

ROS scavenger decreases basal perfusion pressure, vasoconstriction and NO synthase activity in pulmonary circulation during pulmonary microembolism

Mizera Roman^{1, 2}, Hodyc Daniel¹, Herget Jan¹

¹ Department of Physiology, Second Medical School, Charles University in Prague, Prague, Czech Republic, ² Traumatology-orthopaedic center, Regional Hospital Liberec, Liberec, Czech Republic

Short title: ROS scavenger decreases perfusion pressure during pulmonary embolism

Corresponding author:

MUDr. Roman Mizera
Traumatologicko-ortopedické centrum KNL a.s.
Husova 10, Liberec 1, 46063
Email: roman.mizera@nemlib.cz

Summary:

Two mechanisms contribute in the development of pulmonary hypertension in pulmonary embolism (PE) - obstruction of pulmonary blood vessels and vasoconstriction. We hypothesize that hypoxia, increased shear stress and/or activation of gathered leukocytes in the PE may cause a release of reactive oxygen species (ROS). Therefore our aim was to determine the influence of the ROS scavenger Tempol on pulmonary hypertension and to describe NO synthase activity and production of NO oxidative products (NOx) after PE. In general anaesthesia sephadex microspheres suspended in PSS were applied in right jugular vein as the pulmonary microembolism. Then we measured in isolated salt solution-perfused lungs the changes in perfusion pressure, activity of NO synthase and NOx plasma concentration in 7 groups of rats: **C:** control group (n=5), **CN:** C + sodium nitroprusside (SN) (n=5), **EN:** PE + SN (n=5), **ETN:** Tempol + PE + SN (n=5), **CL:** C + L-NAME (n=5), **EL:** PE + L-NAME (n=5), **ETL:** Tempol + PE + L-NAME (n=5). Tempol was applied intraperitoneally before PE. Animals that received Tempol (groups TN, TL) had significantly lower basal perfusion pressure than those which didn't receive Tempol (EN, EL). Overall we measured a higher decrease of perfusion pressure than in the control group (C) after application of SN. Administration of L-NAME after PE (EL) increased the pressure more than in the control group (NL). NOx concentration was higher after PE. We found that preventive administration of Tempol decreases the increase in perfusion pressure after PE. PE increased NO release and concentration of NOx.

Key words: ROS scavenger, pulmonary hypertension, pulmonary embolism, nitric oxide, free radicals

Introduction

Acute pulmonary embolism (PE) is a serious condition with an incidence of 20/1000 per year and is the third most common cause of death from cardiovascular disease after heart attacks and strokes in Europe. It is the most serious post-operative complication, particularly after long bone and joint surgeries. Pulmonary embolism is a significant cause of mortality and morbidity in hospitalized patients (10% deaths during hospitalization) (Torbicki *et al.* 2008).

Successive pulmonary embolism which develops for a longer time leads to an increase in pulmonary vessel resistance, pulmonary hypertension and chronic right side heart failure.

There are two important mechanisms in the development of pulmonary hypertension after PE - obstruction of pulmonary vessels by the embolus and vasoconstriction. Our hypothesis is that radical substances released in the lungs after PE may participate in some cases of PE induced increase of vascular resistance. Hypoxia, increased shear stress and activation of gathered leukocytes may cause the release of reactive oxygen species (ROS). ROS may be responsible for pulmonary vasoconstriction after PE (Stratmann and Gregory 2003).

Therapy of PE is based mainly on the release of the obstruction by thrombus. The first choice of therapy is tPA thrombolysis combined with heparin anticoagulation. In certain cases a surgical method – embolectomy – is indicated (Torbicki *et al.* 2008).

Though experiments with vasodilatation by NO inhalation or by Sildenafil have been performed (Tanus-Santos *et al.* 1999, Dias-Junior *et al.* 2010), the vasodilatory therapy of PE induced pulmonary hypertension has not been implemented in clinical practice. Experimental evidence indicates that models of PE in laboratory animals are

accompanied by oxidant stress of lung tissue (Dias-Junior *et al.* 2010). The key oxygen radical present in various types of lung injury including PE is superoxide.

In our experiments we used Tempol (4 hydroxy-2, 2, 6, 6-tetramethylpiperidin-1-oxyl), which acts as a superoxide dismutase mimetics, for vasodilatation after experimental lung microembolism. Superoxide dismutase decreases the level of superoxide. In the presence of NO and superoxide these substances readily combine, yielding the biologically very reactive peroxynitrite.

In the present study we questioned the hypothesis that superoxide participates in lung vasoconstriction after pulmonary microembolism by Sephadex particles. In the first part of our study we focused on changes in perfusion pressure and the presence of vasoconstriction after PE with preventive application of Tempol. The second part describes observed changes in NO synthase activity and production of NO metabolic products after PE.

Methods

We used 30 male Wistar rats (aged 7-8 weeks, weight 240 ± 20 g). Work with animals was performed in accordance with European Community guidelines and US National Institute of Health guidelines for the use of experimental animals. All parts of the experiment were approved by the Commission for Work with Laboratory Animals of the Second Medical School, Charles University in Prague.

Pulmonary microembolism

The right internal jugular vein was dissected in general anesthesia using a 40 mg/kg intraperitoneal injection of sodium thiopental. Then 0.2 ml saline solution, which contained 320 mg of Sephadex microspheres (G-26, Superfine per kilogram of body

weight), was applied into the exposed jugular vein. The microspheres had been suspended in the PSS for 12 hours before the application in order to enlarge their diameter by swelling (final particle size was 15 – 50 μm). The heart-lung block was isolated twenty minutes after embolization and the preparation of isolated perfused lung was established (Herget *et al.* 1982).

Preparation of isolated perfused rat lungs

After thoracotomy, the lungs were ventilated by a normoxic gas mixture (21% O₂, 5% CO₂, 74% N₂) by tracheal tube with the following ventilation parameters: PEEP 2 cm H₂O, peak inspiratory pressure 10 cm H₂O. An inflow cannula was inserted into the pulmonary artery and an outflow cannula into the left heart chamber, the lungs had been perfused with saline perfusate containing albumin (4g/100 ml) at a constant flow rate (4ml/min/100g). Isolated lungs were placed into a heated humid chamber. The outflow cannula was set up into a circle through a perfusate reservoir. The perfusion pressure was measured in the inflow cannula. After 20 minutes of stabilization, the changes in pressure corresponded to changes in the pulmonary vascular resistance (Herget and MC Murtry 1987, Hampl and Herget 1990).

Experimental protocol (*Fig. 1*)

Experimental protocol included 4 parts:

1. Basal perfusion pressure

In this part of the experiment, animals were divided into 3 groups: C – control group (n=10), E – pulmonary embolization had been induced in the animals before the isolation of the lungs (n=10), ET – similar as E but the animals had received

50mg/kg of Tempol intraperitoneally before the embolization (n=10). The basal perfusion pressure was measured after 20 minutes of stabilization.

2. Presence of vasoconstriction

Groups of animals were analogous to the previous part: CN – control group (n=5), EN - pulmonary embolization had been performed on the animals before the isolation of the lungs (n=5), ETN - the animals had received 50mg/kg of Tempol intraperitoneally before the embolization (n=5). The isolation of the lungs was followed by a stabilization period of 20 minutes. After reading basal perfusion pressure, 0.5 ml 20uM solution of sodium nitroprusside was added to the perfusate in each group and the basal perfusion pressure rate was noted after 10 minutes. The difference between perfusion pressure before and after sodium nitroprusside reflects the pulmonary vascular tonus.

3. NO release

Rats were divided again into 3 groups: CL – control group (n=5), EL - pulmonary embolization had been performed on the animals before the isolation of the lungs (n=5), ETL - the animals had received 50mg/kg of Tempol intraperitoneally before the embolization (n=5). After the isolation of the lungs and stabilization, the L-Name NO synthase inhibitor was added to the lung perfusate (final concentration 50uM in perfusate). After 10 minutes, perfusion pressure was measured again – the difference between these pressures reflects the effect of block of NO production on pulmonary vasculature.

4. NO oxidation products (NOx) concentration in plasma

Before the embolization, venous blood samples from the left subclavian vein were taken from 11 animals. PE was performed exactly as described in the protocol above,

followed by 20 minutes stabilization. Subsequently, similar venous blood samples were obtained. They were centrifuged for 3 minutes at the speed of 5000 RPM and the acquired plasma was deeply frozen. An NO analyser was used to analyse NOx (Sun *et al.* 2010, Hodyc *et al.* 2012).

Statistical Analysis

All data are shown as mean \pm SE and analysed by Statview software with ANOVA or ANOVA repeated measures and Fisher's PLSD posthoc test. Where indicated a paired t test was used. Differences were considered statistically significant when $p < 0.05$.

Results

Tempol decreases basal perfusion pressure after PE

In lungs isolated from rats that had PE (E), the perfusion pressure was significantly higher than in the control group (C). Animals that received Tempol before the embolization (ET) had significantly lower basal pressure than the animals with PE without a previous Tempol application (E). Basal perfusion pressure did not differ significantly between the control group (C) and animals treated with Tempol (ET) (Fig.2).

Presence of vasoconstriction

In the group with PE (EN), we observed a significantly higher decrease in perfusion pressure after application of sodium nitroprusside than in the control group (CN). Vasodilatation induced by nitroprusside in the group with Tempol application (ETN) was similar as in controls (C) (Fig. 3).

Increased activity of NO synthase after PE

Using similar groups as in the previous experiments, the changes of perfusion pressure were measured 10 minutes after application of L-Name. Significantly bigger increase in perfusion pressure after L-Name administration was found in the group which contained PE exposed animals (EL) than in the control group (CL) and in rats that received Tempol prior to the embolization (ETL) (*Fig. 4*).

Increased concentration of NOx in plasma after PE

In all the animals with PE, concentration of NOx measured after PE was higher (mean 24.62 μM) than before PE (mean 22.3 μM). The concentration of NOx before and after PE was compared separately for each animal and evaluated by paired t-test.

Discussion

The main conclusions of our study are the following:

Vasoconstriction which develops after PE is partly caused by an increase of ROS production. After PE, the activity of NO synthase increases and the production of NO rises; application of Tempol lowers the activity of NO synthase.

Our experiment clearly indicates the possible role of ROS in pulmonary hypertension after acute PE, how ROS is generated after PE has not yet been clarified as well as the actual nature of all ROS which cause vasoconstriction of pulmonary vessels.

Possibilities of ROS generation after PE

All cell elements involved in the process of pulmonary hypertension after PE (thrombocytes, leukocytes, endothelial cells and vascular smooth muscle cells)

participate in the production and release of reactive oxygen species during PE. There are four hypothetical ROS generation mechanisms after acute PE: increased shear stress on the wall of pulmonary vessels in the non-embolized part of pulmonary circulation, decreased shear stress in the embolized part behind the embolus, release from activated leukocytes and finally hypoxia.

There is an increase in mechanical pressure on the wall of pulmonary vessels in non-embolized regions of pulmonary circulation by preserved cardiac output. The result of increased shear stress is the upregulation of NAD(P)H oxidase subunit Nox-1, which is found in the cytosol of smooth muscle cells of the pulmonary vessel wall. It also leads to an increase in expression of membrane subunit p47phox and its translocation on the smooth muscle cell membrane (Grote 2003). NAD(P)H oxidase produces superoxide. In the regions which have been cut out of the circulation by embolization, the level of the shear stress drops down. This leads to the upregulation of NAD(P)H oxidase and production of superoxide similarly as in the regions with increased shear stress (Fisher *et al.* 2002). In the smooth muscle cells of non-perfused pulmonary vessels, the intracellular level of Ca^{2+} rises, which leads to a conversion of xanthine dehydrogenase to xanthine oxidase, which produces ROS (Test *et al.* 1984, Fisher *et al.* 2002).

Numerous experimental studies have proven an increase in ROS during hypoxia (Liu 2003). Furthermore, the upregulation of NAD(P)H oxidase is partly responsible for the increase of ROS in hypoxia (Gupte *et al.* 2005). Another source of ROS in hypoxia is the mitochondrial electron transport chain (Waypa and Schumacker 2005).

Possible roles of ROS in vasoconstriction after PE

Reactive oxygen species have oxidation potential, they oxidize cysteine and methionine residues of voltage-gated potassium channels and so cause changes in their conformation. Inhibition of these channels leads to the depolarization of cell membranes of smooth muscle cells in the pulmonary vessel wall, which is followed by activation of L-type Ca²⁺ channels. The next step is Ca²⁺ ion influx and pulmonary vessel vasoconstriction (Moudgil *et al.* 2005).

The relationship between ROS and NO plays the key role in the generation of vasoconstriction. Superoxide reacts with NO to form peroxynitrite. This reaction occurs very quickly, four times faster than the reaction of superoxide with superoxide dismutase. The peroxynitrite which is created has a vasoconstrictor effect (Belik 2004).

The second possible pathway of increase in pulmonary vascular tonus after lung microembolism is the Ca²⁺ sensitization through ROS-dependent activation of RhoA/Rho kinase signaling (Jernigan *et al.* 2008, Shimokawa and Satoh 2014).

ROS activate matrix metalloproteinases (MMPs) (Grote 2003). MMPs have a significant effect when activated chronically – they induce remodeling of pulmonary vessel walls and finally pulmonary hypertension. After acute PE, activated MMPs are able to break down big endothelin-1 to activated endothelin-1, which has a strong vasoconstrictor potential (Van den Steen *et al.* 2002, Fernandez-Patron *et al.* 2001). The preventive application of an MMP inhibitor reduces hemodynamic changes after acute PE (Palei *et al.* 2005).

Conclusion

Presented results may help to obtain the insight in the pathogenesis of changes of pulmonary hypertension after the multiple pulmonary microembolism.

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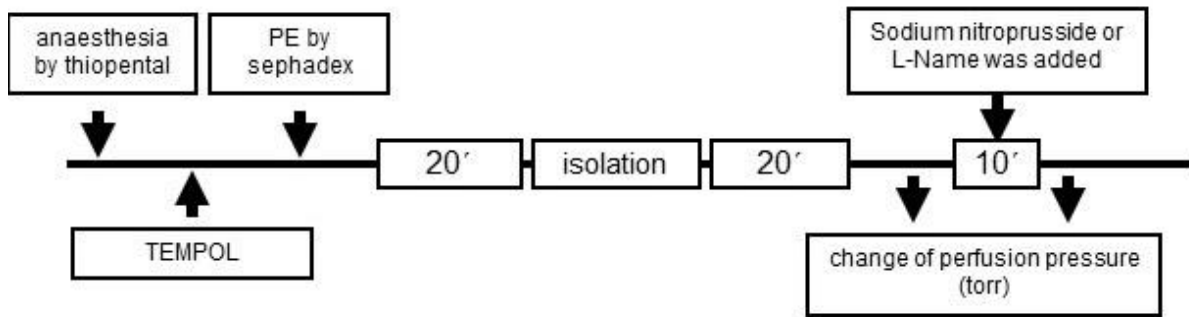


Fig. 1. Experimental protocol

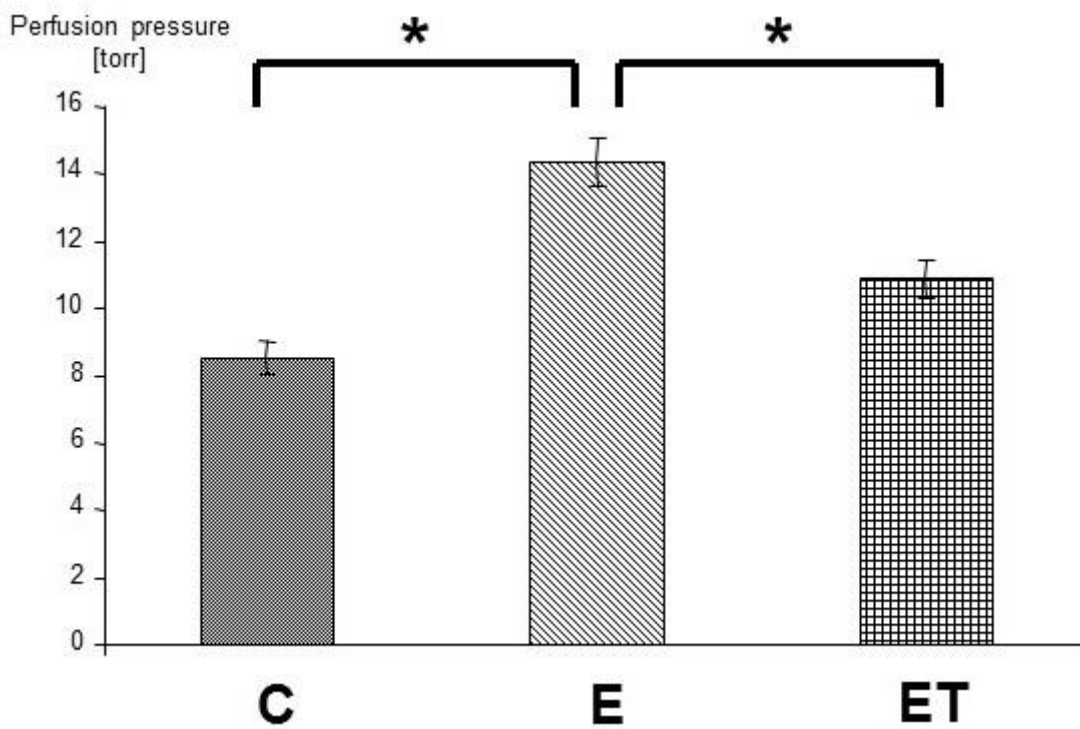


Fig. 2. Tempol decreases basal perfusion pressure after PE. (* $p < 0.01$)

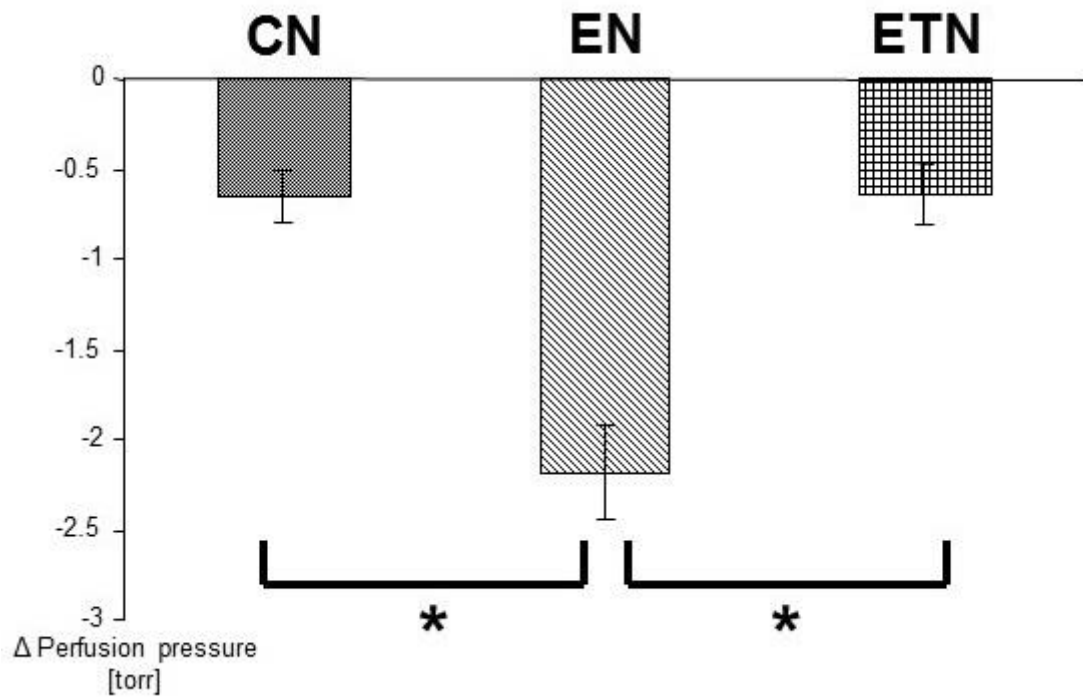


Fig. 3. Effect of sodium nitroprusside on perfusion pressure (higher vasodilatation in EN than in CN group) illustrates the increase of vasoconstriction after PE (* $p < 0.05$)

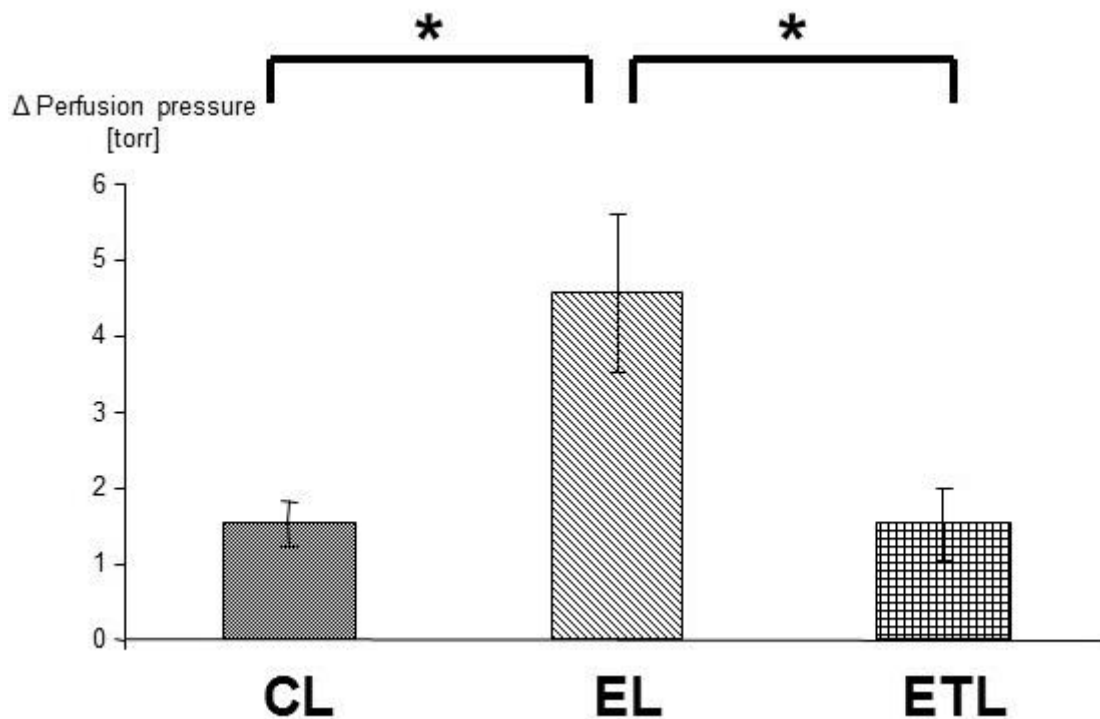


Fig. 4. Higher increase of perfusion pressure after L-Name administration in rats with PE (EL) is caused by higher activity of NO synthase after PE (* $p < 0.01$)