Increased neutrophil mediator release in patients with pulmonary hypertension – suppression by inhaled iloprost

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Summary
Polymorphonuclear neutrophils (PMN) have been implicated in various vascular inflammatory processes. We isolated PMN from venous blood samples of 10 patients with severe primary pulmonary arterial hypertension (PPH), 7 patients with pulmonary hypertension secondary to chronic thromboembolism (CTEPH), and 12 healthy controls. When stimulated with the calcium-ionophore A23187, platelet activating factor (PAF) or the microbial agent N-formyl-Methionyl-Leucyl-Phenylalanine (fMLP), significantly increased release of elastase and superoxide anion was noted in both groups with pulmonary hypertension. Moreover, the neutrophils of CTEPH patients responded with an enhanced liberation of leukotriene (LT) B\textsubscript{4} and 5-hydroxyeicosatetraenoic acid (5-HETE). Inhalation of aerosolized iloprost (5 µg) caused a rapid decline in pulmonary vascular resistance, in both PPH and CTEPH. This hemodynamic response was paralleled by a significant suppression of ionophore- and ligand-induced elastase and superoxide release, as well as LT\textsubscript{B4} and 5-HETE formation. The neutrophil inhibitory effect of the inhalation maneuver was fully reproduced by in vitro incubation of neutrophils with 1-10 pg/ml iloprost for 2 hours. This is the first study to demonstrate that circulating neutrophils from patients with PPH and CTEPH possess an enhanced readiness to respond with inflammatory mediator generation to different stimulatory agents ex-vivo, and that PMN respiratory burst, elastase secretion and leukotriene generation are promptly reduced by an iloprost inhalation maneuver. Neutrophils might participate in the inflammatory processes in pulmonary arterial hypertension.

Keywords
Inflammation, prostaglandins, leukocytes, lung embolism, remodeling

Introduction
Polymorphonuclear neutrophils (PMN) represent an important phagocytic effector cell population at the interface between pulmonary vasculature, lung parenchyma and external environments, playing a pivotal role for pulmonary host defense competence (1). The capacity of these cells to release large quantities of superoxide anions, elastase and lipid mediators, in particular 5-lipoxygenase products of arachidonic acid (AA), is essential for lung antimicrobial defense, but has also been implicated in lung vascular inflammatory and remodeling processes, such as those underlying acute and chronic pulmonary arterial hypertension (2, 3). Superoxide anions and neutrophil elastase may directly provoke constriction of pulmonary arterial smooth muscle cells, independent of endothelial cells. Human neutrophil elastase may cause rearrangement of extracellular matrix and the release of growth factors and further soluble agents, similar to increased vascular elastolytic activity.
activity due to the secretion of endogenous vascular elastase (EVE) (6-10, 11). The secretory lipid mediators leukotriene (LT) B4 and 5-hydroxyeicosatetraenoic acid (HETE) both possess strong neutrophil chemotactic activity, and have been implicated in the development of chronic pulmonary arterial hypertension. Inhalation of nebulized prostaglandin I2 or its long-acting analogue iloprost has been accepted for treatment of acute respiratory failure and chronic pulmonary arterial hypertension (12-17). In several in vitro studies, iloprost was noted to reduce neutrophil-induced lung injury, neutrophil adherence to endothelial monolayers, sequestration and aggregation of leukocytes and platelets as well as coagulation cascade activations (16, 18-21). In the present study, we characterized secretory neutrophil functions in patients suffering from severe primary (PPH) and secondary (chronic thromboembolic (CTEPH)) pulmonary hypertension. Additionally the impact of iloprost inhalation on these functions was investigated. In essence, evidence for enhanced PMN inflammatory mediator release in both PPH and CTEPH was obtained, and dampening of this release response by inhalation of aerosolized iloprost was consistently noted.

Methods

Study populations
Patients with PPH and CTEPH were included in this study. Diagnostic procedures preceding diagnosis included chest x-ray, lung function testing, echocardiography, spiral and high resolution CT-scan of the lung, in some cases pulmonary angiography, clinical chemistry and immunological analysis. On the basis of these results, patients were classified as PPH or CTEPH according to the criteria of the PPH World Conference in Evian. Patients in the PPH and CTEPH group were on chronic treatment with aerosolized iloprost (inhalaion of 5 µg iloprost [dose determined at the mouthpiece] within 4 min by ultrasonic nebulization, 6-9 times per day). Baseline blood samples were collected in the morning before starting the first iloprost inhalation, after a pause of at least 9 h during nighttime. For comparison, an iloprost-naive patient group with both primary and secondary pulmonary arterial hypertension was included, not being previously treated with chronic iloprost inhalation. None of the patients was chronically treated with inhaled nitric oxide. The test protocol for blood sampling before and at preset time intervals after inhalation of iloprost was approved by the local ethics committee of the Justus-Liebig University, and informed consent was obtained from all patients. For control, additional blood samples were taken from healthy people.

Figure 1: Neutrophil elastase (A) and superoxide anion (B) release in PPH, CTEPH and healthy controls. Neutrophils were isolated from venous blood samples of patients with primary pulmonary arterial hypertension (PPH; n=10) and chronic lung embolism (CTEPH; n=7) and of healthy donors (control; n=12). In vitro stimulation was performed with the calcium ionophore A23187 (1µM) and the receptor-operated ligands fMLP (1µM) and PAF (5µM) (10 min each). Data are given as box and whisker plots. The Student-Newman-Keuls test was used as à posteriori test for linear contrasts (#, p < 0.05).
Isolation of human neutrophils

Neutrophils were isolated from patients with pulmonary arterial hypertension as well as healthy donors. Following the standard protocol for patients, PMN were isolated before and 15 minutes after iloprost aerosolization (inhalation of 5 µg iloprost [dose determined at the mouthpiece]). EDTA-anticoagulated venous blood samples were layered over Ficoll-Paque and centrifuged at 400 x g for 35 min, as previously described (22). Cell purity was > 97%, as quantified by flow cytometry, and cell viability was > 96%, as assessed by trypan blue dye exclusion.

Measurement of leukotrienes

LTs and HETEs were extracted from cell supernatants by octadecylsilyl solid-phase extraction columns and detected by the use of conventional UV and photodiode array, as described (23). All data obtained by the different analytical procedures were corrected for the respective recoveries.

Superoxide anion generation

Neutrophil O$_2^-$ generation was assessed as superoxide dismutase-inhabitable reduction of cytochrome $c$ according to Cohen (24).

Release of granular constituents

Elastase was taken as marker for neutrophil degranulation, and enzyme activity in the cell supernatant was measured by monitoring the turnover of L-pyroglutamyl-L-propyl-L-valine-p-nitro-anilide at 405 nm according to standard procedures (25).

Statistics

One-way analysis of variance (ANOVA) was employed to evaluate differences between neutrophil functions. The Student-Newman-Keuls test was used as à après test for linear contrasts. The significance level for the test was set at $p = 0.05$. For each patient group, the response to iloprost inhalation was considered significant if for the 95% confidence interval ($p < 0.05$), the 99% confidence interval ($p < 0.01$) or the 99.9% confidence interval ($p < 0.001$) the pre- and post-exposure difference did not overlap with zero. Differences between baseline hemodynamics and gas exchange parameters in the PPH and CTEPH group were not observed.

Results

Demographics and hemodynamic data

The mean age of the control-, PPH-, CTEPH- and iloprost-naive group was 32, 44, 53 and 62 years, respectively. The average weight was 66, 64, 69 and 71 kg. All patients suffered from severe pulmonary arterial hypertension, and fulfilled the criteria of New York Heart Association (NYHA) classes III and IV. Baseline mean pulmonary artery pressure (PAP) was 58 ± 13, 54 ± 12 and 51 ± 8 mmHg, and mean cardiac index (CI) was 2.1 ± 0.6, 2.1 ± 0.5 and 2.2 ± 0.5 l/min/m$^2$, in the PPH, CTEPH and iloprost-naive patients, respectively. Pulmonary vascular resistance index (PVRI) values were calculated as 2299 ± 1173, 2040 ± 816 and 1723 ± 627 dyne*s*cm$^{-5}$m$^{-2}$ (mean +/- SEM). In response to iloprost inhalation, a decrease in PAP of > 10 mmHg was noted in all groups, measured 15 min after

Figure 2: Neutrophil secretion of 5-lipoxygenase products of arachidonic acid (AA) in PPH, CTEPH and healthy controls. In vitro stimulation of human neutrophils was performed with the receptor-operated ligand fMLP (1µM) in the presence of free AA (10 µM) for 10 min. Data are given for LTB$_4$, 5-HETE and the summed LTA$_4$ decay products, depicted as box and whisker plots. The Student-Newman-Keuls test was used as à posteriori test for linear contrasts (#, $p < 0.05$) (PPH [n=10], CTEPH [n=7], and control [n=12]).
iloprost inhalation, the cardiac index increased consistently, and the PVRI values declined by 30 - 40%. A decline in the PVRI of more than 25% was considered as significant.

**Neutrophils from PPH and CTEPH patients respond with enhanced mediator release**

Incubation of neutrophils from the PPH-group with the calcium ionophore A23187 and the receptor-operated ligands fMLP and PAF caused a rapid degranulation, with an elastase release that was significantly higher than that of the neutrophils isolated from the CTEPH-group and the healthy donors (Fig. 1A). In addition, the elastase values in the CTEPH-group significantly surpassed those of the control group in case of A23187 and fMLP stimulation.

When assaying the respiratory burst in response to fMLP challenge, a markedly enhanced $O_2^-$ release was noted in both groups with pulmonary arterial hypertension as compared to the healthy controls (Fig. 1B).

Incubation of neutrophils isolated from healthy donors and the PPH group with fMLP in the presence of 10µM free arachidonic acid provoked the release of large amounts of LTB₄ and 5-HETE (Fig. 2). In addition, decay products arising from the non-enzymatic hydrolysis of LTA₄ were detected (6t-LTB₄, 6t,12e-LTB₄, 5S,6R-DiHETE, 5S,6S-DiHETE, summed up as LTA₄-decay). Interestingly, the secretory response was significantly more prominent in neutrophils originating from patients with CTEPH as compared to the healthy donor group in case of LTB₄ and 5-HETE. Similar to
fMLP, the receptor operated ligand PAF and the calcium ionophore A23187 provoked the liberation of substantial quantities of LTB₄, 5-HETE and LTA₄-decay products, however, with no significant difference being detected between PPH patients, CTEPH patients and the healthy controls (data not given in detail).

Effect of nebulized iloprost on circulating neutrophils

For testing the effect of iloprost administration on the agonist-induced neutrophil functions, the secretory mediator response was additionally studied in neutrophils collected after inhalation of this prostanoid in patients with PPH and CTEPH. In both groups, a marked suppression of the release response of elastase and all 5-lipoxygenase products after preceding iloprost nebulization was noted (Fig. 3). This was true for stimulation with the ionophore A23187 as well as the receptor operated ligands PAF and fMLP and was detected in both PPH and CTEPH patients. Notably, the suppressor effect surpassed 50% in some of these constellations, as e.g. the PAF induced liberation of 5-HETE, which declined by ≈ 55% in CTEPH and by ≈ 80% in PPH patients after a preceding iloprost inhalation.

In the naive patients not undergoing prior iloprost inhalation, both the initially enhanced elastase and leukotriene release and the suppressive effect of iloprost were fully reproduced (Fig. 4), indicating that chronic iloprost treatment does not affect the reactivity of neutrophils to acute iloprost inhalation.

Iloprost induced suppression of neutrophil inflammatory mediator release – in vitro studies

Neutrophils of healthy donors were incubated in the absence and presence of various concentrations of iloprost for 2 hours. Subsequently, stimulation with A23187 and fMLP was performed, and the liberation of inflammatory mediators was quantified. A significant suppression of the release response was noted for elastase, superoxide anion and all 5-lipoxygenase products, both for challenge with A23187 and fMLP (Fig. 5AC). Interestingly, 1 pg/ml iloprost sufficed to achieve a maximum suppressor effect on superoxide anion and elastase liberation. Maximum efficacy in reducing the synthesis of 5-lipoxygenase products was noted for ≈ 10 pg/ml iloprost, for all metabolites investigated. In control experiments, a 2 hour iloprost incubation period, undertaken in the absence of A23187 or fMLP challenge, did per se not provoke any activation of the neutrophils (data not shown).

Discussion

The current study provided evidence that neutrophils isolated from venous blood samples of patients with severe primary and
Figure 5: Dose-dependent suppression of neutrophil superoxide production, degranulation and leukotriene synthesis by incubation with iloprost in vitro. Neutrophils originating from healthy donors were incubated with different concentrations of iloprost or sham-incubated for 2 h. Subsequently, stimulation with A23187 or fMLP (1 µM each) was performed for 10 min. Data for the release of superoxide anion (5A), elastase (5B) and 5-lipoxygenase products (LTB₄, 5-HETE and LTA₄) (5C) are given (mean +/- SEM, n = 4 independent experiments each). #, p < 0.5 as compared to sham-incubated controls.
secondary pulmonary arterial hypertension possess an enhanced readiness to respond with inflammatory mediator release when challenged with different stimulatory agents. When sampling the neutrophils subsequent to an iloprost inhalation maneuver in these patients, significant decrease in the PMN inflammatory mediator release was noted. This inhibitory effect was reproduced by incubation of neutrophils from healthy volunteers with iloprost in vitro, thus suggesting a direct effect of nebulized iloprost on circulating neutrophils.

When addressing elastase secretion from neutrophils in response to calcium-ionophore challenge and stimulation with the receptor-operated ligands fMLP and PAF, the values obtained for PMN originating from PPH patients consistently surpassed those from PMN of healthy volunteers by ≥ 2-fold. Elastase levels of neutrophils of CTEPH patients were less impressively elevated, but still ranged consistently higher than those of the healthy controls. Concerning fMLP evoked superoxide formation, the levels in both PPH- and CTEPH-neutrophils surpassed those in control PMN by ≥ 2-fold. In contrast, such readiness for enhanced inflammatory mediator release was less obvious for the neutrophil 5-lipoxygenase product formation. As to LTB4 and 5-HETE, enhanced levels were noted for CTEPH-, but not for PPH-neutrophils, and the sum of LTA4 decay products did not differ between controls, PPH patients and CTEPH patients.

What reasons may underlie the enhanced elastase and superoxide anion release of neutrophils from PPH and CTEPH patients, as compared to PMN from healthy controls? Differences in age and gender may be excluded, as gender did not largely differ between groups, and detailed data analysis within the groups of patients did not show any age dependency. Some of the patients were long-term treated with calcium channel blockers (felodipine, verapamil or diltiazem within both groups), which has been noted to affect the inflammatory mediator release of neutrophils (26, 27). However, dampening rather than activation of elastase and reactive oxygen species generation is to be expected in the presence of calcium blockers. Moreover, the comparison of data of calcium channel blocker-treated versus non-treated patients did not forward any evidence for a major impact of these agents on the elastase and superoxide release reaction. Also, all patients were treated with heparin for right heart catheterization, which was not the case in the healthy controls. However, several studies support the view that heparin may reduce, but not increase, neutrophil inflammatory mediator generation (28, 29), and may thus not be responsible for the enhanced readiness of PPH- and CTEPH-neutrophils to release elastase and superoxide. Finally, all patients of the PPH- and CTEPH group were long-term treated with daily inhalation of iloprost (6-9 inhalation maneuvers per day). However, as baseline blood samples were collected in the morning before starting the first iloprost inhalation, after a pause of at least 6 h during nighttime, no circulating iloprost levels are to be expected at this time. Moreover, enhanced neutrophil mediator release was also detected in the iloprost-naïve patients, who had not undergone any previous inhalation of this prostanoid. Thus, the difference between the inflammatory mediator release pattern of PPH- and CTEPH-neutrophils and healthy control PMN may not be ascribed to medical interventions in the patients, but is apparently attributable to the underlying disease. It may be speculated that the irregular intravascular architecture, forcing the neutrophils through narrow-lumen pulmonary vessels, or the intimate contact of the PMN with cell activation phenomena in vascular areas undergoing active remodeling, could be responsible for some kind of “priming” of the cells during their passage through the pulmonary vasculature. Alternatively, some primary type of change of neutrophil function in pulmonary arterial hypertension may not be excluded, but is less probable, as functional changes were not only noted in PPH, but also in pulmonary hypertension due to lung thromboembolism, a disease with different etiology. Further studies are mandatory to elucidate the mechanisms underlying the enhanced readiness of neutrophils for superoxide and elastase liberation in more detail.

Inhalation of iloprost resulted in a rapid and marked change in neutrophil inflammatory mediator release, both in PPH and CTEPH patients. Within 15 min, elastase secretion in response to calcium ionophore-, fMLP- and PAF-challenge was found to be consistently decreased, and this was also true for the liberation of LTB4, 5-HETE and LTA4. Moreover, a marked suppression of the neutrophil respiratory burst was noted. In contrast, iloprost did neither reduce the release of the cyclooxygenase product thromboxane nor the liberation of the inflammatory cytokines interleukin-8 and tumor necrosis factor α, indicating directed modulation of neutrophil mediator release by iloprost (data not given). When asking for the underlying mechanisms of this prompt effect of nebulized iloprost, several aspects are to be considered. First, iloprost inhalation might alter the quantity and/or composition of the circulating leukocyte population, e.g. by some enhanced “washout” of the lung intravascular leukocyte pool due to the iloprost-induced pulmonary vasodilation. However, sequential collection of arterial and venous blood samples after iloprost inhalation did not show any significant change in the number of circulating leukocytes or the composition of the white cell pool, including e.g. neutrophilic versus non-neutrophilic cells, and mature versus immature forms within the PMN pool. Second, the change in neutrophil functional behavior after iloprost inhalation might be secondary to the marked changes in hemodynamics in response to this agent, with a decrease in pulmonary artery pressure and lung vascular resistance and an increase in cardiac output. Though such impact may not be excluded, the studies investigating in vitro neutrophil incubation with iloprost strongly argue against such view: the post-inhalation changes in neutrophil inflammatory mediator release were fully reproduced in these in vitro experi-
ments, with approximate halving of the elastase and superoxide secretion and the liberation of all 5-lipoxygenase products of AA in the optimum iloprost concentration range. This range was ≈ 1-10 pg/ml iloprost, offered for 2 h in vitro. This corresponds well to the recent finding that in patients with iloprost nebulization peak post-inhalation plasma levels range at ≈ 150 pg/ml, leveling off with plasma half-lives of ≈ 9 min over the subsequent 1-2 h (30). Third, long-term effects of daily repetitive iloprost inhalation might trigger some “sensitization” to this type of intervention, thus provoking a strong response to each individual inhalation maneuver which would not be present in non-treated patients (31). However, as mentioned above, a comparable effect of iloprost inhalation was also observed in the “naive” patients, not having undergone any previous prostanoid administration (Fig. 4). And fourth, inhaled iloprost might affect various target cells, e.g. alveolar epithelial cells, macrophages in the alveolar space, and endothelial cells in the intravascular compartment. Neutrophils might thereby be affected in an indirect fashion, via juxtacrine mechanisms. Though not excluding such sequence of action of nebulized iloprost, the mimicry of the alterations of neutrophil function by incubating PMN with iloprost in vitro, again favors a direct impact of the aerosolized drug on the neutrophils.

Such direct impact is well conceivable, as iloprost was previously shown to increase cyclic AMP levels in neutrophils in vitro (20), and may thereby exert various downstream inhibitory effects on inflammatory leukocyte functions. Correspondingly, suppression of neutrophil aggregation, beta integrin expression, adherence to endothelial cells and respiratory burst was previously noted in the presence of iloprost (20, 21).

In conclusion, this is the first study to demonstrate that i) circulating neutrophils from patients with PPH and CTEPH possess an enhanced readiness to respond with inflammatory mediator generation to different stimulatory agents, and that ii) such mediator generation (respiratory burst, elastase secretion, lipid mediator synthesis) is promptly decreased by one iloprost inhalation maneuver. Although these data demand further elucidation with the aim to specify the role of PMN in chronic pulmonary arterial hypertension and the vascular remodeling process, it may be considered, that activated neutrophils participate in the pathogenetic sequelae of this disease. Moreover, the beneficial long-term effects of daily repetitive iloprost inhalation (13) might in part be related to the impact of the drug on intravascular leukocytes. This is well imaginable for PMN elastase and superoxide release, repetitively shown to be involved in different types of inflammatory and remodeling processes in the lung vasculature (2, 9, 10). LTβ4 and 5-HETE may promote additional leukocyte recruitment, and PMN-derived LTβ4 undergoes avd transcellular metabolism in the lung vasculature with the appearance of cysteinyl-leukotrienes (32), which again possess strong vasoconstrictor and promitogenic potency.

References