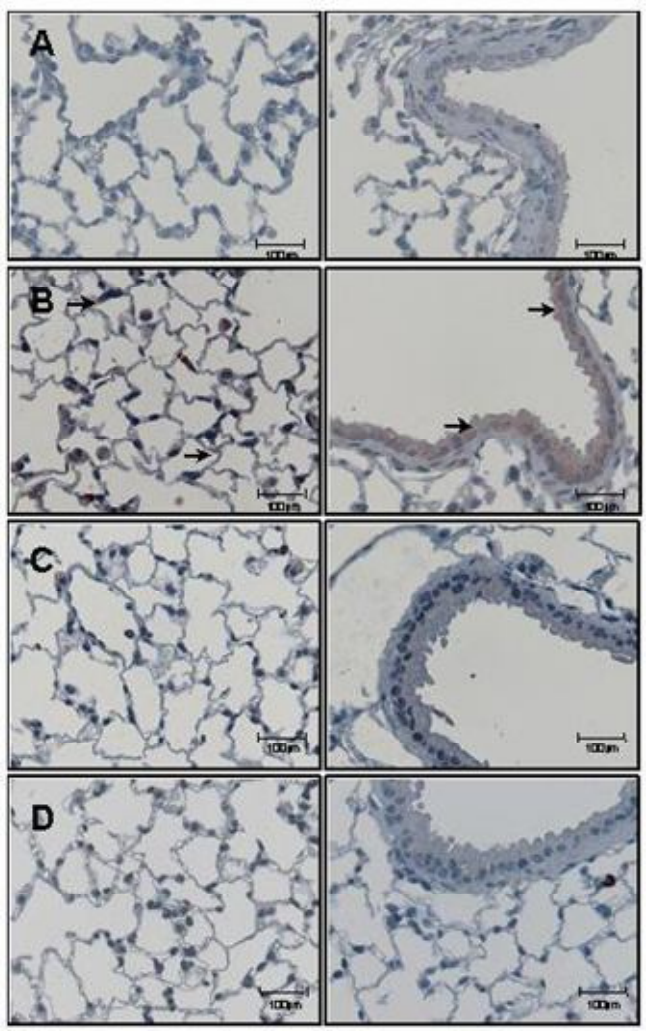


Fig. 10. Immunohistochemical staining for iNOS in mouse lungs after exposure to anoxic ischemia. Lungs from WT (A and B) and iNOS KO (C and D) mice were subjected to 120 min continuous normoxic perfusion (A and C) or 30 min normoxic perfusion followed by 90 min anoxic ischemia (B and D). Brownish staining indicates positively stained cells (arrows). Anoxic ischemia up-regulated iNOS expression in WT mouse lungs. In iNOS KO mouse lungs no iNOS protein was detected (C and D). Representative results from 3 separate experiments are documented. No staining was detected when the primary antibody was omitted from the protocol (data not shown).

protein and mRNA. As expected, no iNOS expression was detected at baseline and after I/R (Figs. 8, 9).

Intravascular ROS release

After onset of reperfusion iNOS KO mouse lungs demonstrated increased ROS release into recirculating buffer. In presence of SOD the increase was considerably reduced confirming that signal derives mainly from superoxide radical. Both, nonspecific NOS and specific nNOS inhibition reduced the increase, whereas selective iNOS inhibition did not demonstrate any effect (Fig. 12).

3.1.4eNOS KO mouse lungs

Permeability and LWG

Lungs from eNOS KO mouse subjected to I/R developed injury to the same extent as

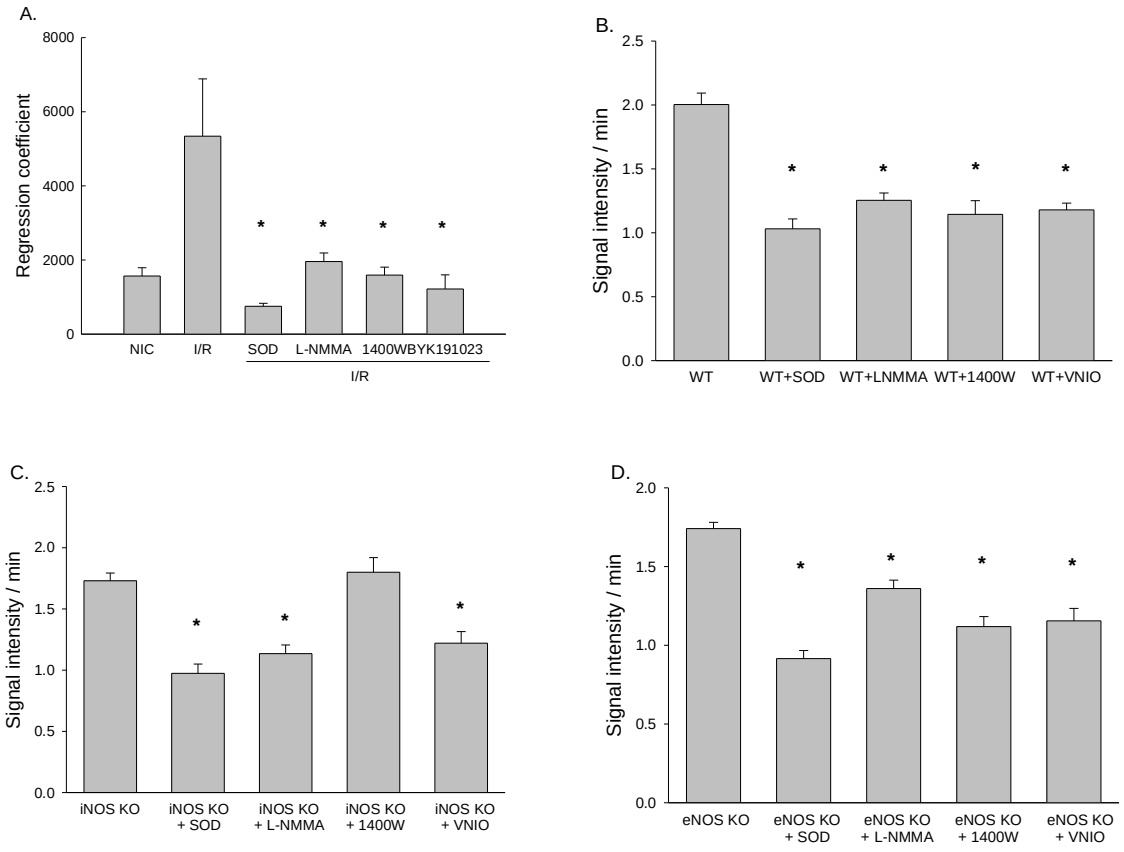


Fig. 11. Influence of NOS inhibition on ROS release in perfusate after reperfusion in the lungs of rabbits, wild type, iNOS KO, and eNOS KO mice. The rabbit lungs underwent 4 h ischemia with following reperfusion. When indicated NO synthase inhibitors were applied 5 min before onset of ischemia. After reperfusion several samples from venous effluent were collected and measured by ESR immediately. From obtained signal curve regression coefficient was calculated. In time matched nonischemic control rabbit lungs spin probe was applied correspondingly at 300^{th} min during perfusion. The mouse lungs were subjected to the 90 min of anoxic ischemia with following reperfusion. When indicated, NO synthase inhibitors were applied 5 min before onset of ischemia. Spin trap CPH 1 mM was applied into perfusate shortly before reperfusion. After starting reperfusion several samples of venous effluent were collected and serial measurements were performed immediately. From obtained signal curve ratio of signal increase was calculated. A. Rabbit lungs. NO synthase inhibition reduced intravascular ROS release ($p<0.05$ vs I/R). B. Wild type mouse lungs. NO synthase inhibition reduced ROS release into recirculating buffer ($p<0.05$ vs I/R). C. iNOS deficient mouse lungs. Treatment with L-NMMA or VNIO reduced ROS release ($p<0.05$). D. eNOS deficient mouse lungs. NO synthase inhibition reduced intravascular ROS release ($p<0.05$ vs I/R). Data presented as mean ± SEM.

the lungs from WT mouse did (Fig. 4). Neither nonspecific NOS inhibition nor specific iNOS inhibition prevented tissue damage. Pre-treatment with nNOS inhibitor VNIO protected against I/R induced Kfc and LWG increase.

Ppa

eNOS KO mouse lungs demonstrated higher Ppa (8.7±0.3 mm Hg, $p< 0.01$ vs. WT) at baseline. Reperfusion induced Ppa increase was similar between WT and eNOS KO mouse lungs. Administration of NO synthase inhibitors did not reduce Ppa increase (Fig. 6D).

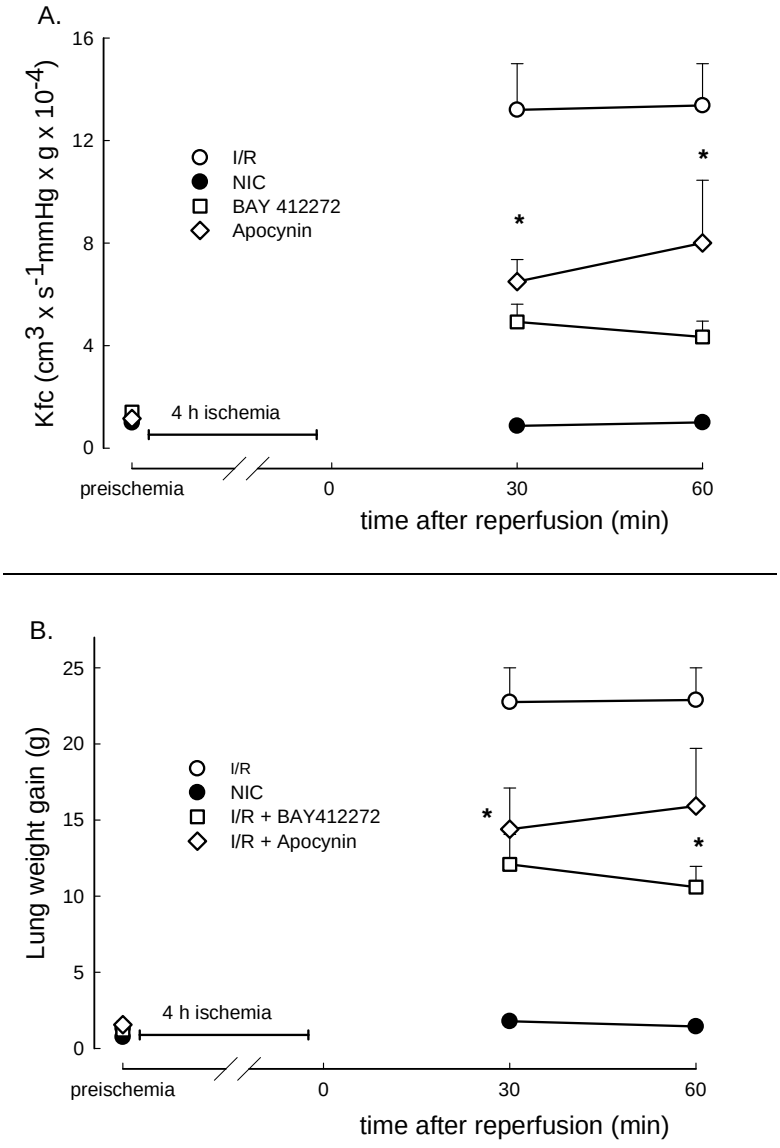


Fig. 12. Impact of sGC stimulation and NADPH oxidase inhibition on capillary filtration coefficient (Kfc) and lung weight gain (LWG) in isolated perfused rabbit lung. sGC stimulator BAY 412272 3µM or NADPH oxidase inhibitor apocynin 0.5 mM were applied 5 min before ischemia. Kfc was determined gravimetrically from the slope of the lung weight gain curve induced by a 7.5-mmHg step elevation of the venous pressure for 8 min. LWG was calculated as the difference in organ weight measured directly before and 5 min after each of these pressure elevation maneuvers. A. Thirty min after onset of reperfusion all treatment groups demonstrated significant reduction of elevated Kfc ($p<0.05$ vs. I/R). B. Thirty min after reperfusion all treatment groups showed significant reduction of elevated LWG ($p<0.05$ vs. I/R). Data presented as mean ± SEM.

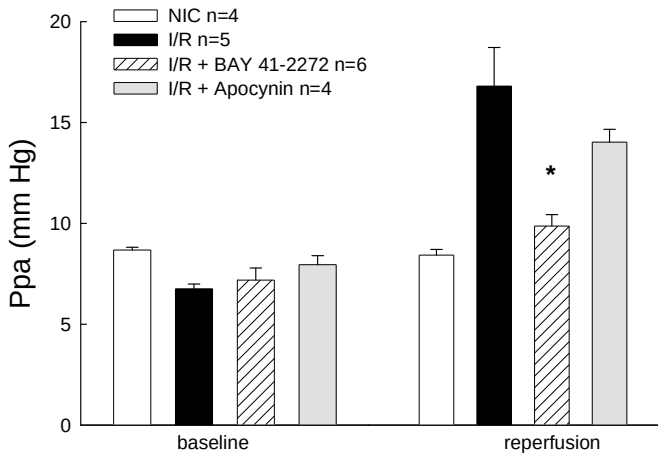


Fig. 13. Effect of sGC stimulation and NADPH oxidase inhibition on reperfusion induced increase in pulmonary arterial pressure. Baseline Ppa represents pressure after steady state period. Reperfusion demonstrates Ppa on 5th min of reperfusion, when perfusion was completely re-established. Reperfusion induced dramatic Ppa increase after reperfusion ($p<0.05$ vs. NIC). BAY 41-2272 reduced extent of the Ppa increase ($p<0.05$ vs. I/R).
Data presented as mean ± SEM

Intravascular ROS release

Reperfusion induced ROS release from eNOS KO mouse lungs was inhibited by L-NMMA, specific iNOS, and nNOS inhibitors (Fig. 11D).

3.2 Role of sGC in I/R injury

BAY 41-2272 application led to significant increase in cGMP concentration in perfusate (Fig. 14). NOex levels were not influenced by administration of the substance (Fig.15). No significant changes in basal Ppa after BAY 41-2272 treatment were observed. However, in these lungs less pronounced Ppa increase after reperfusion was detected (9.9 ± 0.6 mm Hg, $p<0.05$ vs. I/R) (Fig. 13). sGC stimulation significantly attenuated vascular leakage and reduced edema formation (Kfc 4.4E-04 ± 0.5E-04 $cm^3 \times s^{-1} \times mmHg \times g$, $p<0.01$). Changes in weight gain correlated well with that of Kfc values (Fig. 12). These observations were paralleled by reduced ROS release into perfusate in the lungs pre-treated with BAY 41-2272 (Fig. 16).

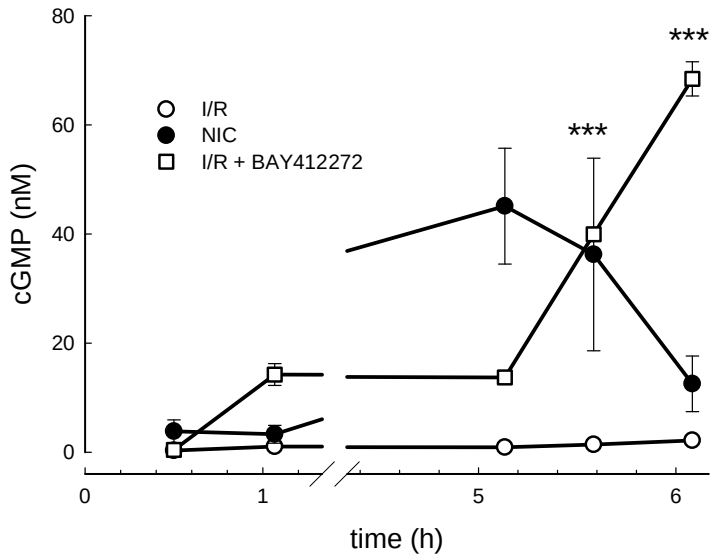


Fig. 14. Effect of sGC stimulation on cGMP release in buffer.
cGMP was measured in recirculating buffer using RIA. Buffer samples were collected during preischemia, after onset of reperfusion and then 30 min. BAY 412272 increased the concentration of cGMP. (***p<0.001 vs. I/R). Data presented as mean ± SEM.

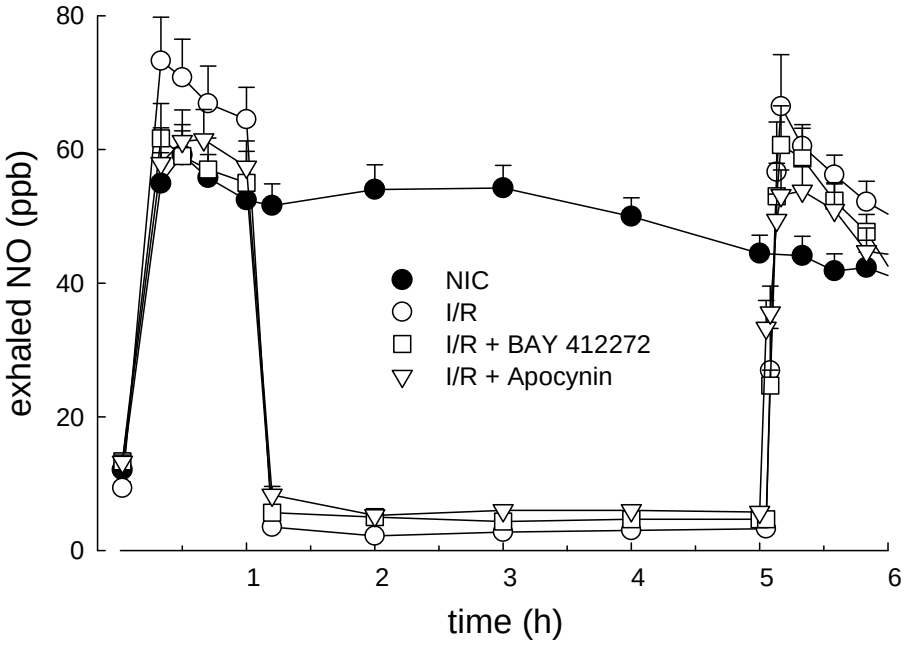


Fig. 15. Impact of BAY 412272 or apocynin application on NO in exhaled gas in rabbit lungs undergoing ischemia/reperfusion. NO was continuously monitored in the exhaled gas mixture. Lungs were exposed to ischemia with anoxic ventilation for 4 hours with following reperfusion. BAY 412272 or apocynin were administered 5 min before onset of ischemia. Values are given as mean ± SEM

3.3 Role of NADPH oxidases in I/R

Application of the NADPH oxidase inhibitor apocynin protected against I/R induced injury (Fig. 13). This substance protected against increase in permeability and reduced weight gain (14.4±2.34 g, $p<0.05$ vs. I/R). Apocynin did not influence NOex levels (Fig. 15). Inhibition of NADPH oxidases was confirmed by detection of reduced ROS release in the perfusate in apocynin treated lungs (Fig. 16).

3.4 Interaction between NO-cGMP pathway and NADPH oxidase

Treatment with PMA, the NADPH oxidase stimulator, resulted in increased intravascular ROS release in isolated lungs, which was accompanied by pronounced increase in Ppa (Fig. 17). BAY 41-2272 inhibited significantly both the increased ROS production (Fig. 17A) and Ppa elevation (Fig. 17B). SOD completely suppressed

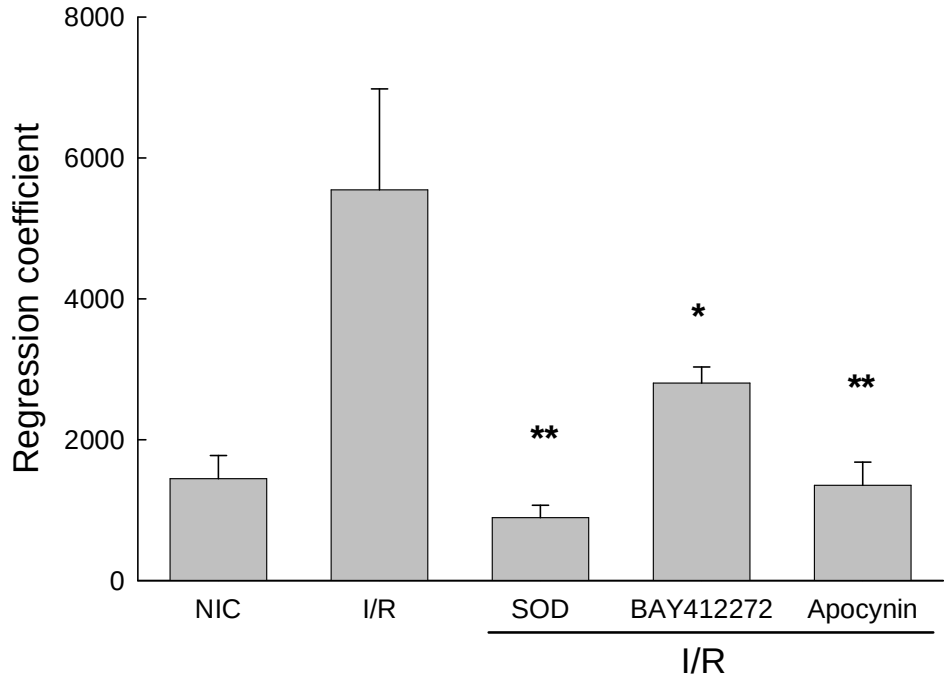


Fig 16. Impact of BAY 412272 and apocynin on ROS release into perfusate. BAY 412272 3 µM or apocynin 0.5 µM were applied in pre-ischemia. Spin trap CPH 1 mM was applied into perfusate 5 min before reperfusion. Aliquots of perfusate were taken from lung effluent and measured by ESR spectroscopy immediately. From serial measurements curves of ROS accumulation in buffer were obtained. Regression coefficients of the slope of curve were calculated. Both BAY 42272 and apocynin demonstrated significant inhibition of ROS release into perfusate ($p<0.05$ vs I/R). Data presented as mean ± SEM

superoxide release accompanied by a reduced pressure response.

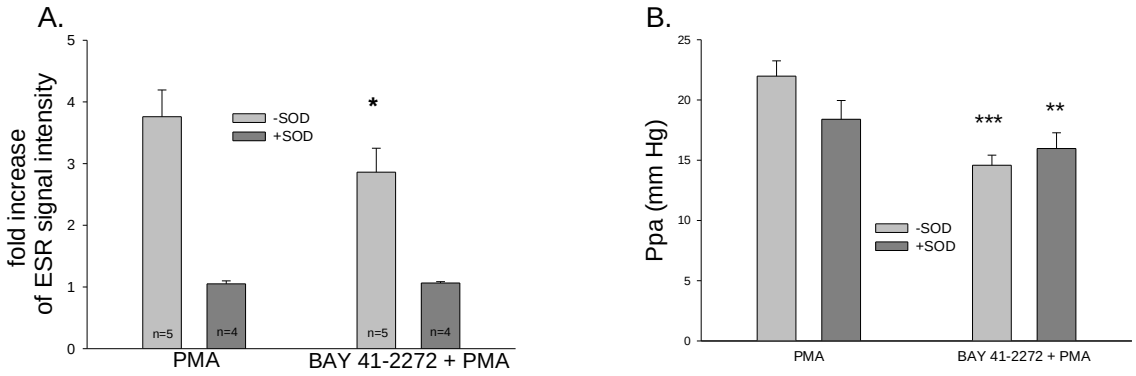


Fig 17. Influence of BAY 412272 on PMA stimulated ROS production and Ppa increase. Lungs were stimulated with PMA 1 µM either in presence or in absence of BAY 412272. Control measurements were performed in presence of SOD 150 U/ml throughout the experiments. A. changes in the increase rate of the ESR signal intensity (fold increase) by comparison of values prior to and after 30 min of PMA stimulation are presented; B. values of Ppa 30 min after PMA stimulation are shown. * $p < 0.05$ vs. PMA group. Data presented as mean ± SEM

4 DISCUSSION

Despite an intensive research, the pathomechanisms of I/R induced lung injury remain unclear. The aim of our investigation was to evaluate the role of NO synthases in mediation of I/R injury and the role of NO-cGMP signalling pathway in regulation of NADPH oxidase activity during the course of I/R.

4.1 The Main findings of this study were following:

- iNOS and nNOS contribute to I/R induced lung injury via ROS production
- there is a complex interaction between NO synthase isoforms
- direct sGC stimulation protects against I/R injury via interaction with activation of NADPH oxidases

I/R induced lung injury is characterized by an increased vascular permeability, which leads to fluid accumulation in pulmonary interstitium and edema formation. Previously, it has been demonstrated that the reperfusion induced transient pressure elevation is not the driving force for subsequent capillary leakage (7). Indeed, similar observations have been made in our study. NO synthase inhibitors prevented I/R induced lung injury without influencing reperfusion induced Ppa increase.

4.2 Role of NO synthase isoforms in I/R injury

In our study, complex changes in expression of NOS isoforms in the course of I/R were observed. Ischemia induced the upregulation of iNOS on the mRNA transcript and protein level in wild type mice. By immunohistochemical staining, localization of the upregulated iNOS was found in small vessels, suggesting the possible involvement of the enzyme in mediation of I/R induced vascular disturbances. In addition, the

downregulation of eNOS and moderate upregulation of nNOS were observed. These findings are in line with other reports (4), where upregulation of iNOS and downregulation of eNOS in the model of rat lung transplantation was observed.

To reveal the contribution of each isoform to I/R injury, NO synthase inhibitors were applied. Unspecific NOS inhibition protected against damage in rabbits and wild type mice, confirming involvement of NO synthases in mediation of I/R injury. Complete NOS inhibition was confirmed by abrogation of NO exhalation after L-NMMA application. Specific iNOS as well as nNOS inhibition prevented I/R induced permeability increase and fluid retention in the lung, suggesting that both isoforms contribute to the tissue damage. Specific iNOS and nNOS inhibition was confirmed by monitoring of NO exhalation. After application of either 1400W or BYK 191023, or VNIO in rabbit lungs no change in the exhaled nitric oxide was observed. Indeed, it has been demonstrated that exhaled NO is derived from eNOS in the setup of isolated perfused rabbit lung (61). These results are in line with the findings of other authors (17), who demonstrated the an upregulation of iNOS in the course of I/R and protective effects of its inhibition.

Interestingly, iNOS KO mice in our study were not protected against I/R injury. In the course of I/R, they demonstrated disturbed permeability and fluid retention comparable with that of wild type animals. Furthermore, iNOS KO mice demonstrated a different pattern of NOS isoform expression at baseline. They had lower eNOS and higher nNOS expression comparing to wild type mice. Ischemia induced further reduction in eNOS expression and downregulation of nNOS. As expected, no expression of iNOS was observed. In these animals unspecific NOS inhibition prevented I/R induced permeability disturbances and lung weight gain. Specific iNOS inhibition did not demonstrate protective efficacy. Selective nNOS inhibition

prevented lung injury, suggesting that nNOS mediates I/R lung injury in the iNOS KO animals.

To determine the contribution of eNOS in development of I/R injury, we employed eNOS KO mice. These animals phenotypically were not different from their WT counterparts. However, the isolated lungs demonstrated higher Ppa at baseline. Lungs from eNOS KO mice subjected to I/R demonstrated increased permeability and lung weight gain. Interestingly, total NOS inhibition in these lungs was not able to protect from injury. Additionally, selective iNOS inhibition did not show any protective effect. Selective nNOS inhibition prevented I/R induced lung injury. These findings may suggest that in these animals, as an adaptation to the absence of crucial eNOS gene, iNOS takes over the function of eNOS and exerts protective role. Hence, complex changes in expression and function of genes may take place in these genetically modified mice.

Furthermore, an increase in the nNOS expression in mouse lungs in the course of I/R was observed. Inhibition of this enzyme reduced intravascular ROS release and protected against I/R injury. Additionally, in iNOS KO and eNOS KO mouse lungs nNOS inhibition prevented permeability disturbances and weight gain. Similarly, nNOS has been found to be involved in mediation of acute lung injury induced by smoke inhalation and bacterial insufflations (62) and its inhibition improved gas exchange, reduced wet to dry ratio, and decreased plasma nitrate level.

Thus, we conclude that NO synthases contribute to I/R induced lung injury and their inhibition prevents tissue damage. The mechanisms of their detrimental effect are not completely clear. NO synthases could contribute to I/R injury by production of ROS by at least two mechanisms: 1) peroxynitrite formation from NO in the oxidative environment, where superoxide is derived from other enzymes, e.g. NADPH oxidases and 2) uncoupling of NOS and direct superoxide production by uncoupled enzyme.

Indeed, in rabbits and wild type mice increased ROS release into recirculating buffer after reperfusion was observed. This increase was inhibited by L-NMMA, suggesting that NO synthases are the source of ROS. Specific iNOS and nNOS inhibitors demonstrated similar efficacy. Lungs from both iNOS and eNOS KO mice demonstrated increased intravascular ROS release after reperfusion. In iNOS KO mouse lungs, nonselective NOS and selective nNOS inhibition prevented this increase, suggesting that nNOS is the source of reactive oxygen species. In eNOS KO mouse lungs selective iNOS or nNOS inhibition led to reduced ROS release. Hence, NO synthases contribute to the development of I/R injury by ROS production.

Thus, our results with pharmacological inhibitors suggest that iNOS and nNOS contribute to I/R induced lung injury. Results obtained from iNOS KO mouse lungs confirm these findings. However, results from eNOS KO mice are difficult to interpret because of complex compensatory changes in expression and function of NOS synthase isoforms.

This is not the first study where the results derived from genetically modified animals do not correlate with the results obtained from studies with pharmacologic enzyme inhibition. For instance, Zeidler et al. (63) demonstrated that iNOS KO mice challenged with LPS and interferon gamma developed inflammatory reaction, which was not different to that of wild type animals. In the light of other studies (64) where anti-inflammatory effect of iNOS inhibition was observed, this is an unexpected result. Instead, resistance of iNOS KO mice against LPS induced injury would be expected. Since NO synthases are crucial genes involved in control of vascular homeostasis, angiogenesis and immunity, deletion of one isoforms should lead to compensatory changes in expression and function of remaining isoforms. In the study of Zeidler et al., the function of nNOS and eNOS has not been elucidated. In contrast,

pharmacological inhibitors were applied to KO mice in our study to elucidate the functions of other expressed NO synthase isoforms.

These experiments revealed transformed function of expressed NOS isoforms in KO mice. In iNOS KO mice, nNOS seems to take over the function of absent iNOS, thus counteracting the potential protective effect of iNOS deletion. In eNOS KO mice, iNOS seems to take over the function of deleted eNOS, thus partly exerting protective effect.

4.3 sGC stimulation in I/R injury

The next question was to study the role of soluble guanylyl cyclase in mediation of I/R injury. But pharmacologic stimulation or genetic over-expression of eNOS would enhance NO production and could contribute to oxidative stress, as it has been demonstrated for eNOS over-expressing mice (23). Therefore, we decided to employ direct stimulation of sGC, the biological target of NO, to stimulate the function of NO-cGMP pathway in I/R lung injury. This approach enabled activation of the signalling pathway as stimulated eNOS would do, without having superoxide and peroxynitrite production by uncoupled NOS.

Direct stimulation of sGC using BAY 41-2272 resulted in substantial sGC stimulation, which was confirmed by cGMP measurement. Stimulation of sGC using BAY 41-2272 protected against permeability disturbances and fluid accumulation in lung tissue. BAY 41-2272 might have an immediate effect on the fluid reabsorbtion from alveolar space via cGMP gated ion channels. Indeed, recent findings (65, 66) suggest that cyclic nucleotide-gated cation channels are an important contributor to adult lung fluid homeostasis.

Additionally, the protective effect could be explained by observation that NO-cGMP signalling pathway interferes with NADPH oxidases by inhibiting assembly (67) and by inhibiting expression (40).

4.4 Interaction between NO-cGMP pathway and NADPH oxidase

Indeed, we observed that BAY 41-2272 inhibited increase in ROS production as well as Ppa increase induced by PMA, a stimulator of NADPH oxidase. Moreover, the effect of BAY 41-2272 on I/R induced lung injury was comparable with that of NADPH oxidase inhibitor apocynin. Consequently, activation of NADPH oxidases plays an important role in mediation of I/R injury and the sGC activator exerts its effect, at least in part, by prevention of activation of NADPH oxidases.

Hence, we found that stimulation of sGC by BAY 41-2272 attenuated I/R induced lung injury. This protective effect of BAY 41-2272 was achieved by restoration of NO-cGMP signalling pathway and preventing activation of NADPH oxidases.

4.5 Conclusion

Thus, we conclude that iNOS and nNOS contribute to mediation of I/R induced lung injury by ROS production. On the other hand, NO-cGMP signalling pathway protects against I/R induced lung injury by interfering with activation of NAPDH oxidases.

5 SUMMARY

Ischemia reperfusion (I/R) lung injury is a complex pathological process, which occurs in many clinical situations. Previous studies reported equivocal results about the role of nitric oxide (NO) synthase isoforms in the mediation of the injury and their possible mechanisms of action. Therefore, the aim of our study was to evaluate the role of NO synthase isoforms and NO-cGMP signaling pathway on I/R injury of the lung in an isolated perfused organ model employing rabbits as well as wild type and iNOS and eNOS knock out (KO) mice.

Lung injury was assessed by measurements of weight gain and microvascular permeability (capillary filtration coefficient (Kfc)). Release of reactive oxygen species (ROS) into the perfusate was measured during early reperfusion by electron spin resonance (ESR) spectroscopy. Unselective NOS inhibitor (L-NMMA), selective iNOS inhibitor (1400W, BYK 191023), selective nNOS inhibitor (VNIO), sGC stimulator (BAY 41-2272), or NADPH oxidase inhibitor (apocynin) were applied 5 min before ischemia according to the protocol.

In untreated lungs dramatic rise in Kfc values and weight gain during reperfusion were observed. This was associated with increased reactive oxygen species (ROS) production. In rabbit and wild type mouse lungs, unselective NOS and selective iNOS or nNOS inhibition protected against I/R injury and was accompanied by reduced intravascular ROS release. iNOS and nNOS were upregulated on protein and transcript level in the course of I/R. In eNOS and iNOS KO mice the contribution of nNOS and iNOS to I/R injury was confirmed. Direct stimulation of sGC using BAY 41-2272 significantly attenuated vascular leakage and suppressed ROS release. In an additional set of experiments BAY 41-2272 diminished phorbol-myristrate-acetate (PMA) induced ROS production by NADPH-oxidases. Involvement of ROS generated

by NADPH-oxidases in I/R was demonstrated by favorable effects of enzyme inhibition by apocynin.

Thus, we conclude that iNOS and nNOS contribute to mediation of I/R induced lung injury by ROS production. On the other hand, NO-cGMP signalling pathway protects against I/R induced lung injury by interfering with NAPDH oxidase activation.

6 ZUSAMMENFASSUNG

Der Ischämie/Reperfusionsschaden (I/R) der Lunge ist ein komplexer pathologischer Prozess, der im Laufe verschiedener klinischer Situationen, wie Organtransplantation und Thrombendarterektomie, auftreten kann. Vorangegangen Studien zeigten unterschiedliche Ergebnisse bezüglich der Rolle von NO Synthase-Isoformen bei der Vermittlung dieser Art des Lungenschadens. Ziel unserer Studie war es, den Einfluss von NO Synthase-Isoformen und des NO-cGMP Signaltransduktionsweges auf den I/R Schaden im Modell der isoliert perfundierten und ventilierten Lungen zu untersuchen.

Die Lungenschädigung wurde mittels Gewichtzunahme- und mikrovaskulärer Permeabilitätsmessungen (Kfc) quantifiziert. Die Freisetzung von reaktiven Sauerstoffspezies (ROS) wurde in rezirkulierendem Puffer mit Beginn der Reperfusion mittels Elektronenspinresonanz (ESR)-Technik gemessen. Pharmakologische Interventionen wurden mit einem unselektivem NOS Inhibitor (L-NMMA), einem selektivem iNOS Inhibitor (1400W, BYK 191023), einem selektivem nNOS Inhibitor (VNIO), einem sGC Stimulator (BAY 41-2272) oder einem NADPH Oxidasen Inhibitor (Apocynin), welche vor Beginn der Ischämie Phase verabreicht wurden, durchgeführt.

In unbehandelten Lungen konnte ein drastischer Anstieg des Kfc-Wertes, eine Gewichtzunahme und eine gesteigerte ROS Produktion nach der Reperfusion beobachtet werden. Sowohl in Kaninchen- als auch in wild-type Mauslungen schützte die unselektive NOS-, oder selektive iNOS- und die nNOS-Hemmung vor einem I/R Lungenschaden. Dieser Effekt war mit einer reduzierten intravaskulären ROS Freisetzung verbunden. Zudem konnte eine erhöhte Protein- und mRNA-Menge der iNOS und nNOS Isoformen während I/R beobachtet werden. In iNOS und eNOS defizienten Mäusen wurde die Beteiligung der nNOS und iNOS Isoformen am I/R

induzierten Lungenschaden bestätigt. Eine direkte sGC-Stimulierung mit BAY 41-2272 reduzierte den vaskulären Schaden und hemmte die ROS Freisetzung. In zusätzlichen Experimenten verminderte BAY 41-2272 die Phorbol-Myristat-Azetat (PMA)-induzierte ROS Produktion durch NADPH Oxidasen. Eine Beteiligung der von NADPH Oxidasen produzierten ROS an der Entwicklung eines I/R Lungenschadens konnte durch eine Inhibierung dieser Schädigung und Sauerstoffradikalproduktion mittels Apocynin demonstriert werden.

Aus diesen Ergebnissen schließen wir, dass sowohl iNOS als auch nNOS über eine ROS-Produktion an der Entwicklung eines I/R Lungenschadens beteiligt sind. Dagegen zeigt eine direkte Stimulation des NO-cGMP-Signaltransduktionsweges eine protektive Wirkung im Hinblick auf eine Reduzierung des I/R Lungenschadens durch eine Beeinflussung der NADPH-Oxidase Aktivität.

7 REFERENCE LIST

1. Eltzschig, H. K., and C. D. Collard. 2004. Vascular ischaemia and reperfusion injury. *Br Med Bull* 70(1):71-86.

2. Ng, C. S., S. Wan, A. A. Arifi, and A. P. Yim. 2006. Inflammatory response to pulmonary ischemia-reperfusion injury. *Surg Today* 36(3):205-14.

3. Pinsky, D. J., Y. Naka, N. C. Chowdhury, H. Liao, M. C. Oz, R. E. Michler, E. Kubaszewski, T. Malinski, and D. M. Stern. 1994. The Nitric Oxide/Cyclic GMP Pathway in Organ Transplantation: Critical Role in Successful Lung Preservation. *Proceedings of the National Academy of Sciences* 91(25):12086-12090.

4. Liu, M., L. Tremblay, S. D. Cassivi, X.-H. Bai, E. Mourgeon, A. F. Pierre, A. S. Slutsky, M. Post, and S. Keshavjee. 2000. Alterations of nitric oxide synthase expression and activity during rat lung transplantation. *Am J Physiol Lung Cell Mol Physiol* 278(5):L1071-1081.

5. Kobzik, L., D. S. Bredt, C. J. Lowenstein, J. Drazen, B. Gaston, D. Sugarbaker, and J. S. Stamler. 1993. Nitric oxide synthase in human and rat lung: immunocytochemical and histochemical localization. *Am J Respir Cell Mol Biol* 9(4):371-7.

6. Sherman, T. S., Z. Chen, I. S. Yuhanna, K. S. Lau, L. R. Margraf, and P. W. Shaul. 1999. Nitric oxide synthase isoform expression in the developing lung epithelium. *Am J Physiol Lung Cell Mol Physiol* 276(2):L383-390.

7. Schutte, H., K. Mayer, H. Burger, M. Witzenrath, T. Gessler, W. Seeger, and F. Grimminger. 2001. Endogenous nitric oxide synthesis and vascular leakage in ischemic-reperfused rabbit lungs. *Am J Respir Crit Care Med* 164(3):412-8.

8. Ischiropoulos, H., A. B. al-Mehdi, and A. B. Fisher. 1995. Reactive species in ischemic rat lung injury: contribution of peroxynitrite. *Am J Physiol Lung Cell Mol Physiol* 269(2):L158-164.

9. Mundy, A. L., and K. L. Dorrington. 2000. Inhibition of nitric oxide synthesis augments pulmonary oedema in isolated perfused rabbit lung. *Br. J. Anaesth.* 85(4):570-576.

10. Barbotin-Larrieu, F., M. Mazmanian, B. Baudet, H. Detruit, A. Chapelier, J. M. Libert, P. Dartevelle, and P. Herve. 1996. Prevention of ischemia-reperfusion lung injury by inhaled nitric oxide in neonatal piglets. *J Appl Physiol* 80(3):782-8.

11. Kemming, G. I., M. J. Merkel, A. Schallerer, O. P. Habler, M. S. Kleen, M. Haller, J. Briegel, C. Vogelmeier, H. Furst, B. Reichart, and B. Zwissler. 1998. Inhaled nitric oxide (NO) for the treatment of early allograft failure after lung transplantation. Munich Lung Transplant Group. *Intensive Care Med* 24(11):1173-80.

12. Botha, P., M. Jeyakanthan, J. N. Rao, A. J. Fisher, M. Prabhu, J. H. Dark, and S. C. Clark. 2007. Inhaled Nitric Oxide for Modulation of Ischemia-Reperfusion Injury in Lung Transplantation. *The Journal of Heart and Lung Transplantation* 26(11):1199.

13. Perrin, G., A. Roch, P. Michelet, M. Reynaud-Gaubert, P. Thomas, C. Doddoli, and J.-P. Auffray. 2006. Inhaled Nitric Oxide Does Not Prevent Pulmonary Edema After Lung Transplantation Measured By Lung Water Content: A Randomized Clinical Study. *Chest* 129(4):1024-1030.

14. Yamashita, T., S. Kawashima, Y. Ohashi, M. Ozaki, T. Ueyama, T. Ishida, N. Inoue, K.-i. Hirata, H. Akita, and M. Yokoyama. 2000. Resistance to Endotoxin Shock in Transgenic Mice Overexpressing Endothelial Nitric Oxide Synthase. *Circulation* 101(8):931-937.

15. Trifilieff, A., Y. Fujitani, F. Mentz, B. Dugas, M. Fuentes, and C. Bertrand. 2000. Inducible Nitric Oxide Synthase Inhibitors Suppress Airway Inflammation in Mice Through Down-Regulation of Chemokine Expression. *J Immunol* 165(3):1526-1533.

16. Squadrito, G. L., and W. A. Pryor. 1995. The formation of peroxynitrite in vivo from nitric oxide and superoxide. *Chemico-Biological Interactions* 96(2):203.

17. Ovechkin, A. V., D. Lominadze, K. C. Sedoris, E. Gozal, T. W. Robinson, and A. M. Roberts. 2005. Inhibition of inducible nitric oxide synthase attenuates platelet adhesion in subpleural arterioles caused by lung ischemia-reperfusion in rabbits. *J Appl Physiol* 99(6):2423-2432.

18. Kaminski, A., C. B. Pohl, C. Sponholz, N. Ma, C. Stamm, B. Vollmar, and G. Steinhoff. 2004. Up-regulation of endothelial nitric oxide synthase inhibits pulmonary leukocyte migration following lung ischemia-reperfusion in mice. *Am J Pathol* 164(6):2241-9.

19. Jones, S. P., J. J. M. Greer, A. K. Kakkar, P. D. Ware, R. H. Turnage, M. Hicks, R. van Haperen, R. de Crom, S. Kawashima, M. Yokoyama, and D. J. Lefer. 2004. Endothelial nitric oxide synthase overexpression attenuates myocardial reperfusion injury. *Am J Physiol Heart Circ Physiol* 286(1):H276-282.

20. Zweier, J. L., and M. A. H. Talukder. 2006. The role of oxidants and free radicals in reperfusion injury. *Cardiovascular Research* 70(2):181.

21. Kuzkaya, N., N. Weissmann, D. G. Harrison, and S. Dikalov. 2003. Interactions of peroxynitrite, tetrahydrobiopterin, ascorbic acid, and thiols: implications for uncoupling endothelial nitric-oxide synthase. *J Biol Chem* 278(25):22546-54.

22. Forstermann, U., and T. Munzel. 2006. Endothelial Nitric Oxide Synthase in Vascular Disease: From Marvel to Menace. *Circulation* 113(13):1708-1714.

23. Elrod, J. W., M. R. Duranski, W. Langston, J. J. M. Greer, L. Tao, T. R. Dugas, C. G. Kevil, H. C. Champion, and D. J. Lefer. 2006. eNOS Gene Therapy Exacerbates Hepatic Ischemia-Reperfusion Injury in Diabetes: A Role for eNOS Uncoupling. *Circ Res* 99(1):78-85.

24. Bucci, M., F. Roviezzo, I. Posadas, J. Yu, L. Parente, W. C. Sessa, L. J. Ignarro, and G. Cirino. 2005. Endothelial nitric oxide synthase activation is critical for vascular leakage during acute inflammation in vivo. *Proc Natl Acad Sci U S A* 102(3):904-8.

25. Holtz, M. L., S. D. Craddock, and L. C. Pettigrew. 2001. Rapid expression of neuronal and inducible nitric oxide synthases during post-ischemic reperfusion in rat brain. *Brain Research* 898(1):49.

26. Scorziello, A., Pellegrini, C., Secondo, A., Sirabella, R., Formisano, L., Sibaud, L., Amoroso, S., Canzoniero, L.M., Annunziato, L., Di Renzo, G.F. 2004. Neuronal NOS activation during oxygen and glucose deprivation triggers cerebellar granule cell death in the later reoxygenation phase. *Journal of Neuroscience Research* 76(6):812-821.

27. Haga, K. K., L. J. Gregory, C. A. Hicks, M. A. Ward, J. S. Beech, P. W. Bath, S. C. R. Williams, and M. J. O'Neill. 2003. The neuronal nitric oxide synthase inhibitor, TRIM, as a neuroprotective agent: effects in models of cerebral ischaemia using histological and magnetic resonance imaging techniques. *Brain Research* 993(1-2):42.

28. Jones, S. P., W. G. Girod, P. L. Huang, and D. J. Lefer. 2000. Myocardial reperfusion injury in neuronal nitric oxide synthase deficient mice. *Coron Artery Dis* 11(8):593-7.

29. Schutte, H., M. Witzenrath, K. Mayer, N. Weissmann, A. Schell, S. Rosseau, W. Seeger, and F. Grimminger. 2000. The PDE inhibitor zaprinast enhances NO-mediated protection against vascular leakage in reperfused lungs. *Am J Physiol Lung Cell Mol Physiol* 279(3):L496-502.

30. King, R. C., V. E. Laubach, R. C. Kanithanon, A. M. Kron, P. E. Parrino, K. S. Shockey, C. G. Tribble, and I. L. Kron. 1998. Preservation with 8-bromo-cyclic GMP improves pulmonary function after prolonged ischemia. *Ann Thorac Surg* 66(5):1732-8.

31. Becker, L. B. 2004. New concepts in reactive oxygen species and cardiovascular reperfusion physiology. *Cardiovasc Res* 61(3):461-470.

32. Fisher, A. B., A. B. Al-Mehdi, and V. Muzykantov. 1999. Activation of Endothelial NADPH Oxidase as the Source of a Reactive Oxygen Species in Lung Ischemia. *Chest* 116(90001):25S-26.

33. Dodd, O. J., and D. B. Pearse. 2000. Effect of the NADPH oxidase inhibitor apocynin on ischemia-reperfusion lung injury. *Am J Physiol Heart Circ Physiol* 279(1):H303-12.

34. Walder, C. E., S. P. Green, W. C. Darbonne, J. Mathias, J. Rae, M. C. Dinauer, J. T. Curnutte, and G. R. Thomas. 1997. Ischemic Stroke Injury Is Reduced in Mice Lacking a Functional NADPH Oxidase. *Stroke* 28(11):2252-2258.

35. Ray, R., and A. M. Shah. 2005. NADPH oxidase and endothelial cell function. *Clin. Sci.* 109(3):217-226.

36. Girouard, H., and C. Iadecola. 2006. Neurovascular coupling in the normal brain and in hypertension, stroke, and Alzheimer disease. *J Appl Physiol* 100(1):328-335.

37. Wang, J. P., L. C. Chang, S. L. Raung, M. F. Hsu, L. J. Huang, and S. C. Kuo. 2002. Inhibition of superoxide anion generation by YC-1 in rat neutrophils through cyclic GMP-dependent and -independent mechanisms. *Biochem Pharmacol* 63(4):577-85.

38. Hwang, T. L., H. W. Hung, S. H. Kao, C. M. Teng, C. C. Wu, and S. J. Cheng. 2003. Soluble guanylyl cyclase activator YC-1 inhibits human neutrophil functions through a cGMP-independent but cAMP-dependent pathway. *Mol Pharmacol* 64(6):1419-27.

39. Duerrschmidt, N., C. Stielow, G. Muller, P. J. Pagano, and H. Morawietz. 2006. NO-mediated regulation of NAD(P)H oxidase by laminar shear stress in human endothelial cells. *J Physiol* 576(2):557-567.

40. Muzaffar, S., N. Shukla, A. Srivastava, G. D. Angelini, and J. Y. Jeremy. 2005. Sildenafil citrate and sildenafil nitrate (NCX 911) are potent inhibitors of superoxide formation and gp91phox expression in porcine pulmonary artery endothelial cells. *Br J Pharmacol* 146(1):109-17.

41. Searles, C. D. 2006. Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression. *Am J Physiol Cell Physiol* 291(5):C803-816.

42. Dudzinski, D. M., and T. Michel. 2007. Life history of eNOS: Partners and pathways. *Cardiovascular Research* 75(2):247.

43. Mungrue, I. N., D. S. Bredt, D. J. Stewart, and M. Husain. 2003. From molecules to mammals: what's NOS got to do with it? *Acta Physiol Scand* 179(2):123-35.

44. Guembe, L., and A. C. Villaro. 1999. Histochemical Demonstration of Neuronal Nitric Oxide Synthase during Development of Mouse Respiratory Tract. *Am. J. Respir. Cell Mol. Biol.* 20(2):342-351.

45. Luhrs, H., T. Papadopoulos, H. H. H. W. Schmidt, and T. Menzel. 2002. Type I nitric oxide synthase in the human lung is predominantly expressed in capillary endothelial cells. *Respiration Physiology* 129(3):367.

46. Kavya, R., R. Saluja, S. Singh, and M. Dikshit. 2006. Nitric oxide synthase regulation and diversity: Implications in Parkinson's disease. *Nitric Oxide* 15(4):280.

47. Kirkeboen, K. A., and O. A. Strand. 1999. The role of nitric oxide in sepsis - an overview. *Acta Anaesthesiologica Scandinavica* 43(3):275-288.

48. Kleinert, H., A. Pautz, K. Linker, and P. M. Schwarz. 2004. Regulation of the expression of inducible nitric oxide synthase. *European Journal of Pharmacology* 500(1-3):255.

49. Lucas, K. A., G. M. Pitari, S. Kazerounian, I. Ruiz-Stewart, J. Park, S. Schulz, K. P. Chepenik, and S. A. Waldman. 2000. Guanylyl Cyclases and Signaling by Cyclic GMP. *Pharmacol Rev* 52(3):375-414.

50. Friebe, A., and D. Koesling. 2003. Regulation of Nitric Oxide-Sensitive Guanylyl Cyclase. *Circ Res* 93(2):96-105.

51. Williams, E. A., G. J. Quinlan, P. B. Anning, P. Goldstraw, and T. W. Evans. 1999. Lung injury following pulmonary resection in the isolated, blood-perfused rat lung. *Eur Respir J* 14(4):745-750.

52. Vural, K. M., and M. C. Oz. 2000. Endothelial adhesivity, pulmonary hemodynamics and nitric oxide synthesis in ischemia-reperfusion. *European Journal of Cardio-Thoracic Surgery* 18(3):348.

53. Niemeier, R. W. 1984. The isolated perfused lung. *Environ Health Perspect* 56:35-41.

54. Seeger, W., D. Walmrath, M. Menger, and H. Neuhof. 1986. Increased lung vascular permeability after arachidonic acid and hydrostatic challenge. *J Appl Physiol* 61(5):1781-9.

55. Schutte, H., A. Lockinger, W. Seeger, and F. Grimminger. 2001. Aerosolized PGE1, PGI2 and nitroprusside protect against vascular leakage in lung ischaemia-reperfusion. *Eur Respir J* 18(1):15-22.

56. Lockinger, A., H. Schutte, D. Walmrath, W. Seeger, and F. Grimminger. 2001. Protection against gas exchange abnormalities by pre-aerosolized PGE1, iloprost and nitroprusside in lung ischemia-reperfusion. *Transplantation* 71(2):185-93.

57. Schutte, H., G. Hermle, W. Seeger, and F. Grimminger. 1998. Vascular distension and continued ventilation are protective in lung ischemia/reperfusion. *Am J Respir Crit Care Med* 157(1):171-7.

58. Spriestersbach, R., F. Grimminger, N. Weissmann, D. Walmrath, and W. Seeger. 1995. On-line measurement of nitric oxide generation in buffer-perfused rabbit lungs. *J Appl Physiol* 78(4):1502-8.

59. Dikalov, S., K. K. Griendling, and D. G. Harrison. 2007. Measurement of Reactive Oxygen Species in Cardiovascular Studies. *Hypertension* 49(4):717-727.

60. Weissmann, N., N. Kuzkaya, B. Fuchs, V. Tiyerili, R. U. Schafer, H. Schutte, H. A. Ghofrani, R. T. Schermuly, C. Schudt, A. Sydykov, B. Egemnazarow, W. Seeger, and F. Grimminger. 2005. Detection of reactive oxygen species in isolated, perfused lungs by electron spin resonance spectroscopy. *Respir Res* 6:86.

61. Vaughan, D. J., T. V. Brogan, M. E. Kerr, S. Deem, D. L. Luchtel, and E. R. Swenson. 2003. Contributions of nitric oxide synthase isozymes to exhaled nitric oxide and hypoxic pulmonary vasoconstriction in rabbit lungs. *Am J Physiol Lung Cell Mol Physiol* 284(5):L834-43.

62. Enkhbaatar, P., K. Murakami, K. Shimoda, A. Mizutani, R. McGuire, F. Schmalstieg, R. Cox, H. Hawkins, J. Jodoin, S. Lee, L. Traber, D. Herndon, and D. Traber. 2003. Inhibition of neuronal nitric oxide synthase by 7-nitroindazole attenuates acute lung injury in an ovine model. *Am J Physiol Regul Integr Comp Physiol* 285(2):R366-372.

63. Zeidler, P. C., L. M. Millecchia, and V. Castranova. 2004. Role of inducible nitric oxide synthase-derived nitric oxide in lipopolysaccharide plus interferon-[gamma]-induced pulmonary inflammation. *Toxicology and Applied Pharmacology* 195(1):45.

64. Han, X., M. P. Fink, T. Uchiyama, R. Yang, and R. L. Delude. 2004. Increased iNOS activity is essential for pulmonary epithelial tight junction dysfunction in endotoxemic mice. *Am J Physiol Lung Cell Mol Physiol* 286(2):L259-267.

65. Junor, R. W., A. R. Benjamin, D. Alexandrou, S. E. Guggino, and D. V. Walters. 1999. A novel role for cyclic nucleotide-gated cation channels in lung liquid homeostasis in sheep. *J Physiol* 520 Pt 1:255-60.

66. Kemp, P. J., K. J. Kim, Z. Borok, and E. D. Crandall. 2001. Re-evaluating the Na(+) conductance of adult rat alveolar type II pneumocytes: evidence for the involvement of cGMP-activated cation channels. *J Physiol* 536(Pt 3):693-701.

67. Fujii, H., K. Ichimori, K. Hoshiai, and H. Nakazawa. 1997. Nitric Oxide Inactivates NADPH Oxidase in Pig Neutrophils by Inhibiting Its Assembling Process. *J. Biol. Chem.* 272(52):32773-32778.

8 APPENDIX

8.1 Curriculum vitae

Bakytbek Egemnazarov

Permanent Address

T.Moldo street 3,
720040 Bishkek,
Kyrgyz Republic
Tel- +996-312-664303

Correspondence Address

Innere Medizin II
Aulweg 130
35392 Giessen
Tel- +49-641-9946803

Email: bakytbek.egemnazarov@innere.med.uni-giessen.de

Personal

Kyrgyz, married, have a child, 31Years (DOB: 28th March 1977)

Work Experience

Year 2002 to June 2002

Research Associate, National Centre of Cardiology and Internal Medicine, Department of Pulmonary Hypertension and Mountain Medicine (Bishkek, Kyrgyzstan)

Educational Background

Year 1994 to June 2000

Kyrgyz State Medical Academy (Bishkek, Kyrgyzstan)
Doctor of General Practice

Year 2000 to August 2002

The Scientific and Methodological Centre for Medical Training, Kyrgyz National Center of Cardiology and Internal Medicine (Bishkek, Kyrgyzstan)
Postgraduate Clinical Training specializing in cardiology

Year 2002 to 2004

Fellowship in research group “Sonderforschungsbereich 547” University of Giessen School of Medicine, Department of Medicine II (Giessen, Germany**)**

Year 2004 to 2007

International Graduate Program “Molecular Biology and Medicine of the Lung”, University of Giessen School of Medicine, Department of Medicine II (Giessen, Germany)

Year 2005 till present

International PhD Graduate Program of the Faculties of veterinary medicine and medicine of the Justus Liebig University Giessen (Giessen, Germany)

Honors

Diploma with Honor of Kyrgyz State Medical Academy

Stipend of Government of Kyrgyz Republic for research work 2002

Bursary of the ERS Lung Science Conference 2007 in Taormina

Professional Memberships:

European Respiratory Society

Languages:

Kyrgyz - native

Russian - excellent

German – advanced

English – advanced

Computer skills:

User: MS Office, MS Publisher, SPSS, Sigma Plot

8.2 Publications

Weissmann N., KuzkayaN., Fuchs B., Tiyerili V., Schafer R.U., Schutte U., Ghofrani H.A., Schermuly R.T., Schudt C., Sydykov A., **Egemnazarov B.**, Seeger W., and Grimminger F. 2005. Detection of reactive oxygen species in isolated, perfused lungs by electron spin resonance spectroscopy. *Respir Res* 2005 Jul 31;6:86.

Egemnazarov B., Sydykov A., Schermuly R.T., Weissmann N., Stasch J.P., Sarybaev A.S., Seeger W., Grimminger F., Ghofrani H.A. Novel soluble guanylyl cyclase stimulator BAY 41-2272 attenuates ischemia/reperfusion induced lung injury. *Am J Physiol Lung Cell Mol Physiol.* 2009 Mar;296(3):L462-9.

Ketabchi F, **Egemnazarov B**, Schermuly RT, Ghofrani HA, Seeger W, Grimminger F, Shid-Moosavi M, Dehghani GA, Weissmann N, Sommer N. Effects of hypercapnia with and without acidosis on hypoxic pulmonary vasoconstriction. *Am J Physiol Lung Cell Mol Physiol.* 2009 Aug 28. [Epub ahead of print] PMID: 19717554

Egemnazarov B., Schermuly R.T., Dahal B., Elliott G.T., Hoglen N.C., Surber M.W., Weissmann N., Grimminger F., Seeger W., Ghofrani H.A. Nebulized sodium nitrite with neutral and acidified pH attenuated acute hypoxic pulmonary vasoconstriction. *In preparation.*

8.3 Conferences

Novel sGC activator BAY 41-2272 attenuates ischemia/reperfusion injury of the lung via inhibition of NADPH oxidase. Egemnazarov B., Sydykov A., Schermuly R., Weissmann N., Seeger W., Grimminger F.,Ghofrani H.A. DGIM conference, Wiesbaden, 2006

Importance of iNOS in the mediation of ischemia/reperfusion injury of the lung. Sydykov A., Egemnazarov B., Schermuly R., Weissmann N., Seeger W., Grimminger F., Ghofrani H.A. DGIM conference, Wiesbaden, 2006

Novel sGC activator BAY 41-2272 attenuates ischemia/reperfusion injury of the lung via inhibition of NADPH oxidase. Egemnazarov B., Sydykov A., Schermuly R., Weissmann N., Seeger W., Grimminger F., Ghofrani H.A. ERS annual congress, Munich, 2006

The NADPH oxidase inhibitor apocynin protects against lung ischemia/reperfusion injury. Egemnazarov B., Sydykov A., Schermuly R., Weissmann N., Seeger W., Grimminger F., Ghofrani H.A. DGIM conference, Wiesbaden, 2007

Role of NO synthase isoforms in ischemia/reperfusion injury of the lung. Egemnazarov B., Sydykov A., Schermuly R., Weissmann N., R.Voswinckel, Seeger W., Grimminger F., Ghofrani H.A. ATS conference, San Francisco, 2007

Role of NO synthase isoforms in ischemia/reperfusion injury of the lung. Egemnazarov B., Sydykov A., Schermuly R., Weissmann N., R.Voswinckel, Seeger W., Grimminger F., Ghofrani H.A. Lung science conference , Taormina, 2007

Role of inducible NO synthase in ischemia/reperfusion injury of the lung. Egemnazarov B., Sydykov A., Schermuly R., Weissmann N., R.Voswinckel, Seeger W., Grimminger F., Ghofrani H.A. SFRBM conference, Washington, 2007

8.4 Acknowledgements

I would like to thank Prof. Seeger for providing me the opportunity to perform this work. Especially, I am thankful for his idea to start my scientific work from experimental studies. This was really great idea, following which I have gained great experience.

I thank cordially Prof. H.A. Ghofrani for guiding me in my work. I think, he is very good teacher helping always with some exciting ideas and practical tips, at the same time stimulating creative work. I have learned a lot with the aid of Prof. Weissmann and Prof. Schermuly. Thank you for your help!

My special thanks to my friend and colleague A. Sydykov, who shared with me his knowledge. His help was especially invaluable at the beginning of my work.

Special thanks to my MBML class and our mentors Prof. O. Eichelberg and M.Rorry. In the course of our discussions I gained many interesting ideas from you. With your help I understand now what do different “shadows” on blots mean.

My colleagues and friends in the Lab Seeger gave an invaluable support by discussing my results and helping to learn new techniques. With them we had not only fruitful work but also fun during these years. Especially, I would like to thank Markus, Natascha, and Manish for their help.

My family was a great support for me all the time. Gulsina and Uluk understood me even if I was not at home at weekends. My mother, whose support and believe brought me to the point where am I now.

8.5 Statement/Erklärung

I hereby declare that the present manuscript is my own work without use of any illegal outside help. All parts of the manuscript containing information from other published and unpublished sources, including oral communications, are marked as citations. All experiments mentioned in the dissertation are done in accordance to principals of high quality scientific performance as described in the „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“.

“Ich erkläre: Ich habe die vorgelegte Dissertation selbständig, ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.“

Bakytbek Egemnazarov

Giessen

15.10.2008