

Novel molecular mechanisms and drug targets in the treatment of pulmonary hypertension

Ph.D. thesis

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List of abbreviations

AEBSF	4-(2-aminoethyl)-benzolsulfonil-fluorid
ALK-1	activin receptor-like kinase 1 gene
AP-1	activator protein-1
APAH	associated pulmonary arterial hypertension
APTT	activated partial thromboplastin time
APT	acute pulmonary thromboembolism
BCR-Abl	break point cluster - Abelson tyrosine kinase
BMPR2	bone morphogenetic protein receptor type2
CaM	calmodulin
CI	cardiac index
CINC-1, CINC-2α/β	cytokine-induced neutrophil chemoattractant
CNTF	ciliary neurotrophic factor
CO	cardiac output
CO₂	carbon dioxide
COPD	chronic obstructive pulmonary disease
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CTEPH	chronic thromboembolic pulmonary hypertension
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EF	ejection fraction
ERA	endothelin receptor antagonist
ERK $\frac{1}{2}$	extracellular signal-regulated kinase
FDA	US Food and Drug Administration
Fract	fractalkine
GM-CSF	granulocyte-macrophage colony-stimulating factor
GSK-3β	glycogen synthase kinase-3 β
HE	hematoxylin and eosin
HIV	human immunodeficiency virus
ICAM	intracellular adhesion molecule
IκB	inhibitor of kappa B
IL-1α	interleukin-1 α
INF	interferon
i.p.	intraperitoneal
IP-10	interferon gamma-induced protein-10
i.v.	intravenous
KH	chronic hypoxic
LDL	low density lipoprotein
LIX	lipopolysaccharide induced CXC chemokine
LMWH	low molecular weight heparin
L-Sel	L-selectin
LV	left ventricle
MAPK	mitogen-activated protein kinase
MCT	monocrotaline
MIG	monokine induced by gamma interferon
MIP-1α, MIP-3α	macrophage inflammatory protein
MLCK	myosin light-chain kinase
MMPs	matrix metalloproteinase

NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitrogen monoxide
Nrf-2	nuclear factor erythroid 2- related factor 2
PA	pulmonary artery
PASMC	pulmonary arterial smooth muscle cell
PAH	pulmonary arterial hypertension
PAP	pulmonary arterial pressure
PARP	poly (ADP-ribose) polymerase
PBS	phosphate buffered solution
PDE-5	phosphodiesterase type 5
PDGF	platelet derived growth factor
PE	pulmonary embolism
PESI	pulmonary embolism severity index
PH	pulmonary hypertension
PI-3K-Akt	phosphatidylinositide 3-kinase-Akt
PKG	protein kinase G
p.o.	per os
PVRI	pulmonary vascular resistance index
PWP	pulmonary wedge pressure
RANTES	regulated on activation normal T cell expressed and secreted
rtPA	recombinant tissue plasminogen activator
RV	right ventricle
SDS	sodium-dodecyl-sulphate
SEM	standard error of the mean
SK	streptokinase
SMC	smooth muscle cell
STAT	signal transducer and activator of transcription protein
Thym Chem	thymus chemokine
TIMP-1	tissue inhibitor of metalloproteinase
TL	thrombolysis
TNF-α	tumor necrosis factor- α
tPA	tissue plasminogen activator
TPG	transpulmonary pressure gradient (mean PAP – mean PWP)
UFH	unfractionated heparin
UH-SK	ultra-high-dose streptokinase
UK	urokinase
VEGF	vascular endothelial growth factor
VSMCs	vascular smooth muscle cells
VTS	venous thromboembolic syndrome
WHO	World Health Organization

Introduction

PH has been defined as an increase in mean pulmonary arterial pressure > 25 mmHg at rest as assessed by right heart catheterization. Pulmonary arterial hypertension is a rare, progressive and incurable condition that often progresses undetected until it reaches the most severe stages. Although it is a rare disease, the number of recognized cases seems to be increasing.

PAH is a progressive disease, it is characterized by obstructive proliferative changes in the lung microcirculation so called vascular remodelling. Our knowledge regarding the multiple pathological processes leading to the evolution of microvascular injury is still limited. Multiple lines of evidence support that vasoconstriction as well as inflammatory processes precede the remodelling of the pulmonary arterioles. Endothelial dysfunction interfere at various levels of the microvascular injury that is characterized by medial hypertrophy, intimal proliferative changes, adventitial thickening with perivascular inflammatory infiltrates. These alterations as well as the increased level of reactive oxygen species account for decreased apoptosis and increased proliferative vascular remodelling has significant role in the pathogenesis and progression of PH.

Several medications were approved in PAH that improve symptoms and slow down progression. However currently there is no effective cure for PAH.

PD5 inhibitors inhibit the cleavage of cGMP and induces pulmonary vasodilation by extending the vasodilator effect of nitrogen monoxid.

Monocrotaline (MCT) is a C11 toxic macrocyclic pyrrolizidine alkaloid derived from plant *Crotalaria spectabilis*' seed. Due to the substance the enlargement of the Golgi apparatus in the endothelial cells and pneumocytes develops, leading to megalocytosis. As the formation of protein structure is inhibited in this organelle lack of the membrane proteins occur resulting in compensatory proliferative and anti-apoptotic factor induction and the NO signal transduction pathway is impaired.

Own experiments in rat PH model

Aim

In our current experiments we observed the pulmonary effects of sildenafil in MCT induced rat PH model. We wanted to explore new mechanisms, contributing the known positive vasodilative effect of sildenafil in PH.

We examined the mechanisms by which sildenafil can contribute to the attenuation of MCT induced inflammatory processes.

We aimed to reveal the changes of the cytokine network, for which we used a broad spectrum cytokine array analysing the expression of 29 cytokines.

We studied the activation pathway of nuclear factor kappa-light-chain-enhancer of activated B-cells, mitogen-activated protein kinases (MAPK) and the phosphatidylinositol 3-kinase-Akt (PI-3k-Akt) pathway, thus revealing the gene expression related signaltransduction pathways.

Morphological changes were detected by histological studies, while biochemical changes were examined by Western blot analysis and immunohistological studies in addition to cytokine array.

Experimental protocol

In our experiments we used male Wistar rats (250 – 300 g). All animals were housed one or two per cage, under optimal laboratory conditions (controlled temperature, humidity and 12:12 h- light-dark cycles) with free access to water and standard rodent chow. The animals were randomly assigned into four groups.

- Sham group (n= 6) receiving i.p. injection of isotonic saline (0.1 ml/kg) on day 0.
- Sham+ sildenafil (SLD) group (n= 8) receiving i.p. injection of isotonic saline (0.1 ml/kg) on day 0 and sildenafil (2 mg/kg per day, per os, in the drinking water) from day 0 to day 28.
- Pulmonary hypertension (PH) group (n= 8) receiving 60 mg/kg i.p. injection of MCT on day 0.

- Pulmonary hypertensive + sildenafil (PH+SLD) group (n= 8) receiving 60 mg/kg i.p. injection of MCT on day 0 and sildenafil (2 mg/kg per day, per os, in the drinking water) from day 0 to day 28.

After 4 weeks animals were sacrificed by overdosing isoflurane and organs were removed and their weight was measured. Samples for molecular biological testing were frozen in liquid nitrogen and were stored at -80 °C.

Survival studies were performed by using 10-10 animals.

Lung histology and morphometric analysis

Lungs were fixed in 6% formalin, embedded into paraffin and 5 µm thin sections were cut with microtome (Leica 2135). Sections were stained with haematoxylin–eosin and digital photos were taken (Olympus E-450 Digital SLR) at 200-fold-magnification. Average wall thickness of alveolar sac was determined at randomly chosen 50 different sites per section. Macrophages were counted in 5 non-overlapping high power fields in each sections.

Inflammatory cytokine and adhesion molecules measurements using rat cytokine array kit

The cytokine array assay was performed on lung homogenates, using rat cytokine array kit (R&D Systems; Biomedica Hungaria, Hungary). The list of measured cytokines are shown in Table 1. These arrays are based on binding between sample proteins and carefully selected capture antibodies spotted on nitrocellulose membranes. We examined tissue samples from all the 4 groups: Sham, Sham+ SLD, PH and PH+SLD groups. The array was performed as described by the manufacturer. Developed films were scanned and analysed by densitometry. The pixel volumes of the bands were determined using the NIH ImageJ 1.40 software.

Immunohistochemical staining

Histological sections were prepared followed by incubation with rabbit clonal antibodies (anti-CD34 - cat# 10097.10, clone: Q19-E, antibody 1:200 dilution and anti-NF-κB antibodies - Abcam, cat#ab86299 in 1:20 dilution) as primary antibodies. Following incubation with the primary antibodies sections were incubated with the secondary anti-rabbit antibodies

(HISTOLS –R Detection System, anti-rabbit, Histopathology Ltd.) at room temperature than washed and cleared in xylene followed by fixation process.

Immunoblotting / Western blot analysis

Lung tissue samples (50 mg each) were homogenized, proteins were precipitated by trichloroacetate. Prepared samples were separated on 10% sodium-dodecyl-sulphate (SDS)–polyacrylamide gels, and transferred to Protran nitrocellulose membranes. After blocking membranes were probed overnight at 4 °C with antibodies recognizing the following antigens: total-p38MAPK, phosphop38 MAPK (Thr180/Tyr182), phospho-extracellular signal-regulated kinase (ERK)1/2 (Thr202/Tyr204), total ERK1/2, total GSK-3b, phospho-GSK-3b (Ser9), phospho-Akt (S473), total Akt, total-NF-κB, phospho-NF-κB (Ser536) (each 1:500 dilution, Cell Signaling Technology). After incubation the nitrocellulose membranes were incubated with goat anti-rabbit horseradish peroxidase conjugated secondary antibody (1:3000 dilution; Bio-Rad) at room temperature. The antigen-antibody complexes were visualized with enhanced chemiluminescence (ECL) labelling using an ECL immunoblotting detection system (Amersham Biosciences). Developed films were scanned and the pixel volumes of the bands were determined using the NIH ImageJ 1.40 software.

Data analysis

Data were analysed using the Kolmogorov-Smirnov normality test followed by the one-way Anova test and Bonferroni post hoc multiple comparison test. Differences were considered significant at $p < 0.05$. Analyses were performed using IBM SPSS Statistics 20.

Results

Effects of sildenafil treatment on histological changes of the lung

The mean wall thickness of alveolar sac was 3.31 ± 0.88 mm. CD-34 immunohistochemistry that indicates vascularization and vascular remodelling showed sporadic weak positivity. Sildenafil treatment alone did not cause any change neither in the wall thickness of alveolar sac nor in macrophage infiltration. In MCT treated animals, increased vascular wall thickness and due to inflammatory cell recruitment, the appearance of these cells and increased alveolar wall thickness have been detected in pulmonary hypertensive rats. The mean wall thickness of alveolar sac increased to 9.77 ± 2.63 mm vs 3.31 ± 0.88 μm ($p < 0.05$). As a result of MCT treatment a markedly increased macrophage count and macrophage infiltration have also been

	IL-1α	IL-1 β	IL-1ra	IL-2	IL-3	IL-4	IL-6	IL-10	
	IL-13	IL-17	IP-10	LIX	L-Sel	MIG	MIP-1α	MIP-3α	
Ref Spt	<i>RANTES</i>	<i>Thym Chem</i>	TIMP-1	TNF- α	<i>VEGF</i>			Neg Ctr	

Table 1: Rat cytokine array kit

The bold marked cytokines showed the most significant changes where the density of the spots of the PH group increased compared to the sham group, and the density of the PH + SLD group reduced compared to the PH group. In the case of the italic marked cytokines the density of the spots was higher in the sham group than in the PH group. The non-marked cytokines did not show any changes.

Effects of sildenafil treatment on NF- κ B activation

Expression of most the aforementioned cytokines that were significantly induced by MCT treatment are regulated by NF- κ B transcription factor. In our studies we determined NF- κ B activation by immunoblotting and immunohistochemistry utilizing phospho- NF- κ B p65 specific primary antibody among the various groups. Phospho-NF- κ B p65 immunohistochemistry showed sporadic patchy weak background positivity in the Sham group. A very few of the nuclei demonstrated strong positive staining indicating nuclear translocation of NF- κ B. NF- κ B activation and nuclear translocation was basically identical to control among the sildenafil treated animals (without MCT treatment). On the other hand MCT treatment caused a massive activation and nuclear translocation of NF- κ B in the endothelial and epithelial cells of the lung tissues in the pulmonary hypertensive rats. The phosphorylation thereby activation of NF- κ B significantly reduced by sildenafil treatment in MCT induced pulmonary hypertensive animals, as inhibits detaching of I κ B from the NF- κ B molecule leading to the inhibition of NF- κ B translocation to the nucleolus and the complex remains in the cytoplasm. The translation of mRNS of different proinflammatory proteins, growth factors and apoptosis regulating proteins is pNF- κ B dependant gene expression process and due to lack of a very important regulating molecule it cannot happen.

Effect of sildenafil on PI-3K-Akt and MAPK signalling pathway

NF- κ B activation is regulated by various kinase signalling pathways including PI-3K-Akt and MAPK pathways. Therefore, we assessed the activation of these pathways by immunoblotting

utilizing phosphorylation-specific primary antibodies. The phosphorylation thereby activation of Akt was not detectable in control lung tissues. Sildenafil alone slightly increased Akt activation, although, it did not reach the level of statistical significance. MCT treatment significantly increased Akt phosphorylation that was further enhanced by sildenafil administration.

The phosphorylation of Akt's downstream target GSK-3 β showed similar pattern among the different groups as that of Akt verifying Akt activation.

Since cGMP was reported to regulate ERK1/2 and p38 among the MAPKs, we studied the effect of MCT and sildenafil on them. In the control lungs with or without sildenafil treatment, a slight phosphorylation of ERK1/2 (pERK 1 and 2) was observed. In MCT treated lungs a moderate activation of ERK1/2 occurred that was slightly decreased by sildenafil treatment, however, none of these changes reached the level of statistical significance. The p38 MAPK activation/phosphorylation in control lungs was undetectable that was slightly elevated by sildenafil treatment. MCT induced a robust activation of MAPK signalling pathway. The increased level of the phosphorylated form of p38MAPK the pp38MAPK was observed due to oxidative stress in pulmonary hypertension that was significantly diminished by sildenafil as in case of cytokines and chemokines. Because the inhibition of I κ B function is not suppressed by the MAPK, the I κ B remains in complex with NF- κ B which remains under inhibition, thereby promoting cell survival.

Conclusion

Sildenafil has a strong cytoprotective effect; however, it causes vasodilation, thereby optimizing the pulmonary circulation, oxygenation, strongly inhibits apoptosis and proinflammatory biochemical pathways and suppresses these signalling mediators. In summary: in the future sildenafil can receive a greater role in the therapy of inflammatory diseases.

Acute pulmonary embolism (PE)

Acute pulmonary embolism is a relatively common condition that may become critical and associated with increased mortality.

Pulmonary hypertension in acute pulmonary embolism

Emboic masses lead to mechanical obstruction of pulmonary arteries resulting in increased pressure in pulmonary arteries. Nevertheless it is not just the mechanical obstruction which leads to pulmonary hypertension in PE. Due to in situ thrombosis the coagulation cascade, platelets and endothelial cells are activated resulting in endothelial dysfunction. Certain factors such as shear stress, increased pressure, inflammation, certain cytokines, extracellular matrix remodelling, endogenous vascular elastase, plasminogen activator/plasmin system and matrix metalloproteases are also involved in the pathogenesis.

Oxidative stress and leucocyte activation also play role but we have only limited data regarding the pathology of oxidative stress associated with acute pulmonary embolism from human studies.

Further biochemical substances such as matrix metalloproteases (MMPs) are also involved in acute right ventricular failure associated with acute massive pulmonary embolism. Activated neutrophils release increased amounts of reactive oxygen species including superoxide, thus activating MMPs. Particularly important for the pulmonary vasculature, increased mechanical stretch of the vessel wall promotes the formation of reactive oxide species, thus further contributing to enhanced MMP release. Moreover, activated MMPs (particularly MMP-2 and -9) disrupts vascular structure by causing fragmentation of internal elastic laminae, which is an early histopathologic feature of pulmonary hypertension. Another factor mechanism that may play a role in acute pulmonary embolism-induced pulmonary hypertension is the possible involvement of MMPs in the modulation of vascular tone. Activated MMPs may induce the degradation of big endothelin-1 into endothelin 1-32, which has potent vasoconstrictor effects. It is known that fibrinolytic drugs such as tissue plasminogen activator (rt-PA; alteplase), which are used to induce clot lysis, may promote MMP-9 release by neutrophils. Some members of our study group were first to show increased MMP-9 levels following fibrinolysis with alteplase or streptokinase in patients with massive and submassive pulmonary embolism, and this effect was associated with no significant changes in plasma TIMP-1 levels. These findings suggest that thrombolytic therapy increases net MMP activity, and this alteration may increase the risk of intracerebral haemorrhage, which is the most feared bleeding complication of thrombolysis, especially because it is fatal in more than 50% of cases. Although it not known whether increased MMP-9 levels after the use of thrombolytic agents explains intracerebral haemorrhage following fibrinolysis for acute pulmonary embolism, it is possible that the MMP inhibitors may decrease the risk of intracerebral haemorrhage or other bleeding complication after thrombolysis for this condition, as previously shown in rodents.

Imbalance between vasoactive substances may also play role in the development of acute pulmonary hypertension associated with acute pulmonary embolism. More recently, significant haemolysis has been shown in animal models of APT, and increased arginase I and II activity has been implicated in the increased pulmonary vascular resistance commonly found after APT. The authors showed that haemolysis-induced increases in arginase I could deplete L-arginine, therefore impairing endogenous NO formation. Under normal conditions, haemoglobin is confined to red blood cells, and therefore NO produced by endothelial cells reach smooth muscle cells producing vasodilation. Impaired NO availability resulting from increased cell-free haemoglobin concentrations has been shown as a novel important mechanism of human disease involving haemolysis-induced vasoconstriction and endothelial dysfunction resulting of NO scavenging by haemoglobin

Our hypothesis was that lung embolization and thrombolysis increases cell-free haemoglobin concentrations that impair NO bioavailability as a direct result of NO scavenging (consumption), as previously shown in other disease conditions associated with haemoglobin decompartmentalization into plasma Our study incorporated both experimental lung embolization model and clinical study.

Own experiments on animal pulmonary embolism model and in clinical practice

Aim of the study

Our hypothesis was that lung embolization and thrombolysis increases cell-free haemoglobin concentrations and NO consumption by plasma resulting in reduced NO bioavailability leading to increased vascular resistance.

In the present study, we examined whether experimental lung embolization with autologous blood clots or with the infusion of microspheres increase cell-free haemoglobin levels and NO consumption by plasma.

To validate our experimental findings, we compared free haemoglobin concentrations and NO consumption by plasma samples from patients with APT with those from healthy controls.

In clinical study we examined the effects of thrombolysis on cell-free haemoglobin levels and NO consumption.

Materials and methods

Animal model

Measurements were done at Faculty of Medicine of Ribeirão Preto, University of São Paulo, Brasil. Experiments fit in with guidelines of the Faculty of Medicine of Ribeirão Preto, University of São Paulo, and the animals were handled according to the guiding principles published by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Fourteen male lambs weighing 17.2 ± 2.0 kg (mean \pm SD) were used in this study. The animals were anesthetized with intramuscular ketamine and xylazine (10–15 mg/kg and 0.1–0.2 mg/kg, respectively), relaxed with pancuronium (0.1 mg/kg bolus and 0.5–1 mg/hr; IV), tracheally intubated, and their lungs were artificially ventilated with room air using volume control mechanical ventilation. The tidal volume was set at 15 mL/kg, and the respiratory rate was adjusted to maintain a baseline physiologic arterial carbon dioxide tension. Anaesthesia was maintained with intramuscular injections of ketamine (5 mg/kg) and midazolam (0.5 mg/kg) every 30 minutes. Fluid-filled catheters were placed into the left femoral artery and right femoral vein for mean systemic arterial pressure monitoring via a pressure transducer and fluid administration, respectively. A 7.5F balloon-tipped Swan-Ganz thermodilution catheter was placed into the pulmonary artery via the left femoral vein. Its correct location being confirmed by detection of the typical pressure wave of this artery. The catheter was connected to pressure transducers to allow the monitoring of mean pulmonary artery pressure, central venous pressure, and pulmonary artery occlusion pressure. The transducers were zeroed at the level of the right heart and recalibrated before each set of measurements. Thermodilution cardiac output measurements were determined in triplicate by injecting 3 mL of saline and the results recorded (DX2010 Monitor, Dixtal do Brasil, Manaus, Brasil). The heart rate was measured using a surface electrocardiogram (lead I). The cardiac index, systemic vascular resistance index, and pulmonary vascular resistance index (PVRI) were calculated by standard formulae using the thermodilution curves and the results of invasive pressure measurements.

The animals were randomly assigned to one of three experimental groups:

1. Sham group ($n = 4$), in which non-embolized animals received only saline infusions.
2. Acute pulmonary embolism (APT) group ($n = 5$), in which APT was induced by infusing autologous blood clots for 5 to 10 minutes via a large-bore cannula placed in the right atrium. The clots derived from venous blood samples (5 ml/kg) collected and allowed to clot for at least 60 minutes, and then cut into 2- to 3-mm cubes.

3. Embolism with microspheres (EMB) group ($n = 5$), in which the animals received repeated intravenous injections (every 30 s) of 300 μm microspheres (Sephadex G50; Pharmacia Fine Chemicals; Uppsala, Sweden) into the inferior vena cava over 5 to 10 minutes. The amount of microspheres infused in each male lamb was adjusted to induce an increase of 20 mm Hg in mean pulmonary artery pressure.

Hemodynamic evaluation was performed 30 and 180 minutes after lung embolization.

Arterial blood samples were drawn at BL, and 30 and 180 minutes after lung embolization for plasma-free haemoglobin and plasma NO consumption measurements.

Clinical study

This study was carried out in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and the permission of the Institutional Scientific and Human Research Ethics Committee of the University of Pécs (810/2001). Each patient provided written informed consent and was informed clearly about the details of study and blood sampling.

We prospectively screened patients with high and intermediate risk of pulmonary embolism, according to the latest European Society of Cardiology guidelines. A group of 28 healthy control subjects were randomly selected from the local population. About 14 Caucasian patients were divided into two therapeutic groups using block randomization: 7 patients received ultra-high-dose (9 million units/6 hours) streptokinase (UH-SK), according to previous studies and to our hospital experience, and 7 patients received alteplase (tPA) 100 mg/2 hours to induce thrombolysis.

The inclusion criteria were:

- Extension of the unperfused lung area $>50\%$, which were verified with spiral CT and/or perfusion lung scan
with
- hemodynamic instability (systolic blood pressure < 90 mm Hg)
or
- with stable haemodynamic with echocardiographic findings such as D-sign, increased right ventricular pressure (>40 mm Hg), and elevated troponin I levels (>0.11 ng/l).

Exclusion criteria:

- Patients who declined to give consent

or

- advanced malignant disease
- or
- absolute contraindication to thrombolysis.

A single 5000 IU intravenous bolus of unfractionated heparin (UFH) was administered to patients with suspected APT if the patient had not already received low-molecular weight heparin previously. The patients received immediate oxygen therapy at 50% or 100% with a face mask. Morphine was given as IV bolus of 2 mg for pain relief. Patients with circulatory shock received inotropic support according to their requirement to keep the mean arterial pressure at 70–80 mm Hg. Norepinephrine was used at 5–20 µg/min, whereas dobutamine was used at 5–10 µg/kg/min. Dobutamine was added to norepinephrine when norepinephrine had to be used greater than 10 µg/min. None of the patients required mechanical ventilation. Patients with suspected hypovolemia received 20 ml/kg/30 min crystalloids. Maintenance crystalloid infusion was 1.5– 2 ml/kg/hr. Further supportive treatment was homogenous: pantoprazole 40 mg IV twice a day, and noradrenaline and/or dobutamine were given as positive inotropic drugs if required. In patients treated with streptokinase, UFH infusion was continued according to heparin adjustment nomogram only after thrombolysis, whereas continuous UFH infusion was given simultaneously with alteplase. UFH was continued in the first 48 hours, or therapeutic dose of low-molecular weight heparin was commenced. The effectiveness of thrombolytic treatment was controlled by a second-look spiral CT or perfusion lung scan. If any of the examinations did not verify at least 30% decrease in the size of unperfused lung area after the first treatment cycle, thrombolysis was repeated after 24 hours. If the fibrinogen level had been lower than 2 g/l before the second thrombolytic cycle, fresh frozen plasma was administered. Arterial blood samples were collected before thrombolysis as a BL value and then at 8 hours, 1, 3, 5, and 30 days after thrombolysis to measure plasma-free haemoglobin concentrations and NO consumption by plasma.

Samples from healthy subjects ($n = 28$) were collected to compare plasma-free haemoglobin concentrations and NO consumption by plasma from APT patients with those found in healthy subjects.

Measurement of haemoglobin concentrations

The plasma-free haemoglobin concentrations measured with a commercially available haemoglobin detection kit (Cat.#K013H1, Arbor Assays, Ann Arbor, MI) according to manufacturer's instructions.

NO consumption assay

We used a previously described (14, 24) NO consumption assay to assess NO consumption by plasma samples. Briefly, a solution of 40 μ M DETA NONOate (Cayman Chemical, Ann Arbor, MI) in phosphate-buffered saline (pH 7.4) was prepared in a glass vessel purged with nitrogen inline with an NO chemiluminescence analyser (Sievers Model 280 NO Analyser, Boulder, CO) to produce a steady-state NO signal of about 40–60 mV. This signal is generated by the decay of DETA NONOate and the release of NO from DETA NONOate, thus producing a stable BI signal. Thereafter, we injected 50 μ l of plasma samples in triplicate, which produced decreases in BI NO signal (NO consumption, mV). Data were analysed using the software program ORIGIN Version 6.1 (Originlab, Northampton, MA), and the area under the curve of decreasing NO signal over time was measured for each plasma sample.

Statistical Analysis

Two-way (embolization vs. time) analysis of variance (ANOVA) and one-way ANOVA for repeated measures followed by Bonferroni post-tests were used to compare hemodynamic or biochemical parameters. The Spearman correlation (r_s , p) was calculated for associations between plasma-free haemoglobin concentrations and NO consumption or other hemodynamic parameters. Plasma-free haemoglobin concentrations and NO consumption by clinical samples were compared by the Student t -test, and the changes induced by thrombolysis in these parameters were evaluated by one-way ANOVA for repeated measures and Bonferroni post-test. The data are shown as mean \pm SEM, and a p value

RESULTS

Experimental embolism studies

In order to examine whether experimental pulmonary embolism increases NO consumption by plasma secondarily to increased plasma levels of free haemoglobin, we carried out measurements in plasma samples from embolized animals. We tested this hypothesis in two different animal models of pulmonary embolism (thromboembolism and microsphere embolism) because thromboembolism could promote increases in plasma-free haemoglobin concentrations different from those found in microsphere embolism.

As expected, lung embolization increased both PVRI and mean pulmonary arterial pressure in both animal models ($p < 0.05$), and these hemodynamic alterations were associated with increased plasma heme concentrations ($p < 0.05$) and NO consumption by plasma samples (p

< 0.05). We found significant correlation between NO consumption by plasma samples and heme concentrations ($p < 0.001$). In parallel with these results, we found significant correlations between PVRI ($p < 0.001$) or MPAP ($p < 0.001$) and NO consumption by plasma samples.

Clinical findings

In order to validate our experimental results, we examined whether clinical acute pulmonary embolism increases NO consumption by plasma in association with increased plasma levels of free haemoglobin. Therefore, we measured these parameters in plasma samples from patients with APT. In agreement with our experimental results, we found higher plasma heme concentrations and NO consumption by plasma samples from patients with APT (both $p < 0.05$) compared with healthy subjects. Moreover, both parameters increased immediately after thrombolysis with alteplase or streptokinase (both $p < 0.05$). Interestingly, these alterations apparently peaked earlier in patients treated with streptokinase (at day 1 after thrombolysis), as compared with patients treated with alteplase (at day 3 after thrombolysis). Again, we found significant correlation between NO consumption by plasma samples and heme concentrations ($p < 0.001$).

Conclusion

Our findings support the suggestion that the physiological control of vascular function may be impaired during acute PE. Our study was the first to show that both experimental and clinical PE increases NO consumption by plasma in association with increased haemolysis. However, our present results expand those previous findings that impaired endogenous NO formation is a mechanism exerting a very relevant protective role in the cardiovascular responses to acute PE and show that haemoglobin decompartmentalization during acute PE consistently increases NO consumption by plasma and may result in lower vascular NO bioavailability. Our results revealed a new mechanism involving increased haemoglobin decompartmentalization and augmented NO consumption, possibly contributing to the hemodynamic derangement of acute PE. Moreover, we showed for the first time that thrombolytic therapy transiently further aggravates this alteration in patients with acute PE. Our findings may have several clinical implications and help to understand complex mechanisms that play role in the hemodynamic changes and pulmonary hypertension associated with acute PE.