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Exercise decreases plasma antioxidant capacity and increases urinary isoprostanes of IPF patients

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Summary
We tested whether markers of systemic oxidant stress were detectable in 29 typical IPF patients, and whether these increased after low level exercise. We obtained resting plasma for measurement of amino terminal pro brain natriuretic peptide (NT-proBNP), and plasma and urine samples for isoprostanes and total nitrite. Total antioxidant capacity (TAC) was measured in plasma, and H_2O_2 was measured in urine. Subjects exercised at ~50 W on a semi recumbent bicycle until limited by dyspnea. Samples were obtained immediately after exercise for measurement of the same variables.

Plasma and urine samples were also obtained at rest from 6 normal individuals over 40 years of age solely to establish comparison values for NT-proBNP, nitrite, H_2O_2 and TAC assays.

Plasma NT-proBNP was high at rest and after exercise, suggesting pulmonary arterial hypertension. IPF patients' resting NT-proBNP concentrations apparently exceeded those of normal controls. IPF plasma isoprostanes at rest exceeded the normals. IPF urine isoprostanes increased significantly after exercise ($P = 0.047$ by signed rank test); and, plasma TAC decreased significantly after exercise ($P < 0.001$ by signed rank test). Neither plasma nor urine nitrite changed significantly after exercise. H_2O_2 concentration was quite high after exercise in some IPF subjects' urine.

IPF patients demonstrate systemic oxidant stress at rest detectable as increased isoprostanes in the circulation. An increase in urine isoprostanes and a decrease in plasma TAC after exercise suggest that reactive oxygen species (ROS) are produced during low level exercise done by IPF patients.
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Background

Idiopathic pulmonary fibrosis (IPF) is a progressive, fibrotic lung disease of unknown etiology prevalent in males over 50 years of age.[1,2] Patients with IPF have evidence of oxidant stress within the bronchoalveolar compartment and circulation.[3,4]

Several structurally unrelated antioxidants inhibit bleomycin (BLM)-induced lung injury. N-acetylcysteine (N-Ac) protects rats from bleomycin-induced fibrosis.[5,6] Transgenic mice that over express extracellular superoxide dismutase (EC-SOD) are protected from BLM-induced lung fibrosis.[7] A catalytic metalloporphyrin with SOD and catalase activity also attenuates bleomycin-induced pulmonary fibrosis.[8] Suppression of the transcription factor Nrf-2, which regulates many aspects of the antioxidant response, in mice worsens BLM-induced pulmonary fibrosis.[9] Perhaps most important, N-Ac has been found to minimize significantly the progression of pulmonary function worsening in IPF patients, who were also treated with prednisone and azathioprine.[10] N-Ac has been shown to prevent epithelial-mesenchymal transition of alveolar epithelial cells while repleting cellular glutathione and decreasing reactive oxygen species (ROS) production stimulated by transforming growth factor-beta (TGF-β).[11]

Fluid recovered from IPF lungs by bronchoalveolar lavage (BAL) contains high concentrations of myeloperoxidase (MPO), and alveolar inflammatory cells release increased quantities of superoxide anion (O_2^-) and its dismutation product, H_2O_2.[12] Alveolar ELF GSH concentration was decreased by 77% in IPF patients compared to normal controls (where [GSH] $=$ 429 $\pm$ 34 μM).[13] Suppression of oxidant stress is in part responsible for the apparent clinical effectiveness of N-acetylcysteine.[14]

Oxidative stress may trigger production or activation of growth factors and cytokines and regulate matrix metalloproteinases (MMP) that promote fibrosis.[15] ROS, which may come from activated inflammatory cells, increase release of transforming growth factor-beta (TGF-β) from epithelial cells and activate it. TGF-β activation decreases cellular glutathione, and its activation is a pivotal event in the pathogenesis of fibrosis.[16]

Exercise limitation of IPF patients is also related to a combination of decreased oxygen delivery and increased pulmonary vascular resistance. Hypoxia causes increased O_2^- production by mitochondrial complex III, so H_2O_2 would be anticipated to diffuse from muscle cells if scavenging mechanisms were overwhelmed. An underlying rationale for the present study is therefore that oxidant stress may be detected in the systemic circulation, when IPF patients exercise at a low workload and develop hypoxemia.

We designed this pilot study to measure markers of systemic oxidant stress in clinically typical IPF patients who were to be enrolled in a randomized controlled trial of sildenafil (see http://clinicaltrials.gov/ct2/show/NCT00359736?term=ipf+AND+sildenafil&rank=4). Previous studies have not examined well defined IPF cohorts.[4] Detailed demographic data and results of that trial are reported elsewhere.[17] We hypothesized that classical markers of reactive oxygen species (ROS) in the plasma and urine, including isoprostanes and total antioxidant capacity (TAC) would demonstrate oxidant stress at rest and be increased in IPF subjects by low level exercise. We measured amino terminal pro brain natriuretic peptide (NT-proBNP) and estimated systolic pulmonary artery pressures (PAP_{sys}) by echocardiography to determine if the subjects were at risk of pulmonary hypertension.[18] Our results demonstrated that typical IPF patients, who often have elevated NT-proBNP levels, develop hypoxemia and mild lactic acidosis that is associated with increased urinary isoprostanes and decreased plasma total antioxidant capacity during exercise at a low power output.

Methods

Experimental design

IPF was defined according to the American Thoracic and European Respiratory Societies (ATS-ERS) clinical diagnostic criteria.[1] We studied 29 IPF patients who were to begin a double blind, randomized, placebo controlled trial of sildenafil as a potential therapy for patients with moderate impairment of pulmonary function and mild to moderate increases in echocardiographically estimated systolic pulmonary artery pressure (range 25–50 mm Hg). Patients were studied in this protocol before they received the experimental drug or placebo. Subjects did not undergo right heart catheterization. Pulmonary function tests, including arterial blood gases, were obtained before entry into the trial. Patients were recruited between August 2006 and November 2008. All subjects and normal controls provided written informed consent.

We also obtained plasma and urine samples from six volunteers, who were laboratory employees. Two of the six were female. The average age was 50 $\pm$ 6 (SD) years, and the average weight was 76 $\pm$ 16 (SD) kg. None of the volunteers smoked, and none had any evident pulmonary disease. Plasma and urine samples from the controls were used only to standardize the assays and provide a range of expected values. The controls did not participate in the exercise protocol.

This study was reviewed and approved by the Miami VAHS Institutional Review Board as protocol 4549.04.

Exercise test protocol to quantify dyspnea

Subjects underwent standardized 50-W exercise bicycle challenges immediately before the 6-month drug intervention. We chose this constant low level power output to test endurance. The subjects had also completed 6-min walk tests, which are reported elsewhere.[17] Patients used sufficient supplemental oxygen to maintain pre-exercise oxygen saturation at 90% or greater, only if needed. Exercise was done in the sitting position on a semi recumbent bicycle (Life Fitness 95Ri, Life Fitness USA). It consisted of pedaling at 50–60 revolutions/minute at a constant workload of 50 W. This approximates an energy expenditure of three metabolic equivalents (MET; where 1 MET $=$ 3.5 mL O_2/kg/minute). Exercise continued until patients were limited by dyspnea or fatigue. O_2 saturation, pulse rate, blood pressure and Borg dyspnea score were monitored and recorded before and immediately after pedaling. Total exercise time (seconds) was recorded.

Sample collections

We collected blood and urine samples in the resting state before and immediately after the standardized exercise. Blood samples were collected by venipuncture in two 10 mL tubes containing sodium heparin (BD Vacutainer 366480, Franklin Lakes, NJ). Plasma was separated by centrifugation (1500 × g for 10 min at 4 °C) and multiple aliquots were stored at −80 °C until analysis. Urine samples were stored in multiple aliquots at −80 °C until analysis. Samples were thawed only once before assays.

BNP assays

NT-proBNP was assayed using the automated assay described in detail by Elin and Winter.[19]

Plasma lactate assays

The concentration of lactate in plasma samples obtained before and after exercise was determined using an enzymatic assay as described.[20] Blood samples were placed on ice and plasma was separated as rapidly as possible. Samples were stored frozen at −80 °C until assays were done.

Urine creatinine assays

Urine creatinine was assayed by the clinical laboratory at the Miami VAHS, using an automated assay.

Isoprostane measurements

We quantified F_2-isoprostanes in plasma and urine using high performance liquid chromatography (HPLC) tandem mass spectroscopy (MS—MS) as described in detail.[21] Plasma and urine samples were stored at −80 °C until analysis (although isoprostanes are resistant to degradation during handling). The HPLC system consisted of Agilent Series 110 components (Agilent, Waldbronn, Germany). The HPLC system was interfaced with a triple quadrupole mass spectrometer (ADI 4000, Applied Biosystems, Foster City, CA) run in the negative multiple reaction model. HPLC systems and mass spectrometer were controlled by Analyst software (Version 1.3.1, Applied Biosystems). 500 μL samples were injected. The assay has been validated according to FDA Center for Drug Evaluation and Research Guidelines for bio analytical method validation.

Assays were done at the University of Colorado Health Sciences Center. Normal values for 15-F_2 -isoprostanes have been established by this laboratory for plasma (range 3—25 ng/L; $n = 16$) and urine (55—348 ng/mg creatinine; $n = 16$) using this technique and equipment. These values were used for comparison with the pre- and post-exercise isoprostane data obtained in our subjects.

Nitrate/nitrite assays

We measured total nitrite in plasma and urine as a marker of $^{\bullet}NO$ production at rest and after exercise using a nitrate/nitrite colorimetric assay (Kit No. 780001, Cayman Chemical Company, Ann Arbor, Michigan). Nitrate reductase was used to reduce all nitrate to nitrite before measurement.[22,23] Plasma samples were ultrafiltered through a 10 kDa molecular weight cut-off filter (Amicon) that had been rinsed by centrifugation with ultrapure water. The detection limit of the assay is about 1 μM nitrite. Urine typically contains 200—2000 μM nitrite, and so urine samples were diluted before assay. Urine nitrite concentration was expressed as nmoles/milligram creatinine.

Total antioxidant capacity

We determined the Trolox equivalent total antioxidant capacity (TAC) of plasma before and after each standardized exercise test. This assay quantifies the ability of the sample to inhibit an *in vitro* oxidation assay, and compares the degree of inhibition by plasma to known quantities of Trolox (Antioxidant Assay Kit No. 709001, Cayman Chemical Company, Ann Arbor, Michigan). It relies on the ability of low molecular weight antioxidants in plasma to inhibit oxidation of 2, 2′-azino-di-[3-ethylbenzthiasoline sulphonate] (ABTS) to $ABTS^{+}$ by metmyoglobin. Inhibition of absorption at 750 nm is measured and compared to that of the water soluble tocopherol analog, Trolox. The data therefore reflect the net antioxidant capacity of proteins (*e.g.*, albumin) and small molecules (*e.g.*, GSH, vitamin E, and vitamin C) normally present in plasma.[24]

H_2O_2 assays

We assayed urine H_2O_2 using the xylenol orange detection method for quantifying oxidation of ferrous to ferric ions by H_2O_2 (Urinary Hydrogen Peroxide Assay, Catalog No. 706011, Cayman Chemical Company, Ann Arbor, MI).[25] Catalase was added to duplicate assay wells to confirm the specificity for H_2O_2. Ferric ion binds to the dye xylenol orange to form a stable colored complex. Urine samples with or without catalase were mixed with reagent and rocked at room temperature for 60 min. Absorbance was recorded at 595 nm.

Statistical analyses

Data were expressed as arithmetic means ± SD or medians with [25—75th] percentiles indicated, if the distribution of values were non Gaussian. Pre- and post-exercise BNP and markers of oxidant stress were compared using the signed rank test (i.e., pre- and post-exercise data were paired). Comparisons of IPF pre-exercise values to normal controls were not tested statistically, because the groups were not studied contemporaneously. Data from the control groups are included in the figures to illustrate the distribution of the data. In *post hoc* exploratory analyses, Pearson product moment correlations were calculated to find whether any of the plasma or urine markers of oxidant stress correlated with the post-exercise (nadir) S_aO_2 or the decrease in S_aO_2 during exercise. Statistical calculations were done using SigmaStat (Systat Software, San Jose, CA).

Results

Characteristics of the subjects and controls

Demographic and physiological characteristics of the 29 IPF subjects are given in Table 1. Normal controls recruited from our laboratory staff were in apparently good health. We used plasma and urine from these individuals to validate the assay protocols in our laboratory. By design, normal controls did not complete the exercise protocol. Only data from volunteers who were over 40 years of age (an entry criterion for the study) are shown here. Those individuals ($n = 6$; 2 were female) averaged 50 ± 6 (SD) years of age.

Standardized exercise tests

Twenty-nine IPF subjects exercised in the sitting position for 222 ± 170 (SD) seconds on the recumbent bicycle at a constant workload of 50 W. Exercise was limited by dyspnea or fatigue, i.e., the subjects chose when to stop. No adverse events occurred during exercise. Blood and urine samples were obtained immediately before and after exercise as described above.

Plasma lactate assays and oxygen saturation data

The 29 subjects' median [25—75th percentiles] plasma lactate concentration before exercise was 1.4 [1.1—2.0], and it increased significantly after exercise to 2.3 [2.0—2.9] mM ($P < 0.001$ by signed rank test). The median S_aO_2 before exercise was 97 [96—98] %, and after exercise it decreased significantly to 91 [88—95] % ($P < 0.001$ by signed rank test).

NT-proBNP assays

The pre-exercise median NT-proBNP [25—75th percentiles] was 108 [48—329] ng/mL, and the post-exercise median was 112 [51—314]. Some subjects' NT-proBNP levels increased substantially after exercise. Normals' ($n = 6$) NT-proBNP levels were 36 [23—53] pg/mL. These data are shown in Fig. 1. A number of individuals in this study had NT-proBNP levels (after conversion to the equivalent BNP concentration) in the range that Leucthe et al. report for IPF patients with pulmonary hypertension.[18]

Urine creatinine assays

The subjects' median urine creatinine concentration [25—75th percentiles] was 114 [60—179] mg/dL and the normals' urine creatinine concentration was 85 [53—179] mg/dL.

Plasma isoprostane assays

Isoprostanes are produced *in vivo* by free radical catalyzed oxidation of membrane bound polyunsaturated fatty acids.[26] IPF plasma isoprostane values did not change significantly after exercise, but they clearly exceeded the normal control range for the assay. These data are shown in Fig. 2.

Table 1 Baseline characteristics of the subjects.

Variable	Overall mean at baseline ($n = 29$)
Male gender	23
Age (years)	70±9[a]
Months after diagnosis	24 ± 12
Previous smokers	25 (86%)[b]
Oxygen use	15 (52%)
6-MWD (meters)	347 ± 71
Bicycle exercise time (seconds)	220 ± 170
Borg dyspnea index before exercise	1.0 ± 1.4
Borg dyspnea index after exercise	3.9 ± 1.9
FVC (% predicted)	62 ± 12
FEV_1/FVC (ratio)	0.87 ± 0.14
DLCO (% predicted)	42 ± 9
TLC (% predicted)	61 ± 10
PaO_2 (mm Hg)	81 ± 10
SaO_2 (mm Hg) before exercise	97 ± 2
SaO_2 (mm Hg) after exercise	92 ± 5

[a] Data are means ± SD.
[b] Data are numbers of individuals (percent of total).

Urine isoprostane assays

IPF urine isoprostane concentrations increased significantly after exercise ($P = 0.047$ compared to pre-exercise by signed ranks test). These data are shown in Fig. 3. Increased post-exercise urine isoprostanes correlated significantly with decreased S_aO_2 after exercise ($r = -0.434$; $P = 0.0438$).

Plasma total antioxidant capacity assay

After standardized bicycle exercise, the IPF plasma TAC decreased significantly, demonstrating increased systemic

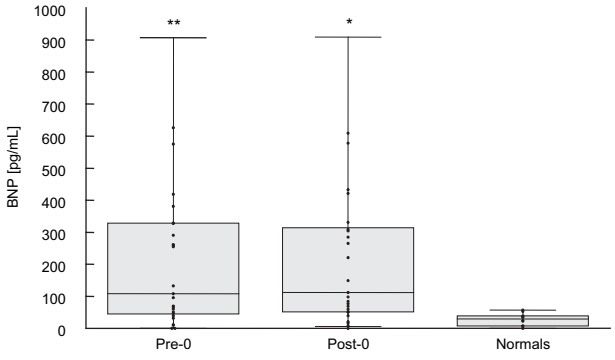

Figure 1 Plasma BNP concentrations before and after exercise. Plasma NT-proBNP values are shown as the concentrations in pg/mL. The median, 25—75th percentiles (box) and 5—95th percentiles (whiskers) for 29 IPF subjects are shown. The corresponding values in plasma established from 6 normal controls averaging 50 years of age are shown by the box-whisker plot on the right. Pre-0, before exercise; post-0, after exercise.

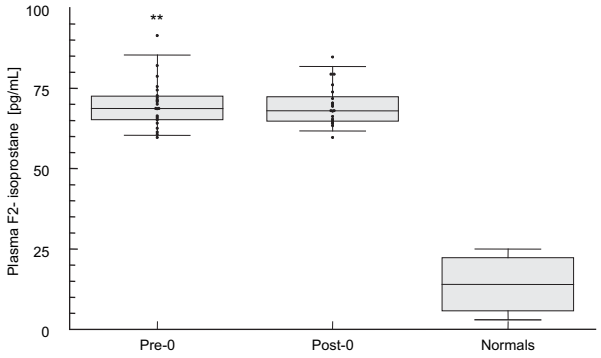

Figure 2 Plasma isoprostane concentrations before and after exercise. Data shown are plasma isoprostane concentrations in pg/mL. The median, 25—75th percentile (box) and 5—95th percentiles (whiskers) for 29 IPF subjects are shown. The corresponding values in plasma established from 16 normal controls described previously (see reference 20) are shown by the box-whisker plot on the right. The plasma isoprostane values did not change significantly after exercise, but they apparently exceeded the normal control range for the assay. Pre-0, before exercise; post-0, after exercise.

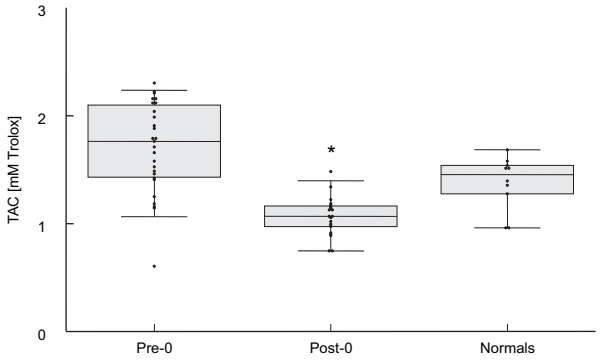

Figure 4 Plasma total antioxidant capacity before and after exercise. Data shown are plasma total antioxidant capacity in Trolox equivalents [mM]. The median, 25—75th percentile (box) and 5—95th percentiles (whiskers) for 29 IPF subjects are shown. The corresponding values in plasma established from 6 normal controls averaging 50 years of age are shown by the box-whisker plot on the right. The plasma TAC decreased significantly after exercise (*, $P < 0.001$ post-exercise compared to pre-exercise by signed rank test). Pre-0, before exercise; post-0, after exercise.

oxidant stress ($P < 0.001$ compared to pre-exercise by signed ranks test). As shown above, the decrease in TAC was accompanied by corresponding increases in lactate and hypoxemia after exercise. These data are shown in Fig. 4.

Nitrate/nitrite assays

IPF patients have a range of total nitrite concentrations in the urine and blood that is easily measurable using the Griess reaction. The values are quite comparable to normal human data in the literature.[27] These data are shown in Table 2.

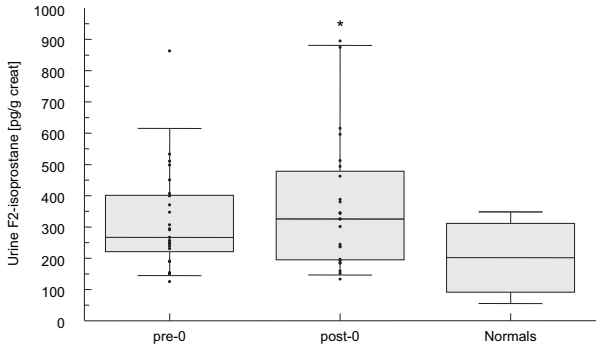

Figure 3 Urine isoprostane concentrations before and after exercise. Data shown are urine isoprostane concentrations in ng/g creatinine. The median, 25—75th percentile (box) and 5—95th percentiles (whiskers) for 29 IPF subjects are shown. The corresponding urine values established from 16 normal controls reported previously (see reference 20) is shown by the box-whisker plot on the right. IPF urine isoprostane concentration increased significantly after exercise. Pre-0, before exercise; post-0, after exercise. (*, $P = 0.047$ post-exercise compared to pre-exercise by signed rank test).

Urine H_2O_2 assay

A wide range of H_2O_2 values was found; some IPF patients have post-exercise urine H_2O_2 concentrations several fold above the pre-exercise and normal values. However, because of the high variability, no significant differences were found. These data are shown in Fig. 5.

Discussion

Summary

An underlying rationale for the present study was the previously reported oxidative and nitrosative stress in both epithelial lining fluid and circulation of IPF patients.[4,28,29] We reasoned that exercise would exacerbate systemic oxidant stress in IPF patients, perhaps related to impaired oxygenation and resulting cellular hypoxia as in COPD.[30,31]

We studied patients selected for enrollment in a randomized clinical trial of sildenafil, a pulmonary vasodilator, for IPF.[17] Enrolled patients were quite typical of IPF patients followed in the outpatient clinic, and their diagnoses were based on the presently accepted ATS-ERS case definition.[1,2]

Plasma NT-proBNP is elevated in IPF patients

Despite the modest increase in pulmonary artery systolic pressure (estimated echocardiographically in the mid thirties) required for entry into the trial, we found that IPF patients had plasma NT-proBNP levels clearly above the six normals, as shown in Fig. 1. Elevated brain natriuretic peptide (BNP) levels correlate well with significant pulmonary hypertension in patients with chronic lung diseases, including idiopathic pulmonary fibrosis.[18] None of our subjects had clinically or echocardiographically evident left

Table 2 Plasma and urine nitrite concentrations.[a]

	Pre-exercise	Post-exercise	Controls	P[b]
Plasma total nitrite [μM]	12 [8—15]	10 [7—14]	7 [4—11]	0.314
Urine total nitrite [μmol/g creatinine]	216 [156—341]	218 [133—353]	293 [190—542]	0.509

[a] Data from 29 subjects and 6 controls are medians with the [25—75th] percentiles shown. Total nitrite represents the sum of nitrite and nitrate after reduction *in vitro* with nitrate reductase.
[b] Pre-exercise compared to post-exercise value by signed rank test.

ventricular failure, confirming that high NT-proBNP levels were likely related to the increase in pulmonary artery pressure that typifies IPF. The range and median of post-exercise NT-proBNP values appeared similar to the pre-exercise values as shown in Fig. 1. Nineteen of 29 individual subjects had detectable increases in their NT-proBNP values after exercise. While some individual patients had both high post-exercise NT-proBNP and elevated systolic pulmonary artery pressure (RVSP) by echocardiography, no significant statistical correlation could be found by regression analysis. The lack of correlation was due to individual variability and the small number of subjects in the trial. Increased pulmonary vascular resistance and impaired right ventricular pressure appear to be key factors in exercise limitation of IPF patients.

Oxidant stress is an important mechanism in IPF

Oxidative stress in IPF is detectable using various biomarkers (*e.g.*, protein carbonyls, antioxidant capacity, thiobarbituric reactive substances) in bronchoalveolar lavage fluid (BALF) and plasma.[4,28] For example, 8-isoprostanes (i.e., F_2-isoprostanes), a product of non enzymatic, radical catalyzed lipid peroxidation are found in BALF of patients with interstitial and other chronic lung diseases.[26] Depletion of lung GSH is relevant to the pathogenesis of IPF,[32,33] as is implied by a recent clinical trial. Deterioration of vital capacity (VC) and diffusing capacity (D_LCO) were delayed significantly in the N-acetylcysteine group.[10]

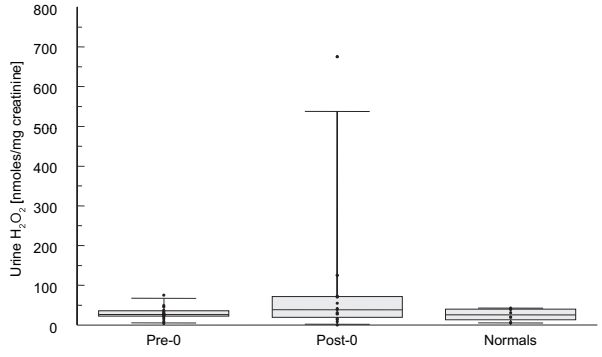

Figure 5 Urine hydrogen peroxide concentration. Data shown are urine H_2O_2 concentrations in nmol/mg creatinine. The median, 25—75th percentile (box) and 5—95th percentiles (whiskers) for 29 IPF subjects are shown. The corresponding values in urine established from 6 normal controls averaging 50 years of age is shown by the box-whisker plot on the right. A range of values was found; some IPF patients have post-exercise urine H_2O_2 concentrations several fold above the pre-0 and normal values. However, because of the variability, no significant differences were found. Pre-0, before exercise; post-0, after exercise.

Plasma and urine isoprostanes indicate systemic oxidant stress in IPF

F_2-isoprostanes are prostaglandin-like compounds, derived from esterified arachidonic acid by non enzymatic, free radical catalyzed reactions. They have provided evidence of oxidative stress in a number of disease states including atherosclerosis, chronic obstructive pulmonary disease and neurodegenerative conditions (for review, see reference 26).

We detected significantly increased urine isoprostanes after low level exercise. Elevated urine isoprostanes reflect rapid renal clearance of lipid peroxidation products from the circulation. While we cannot eliminate renal production of isoprostanes as the source, the kidney presumably remains well perfused during exercise and well oxygenated compared to skeletal muscle.

Plasma isoprostane levels in IPF patients are several fold higher than those in controls' plasma, as shown in Fig. 2. We did not detect an increase after exercise, perhaps because of the already high levels. While we and others [21,26] take this to represent convincing evidence of oxidant stress, the potent vasoconstrictor activities F_2-isoprostanes are also plausibly related to the increase in pulmonary artery pressure found on echocardiography. Isoprostanes are potent vasoactive thromboxane receptor agonists, demonstrating also a potential pathophysiological role as mediators of pulmonary hypertension.[26] Thus, finding increased isoprostanes is mechanistically relevant to exercise induced increase in pulmonary vascular resistance and associated right ventricular dysfunction.

Plasma antioxidant capacity decreases after exercise

IPF patients at rest had plasma antioxidant capacity (TAC) comparable to or higher than normal controls. This result obviously differs from the findings of Rahman *et al.*, who reported that IPF patients had diminished TAC at rest.[4] Over one-half of the subjects in their study were current cigarette smokers (while none of our subjects were active smokers), and this may represent the greatest difference between our study populations. Our patients were strictly classified using contemporary criteria [1,2] and none were treated with corticosteroids or experimental therapy at the time of these

measurements, so the patient population we studied differed significantly from that described by Rahman *et al*.[4]

IPF patients developed a large and significant decrease in plasma TAC after exercise as shown in Fig. 4, reminiscent of exercise induced oxidant stress described in chronic obstructive pulmonary disease (COPD) patients.[29] This oxidant stress is accompanied by hypoxemia and lactate production, indicating that low level exercise has significant metabolic effects in IPF subjects. TAC represents the capacity of plasma to inhibit an *in vitro* oxidation reaction. It is a nonspecific functional assay and likely reflects the availability of reduced sulfhydryl groups on albumin in addition to low molecular weight antioxidants such as nonprotein sulfhydryls, ascorbic acid, tocopherol and membrane lipids that compete as targets of oxidation.[24]

Changes in TAC after exercise are not specific for IPF but rather represent a general indication of oxidant stress that may occur in various disease states. For example, asthma patients are found to have significantly decreased total antioxidant status during acute exacerbations.[34] Similarly, TAC is lower than normal in COPD patients' plasma, and it correlates with the severity of airway obstruction.[35] TAC is decreased in a number of other pathologies, including severe sepsis.[36]

Urinary hydrogen peroxide as a marker of oxidant stress

While we found no significant increase in urinary H_2O_2 after exercise, some individual post-exercise values were high compared to those at rest and to the normal controls as shown in Fig. 5. Because not all urine samples contained measurable H_2O_2, exploratory analysis was limited to those samples in which we could measure H_2O_2. The urinary concentration of hydrogen peroxide is increased in patients with malignancies, where it correlates with increased plasma hydrogen peroxide and erythrocyte malondialdehyde.[37] Although auto oxidation reactions in urine could generate H_2O_2,[25] elevated urinary H_2O_2 is regarded as a marker of systemic oxidant stress in malignancies.[37]

Nitrosative stress in IPF

Reactive nitrogen species (RNS), which interact with ROS, also contribute importantly to epithelial injury in IPF. Myeloperoxidase (MPO) and nitrotyrosine (NT) co-localize in a number of inflammatory lesions.[38] Finding MPO and NT together suggests that NO_2^- (the major end product of $^{\bullet}NO$ metabolism) and HOCl react to produce NO_2, which accounts for tyrosine nitration. Lung sections from IPF patients stain strongly positive for nitrotyrosine (NT) and inducible nitric oxide synthase (iNOS) in alveolar macrophages, neutrophils and alveolar epithelium.[29]

Although nitrosative stress is evident in IPF lung sections,[29] we found no increase in IPF plasma total nitrite concentration compared to controls as shown in Table 2. We likewise found no differences in urine nitrite concentration after exercise. This observation reflects restriction of the well described nitrosative stress found in IPF patients to alveolar compartment.

Systemic oxidant stress and exercise in IPF and other chronic lung disease

We found a significant increase in urine isoprostane concentration after low level exercise as shown in Fig. 3, confirming our hypothesis that low level exercise would exacerbate oxidant stress in IPF patients. Since urine isoprostanes were negatively correlated with post-exercise S_aO_2, the observed increase in isoprostanes is associated with evidence for hypoxemia.

We found a significant decrease in hemoglobin oxygen saturation and a small but significant increase in lactate after low level exercise, suggesting that some subjects exercised at near maximal capacity. Lactate production reflects the integration of ATP production and its cellular utilization, so that efficient lactate uptake may result in relatively low circulating concentrations that reflect adaptation to hypoxia. Plasma lactate may not reflect anaerobic glycolysis in muscles, as aerobic overproduction of pyruvate may occur early during maximal exercise.[39]

IPF patients we studied developed hypoxemia as shown by decreased S_aO_2 and increased lactate concentrations, along with increases in oxidant stress after exercise. While an increase in lactic acid production is typical of severe exercise, it occurred here at a rather low level of exertion (50 W). The increase in lactate may have been blunted because of the subjects' exercise limitation due to increased pulmonary vascular resistance and right ventricular dysfunction.

Exercise as well as hypoxia *per se* imposes oxidative stress on skeletal muscle.[40,41] Increases in myocardial and skeletal muscle free radical concentration confirm that excess ROS produced during exercise precede fatigue.[41] While the source of ROS produced by muscle cells is not entirely clear, it is likely that mitochondria, enzymes and activated phagocytes all contribute.[42] Significant muscle cell hypoxia may occur during exercise in patients with impaired gas exchange and systemic hypoxemia.[30] Hypoxia increases production of ROS by mitochondria, yet indirect evidence from manganese-containing superoxide dismutase heterozygous knockout mice indicates that mitochondria may not be the only source of O_2^-.[42]

Glutathione metabolism is also clearly affected by exercise. Prolonged submaximal exercise by humans causes a decrease in reduced glutathione and a concomitant increase in GSSG.[43] Allopurinol inhibited the increase in GSSG/GSH and lipid peroxidation, otherwise observed in exercising COPD patients.[44] Changes in exercise capacity were not measured, although inhibition of ROS production, *e.g.*, with allopurinol, would conceivably prevent skeletal muscle dysfunction and increase exercise capacity.

Redox signaling may also be required for adaptation to exercise. Endurance exercise training can reduce oxidative stress after exhaustive exercise and may permit increased functional capacity.[45,46] Exercise training increases CuZn-containing superoxide dismutase and decreases $p67^{phox}$, ERK phosphorylation and malondialdehyde production.[40]

Although we obtained novel and valuable data from this study, we recognize several intrinsic limitations in its design and execution. The study population was small, as would be expected in a single center trial. By design, each subject

served as his or her own control, so we did not compare responses of IPF patients to those of disease controls (*e.g.*, COPD or sarcoid) or to normals. We chose rather to assess endurance at a low, constant workload as the stimulus to oxidant stress. Maximum oxygen consumption was not measured, although it is likely that protocol would have provoked a greater degree of oxidative stress. The controls did not participate in the exercise protocol because of the planned, paired nature of the design. The controls tended to be younger and healthier than the IPF patients we studied in the exercise protocol. Despite these limitations, which themselves raise testable hypotheses, the paired design effectively allowed us to detect significant intra-group differences after exercise.

Conclusions

A number of important new observations came from this trial. Finding that NT-proBNP is elevated in typical IPF patients and that two markers of oxidant stress, decreased plasma TAC and increased urine isoprostanes occur after low level exercise and are associated with hypoxemia, is important. Such observations could lead to testing the hypotheses that systemic oxidant stress may be due to cellular hypoxia and that oxidant stress during exercise may be a factor that limits endurance of IPF patients. Taken together, these results show that IPF patients develop hypoxemia and oxidant stress at low levels of exercise and suggest that isoprostanes could therein contribute to increased pulmonary vascular resistance.

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Abbreviations

ATS American Thoracic Society
BLM bleomycin
BNP brain natriuretic peptide
COPD chronic obstructive pulmonary disease
CuZn-SOD copper-zinc superoxide dismutase
EC-SOD extracellular superoxide dismutase
ELF epithelial lining fluid
GSH reduced glutathione
GSSG glutathione disulfide
HOCl hypochlorous acid
cGMP cyclic guanosine monophosphate
ERS European Respiratory Society
IPF idiopathic pulmonary fibrosis
ILD interstitial lung disease
IPAH idiopathic pulmonary arterial hypertension
MMP matrix metalloproteinase
MPO myeloperoxidase
N-Ac N-acetylcysteine
NO nitric oxide radical
NT nitrotyrosine
NT-proBNP amino terminal pro brain natriuretic peptide
PAPsys systolic pulmonary arterial pressure
PDE5 phosphodiesterase five
PAH pulmonary arterial hypertension
O_2^- superoxide anion
RCT randomized controlled trial
RVESP right ventricular end systolic pressure
S_aO_2 arterial oxygen saturation
SD standard deviation
SOD superoxide dismutase
TAC total antioxidant capacity (of plasma)
TGF-β transforming growth factor-beta

Authors' contributions

RJ designed the study, obtained funding, directed all clinical trial procedures and wrote the manuscript in collaboration with the biostatistician. CR served as study coordinator, recruited subjects, did all clinical trial procedures and collected primary data. CG acquired samples and completed assays of the oxidant stress markers. OG served as biostatistician, assisted in study design and co-wrote the manuscript. All authors read and approved the final manuscript.

Conflict of interest statement

The authors declare that they have no conflicting interests.

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