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Research Article

PGC-1α Induction in Pulmonary Arterial Hypertension

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Idiopathic Pulmonary arterial hypertension (IPAH) is characterized by the obstructive remodelling of pulmonary arteries, and a progressive elevation in pulmonary arterial pressure (PAP) with subsequent right-sided heart failure and dead. Hypoxia induces the expression of peroxisome proliferator activated receptor γ coactivator-1α (PGC-1α) which regulates oxidative metabolism and mitochondrial biogenesis. We have analysed the expression of PGC-1α, cytochrome C (CYTC), superoxide dismutase (SOD), the total antioxidant status (TAS) and the activity of glutathione peroxidase (GPX) in blood samples of IPAH patients. Expression of PGC-1α was detected in IPAH patients but not in healthy volunteers. The mRNA levels of SOD were lower in IPAH patients compared to controls (3.93 ± 0.89 fold change). TAS and GPX activity were lower too in patients compared to healthy donors, (0.13 ± 0.027 versus 0.484 ± 0.048 mM and 56.034 ± 10.37 versus 165.46 ± 11.38 nmol/min/mL, resp.). We found a negative correlation between expression levels of PGC-1α and age, PAP and PVR, as well as a positive correlation with CI, PaO_2, mRNA levels of CYTC and SOD, TAS and GPX activity. These results taken together are indicative of the possible role of PGC-1α as a potential biomarker of the progression of IPAH.

1. Introduction

Pulmonary arterial hypertension (PAH) is a complex disorder characterized by the obstructive remodelling of pulmonary arteries, leading to a progressive elevation of pulmonary arterial pressure (PAP) and subsequent right-sided heart failure and death [1]. There are five categories in which pulmonary hypertension (PH) diseases can be grouped according to specific therapeutic interventions directed at dealing with the cause of (1) PAH, (2) pulmonary hypertension with left heart disease, (3) PH associated with disorders of the respiratory system or hypoxemia, (4) PH caused by thrombotic or embolic diseases, and (5) PH caused by multifactorial mechanisms [2]. Idiopathic PAH (IPAH) is included in group 1 and within patients with a mean pulmonary artery pressure (PAPm) ≥ 25 mmHg, and a pulmonary capillary wedge pressure (PCWP), left atrial pressure, or left ventricular end-diastolic pressure ≤ 15 mmHg, and a pulmonary vascular resistance greater than three Wood units [3]. Remodelling of pulmonary arteries leads to an increase of pulmonary vascular resistance (PVR) which produces right ventricular (RV) overload, hypertrophy and dilatation, and eventually RV failure and death [4]. These changes are due to an inadequate adaptation of myocardial contractility [5].

Although physiopathology of IPAH remains under investigation, the role of radical oxygen-mediated events, including myocardial ischemia, seems clear [6]. During the progression of PAH, there is a progressive hypoxia situation originated as a consequence of an increase in the demand of oxygen by hypertrophied cardiomyocytes, as well as a reduction in the capillary density [7, 8]. This hypoxia situation leads to an imbalance in oxidative/antioxidative status with

subsequent cellular damage which contributes to RV failure [9, 10].

The increase in the production of the reactive oxygen species has been established in different experimental animal models of PH and in IPAH-diagnosed patients [11, 12]. The main source of these species, in particular $O_2^{\bullet -}$, is the injured vasculature which results in impaired nitric oxide (NO) signaling and the development of pulmonary vascular remodeling [13, 14]. In this context, the role of superoxide dismutase (SOD) is relevant, because it is involved in the regulation of NO metabolism and in preventing PH, as it has been described in adult animal models [15]. Another important antioxidant enzyme involved in oxidative enzymopathies (including PH) is the glutathione peroxidase (GPX). A deficit in this enzyme is associated with an increase of reactive oxygen species and a decrease of $NO^{\bullet}$ which leads to endothelial dysfunction and impaired vascular reactivity [16].

Peroxisome proliferator-activated receptor (PPAR) γ coactivator-1α (PGC-1α) is a well-known regulator of the transcription of genes involved in oxidative metabolism and mitochondrial biogenesis, including the mitochondrial respiratory chain CYTC [17]. This transcriptional coactivator plays a key role in the metabolic control of the cardiac muscle and participates in cardiomyocyte differentiation [18]. PPAR agonists (pioglitazone and rosiglitazone) preserve both ventricular function and PGC-1α levels [19–21]. The tissue's capacity to produce PGC-1α after an hypoxic event, could predict the regenerative capacity of the tissue. In fact, we have recently reported that expression levels of PGC-1α in blood samples of patients with myocardial infarction can be correlated with the size of the hypoxic area, supporting the role of this protein in protecting myocardiocytes after hypoxia injury [22].

The main objective of this study is to analyze the expression levels of PGC-1α in 12 IPAH-diagnosed patients and in 15 healthy volunteers. These levels are correlated with the progression of the disease, with cytochrome c (CYTC) and superoxide dismutase (SOD) mRNA levels and with total antioxidant status (TAS) and glutathione peroxidase (GPX) activity.

2. Materials and Methods

2.1. Patients. In this study 12 IPAH-diagnosed patients were compared with 15 healthy volunteers. Inclusion criteria for the 12 diagnosed patients included an mPAP > 25 mmHg, a PWP less or equal to 15 mmHg and a PVR > 3 Wood units measured by catheterization. Clinical features of patients included in this study are summarized in Table 1. All patients received different combinations of bosentan, treprostinil, nifedipine, and iloprost before sample collection. Healthy volunteers were paired in age with patients (51.34 ± 8.28 and 56.5 ± 3.23 years old, resp.).

All experiments were approved by the local ethics committee and informed consent was obtained. On the one hand 2.5 mL of peripheral blood were collected in PAX gene RNA collection tubes (Qiagen, Valencia, CA, USA) and stored at −80°C until its analysis, as recommended by the manufacturer. On the other hand, 4 mL of peripheral blood was collected in EDTA vacutainers (Becton Dickinson, NJ, USA). Plasma was isolated by centrifugation (15 minutes at 2500 rpm) and stored at −80°C in 500 μL aliquots.

2.2. Determination of PGC-1α, CYTC, and SOD mRNA Expression. Total RNA was extracted from peripheral blood stored in PAX gene collection tubes using the PAXgene blood RNA kit (Quiagen, Valencia, CA, USA), according to manufacturer instructions. RNA concentration was determined by spectrophotometry using the Nanodropt 200 spectrophotometer (Fischer Scientific, Madrid, Spain) at 260 nm. Only extractions with a ratio 260/280 nm >1,4 were considered in this study. RNA integrity was evaluated by electrophoresis using the Bioanalizer (Agilent technologies, Santa Clara CA, USA). Only those extractions with an RIN near 10 were used for gene expression studies.

cDNA was synthesized using the TaqMan RT reagents (Applied Biosystems, Foster City, CA, USA) following manufacturer's instructions. Reactions were 1/2 diluted and preamplification was carried out using the TaqMan preamp master mix (Applied Biosystems, Foster City, CA, USA) according to the supplier instructions. Assays on demand against PGC-1α, CYTC, SOD, and GAPDH were purchased from Applied Biosystems and gene expression was carried out in a 7900HT real-time thermocycler (Applied Biosystems, Foster City, CA, USA). The comparative ΔC_t method was used to calculate relative expression levels of the genes included [23].

2.3. Determination of Total Antioxidant Status (TAS). TAS was determined in plasma samples using the Total Antioxidant Assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) following the manufacturer's instructions. This assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulfonate]) to ABTS+ by metmyoglobin. Capacity of antioxidants in the sample to prevent ABTS oxidation is compared with that of Trolox, a water-soluble tocopherol analog. Results are expressed as mM Trolox equivalents.

2.4. Analysis of Glutathione Peroxidase (GPX) Activity. GPX activity was estimated in plasma samples using the GPX assay kit (Cayman Chemical Company, Ann Arbor, MI, USA), according to supplier instructions. Plasma samples were 1/2 diluted in sample buffer (provided with the kit) and the GPX activity was evaluated calculating the change in absorbance at 340 nm (ΔA_{340} nm/min) as it is described in the user's manual included in the kit. Results are presented as nmol/min/mL.

2.5. Data Analysis. Data are presented as the mean ± SEM. Statistical analysis of the results was carried out by nonparametric Mann-Whitney test and nonparametric Spearman correlation analysis using the GraphPad software (GraphPad Software Inc., San Diego, CA). Significance was accepted when $P < 0.05$.

Table 1: Clinical, molecular, and biochemical features of IPAH patients.

ID	Age (years)	Sex	PaO_2 (mmHg)	6 MWT (m)	PAP (mmHg)	CI (L/min/m^2)	PVR (dyn/sec/cm^2)	VR	PGC-1α RE	CYTC RE	SOD RE	TAS (mM)	GPX (nmol/min/mL)	Treatment
HP1	45	F	67	595	48	1.3	12.4	No	2.87	2.45	4.12	0.21	70.23	e + b
HP2	75	M	62	255	70	1.8	11	No	0.34	0.42	0.24	0.05	23.76	a + s + i
HP3	34	F	85	554	35	2.8	6.2	YES	67.00	38.50	7.45	0.28	119.05	n
HP4	58	F	70	380	48	2.2	12	No	6.07	5.47	2.29	0.19	67.89	b + t
HP5	38	F	83	450	38	3.24	6.1	YES	8.96	6.95	26.77	0.15	91.27	n
HP6	63	F	68	360	40	2.1	8.5	No	3.22	2.59	4.64	0.25	85.91	i + b + s
HP7	64	M	60	240	53	2.3	7.7	No	0.34	0.48	0.14	0.04	20.22	b + s + t
HP8	66	M	57	334	50	1.8	8.6	No	1.41	1.01	0.13	0.11	45.42	s + i + b
HP9	60	F	58	120	49	1.1	18.9	No	0.34	0.08	0.13	0.02	24.26	s + i + b
HP10	55	F	63	320	31	2.5	7.5	YES	7.76	8.34	3.47	0.22	90.85	b + s
HP11	48	F	62	534	59	1.4	13.6	No	0.33	0.36	0.17	0.06	19.21	e + s + b
HP12	72	F	60	280	65	2.2	12.5	No	0.22	0.21	0.50	0.01	14.34	i + b

PaO_2: partial pressure of oxygen in arterial blood, 6MWT: 6-minute walk test, PAP: pulmonary arterial pressure, CI: cardiac index, PVR: pulmonary vascular resistance, VR: vasoreactivity, PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1-α, RE: relative mRNA expression, CYTC: cytochrome c, SOD: superoxide dismutase, TAS: total antioxidant status, GPX: glutathione peroxidase, e: epoprostenol, si: sildenafil, i: iloprost, b: bosentan, t: treprostinil, and n: nifedipine.

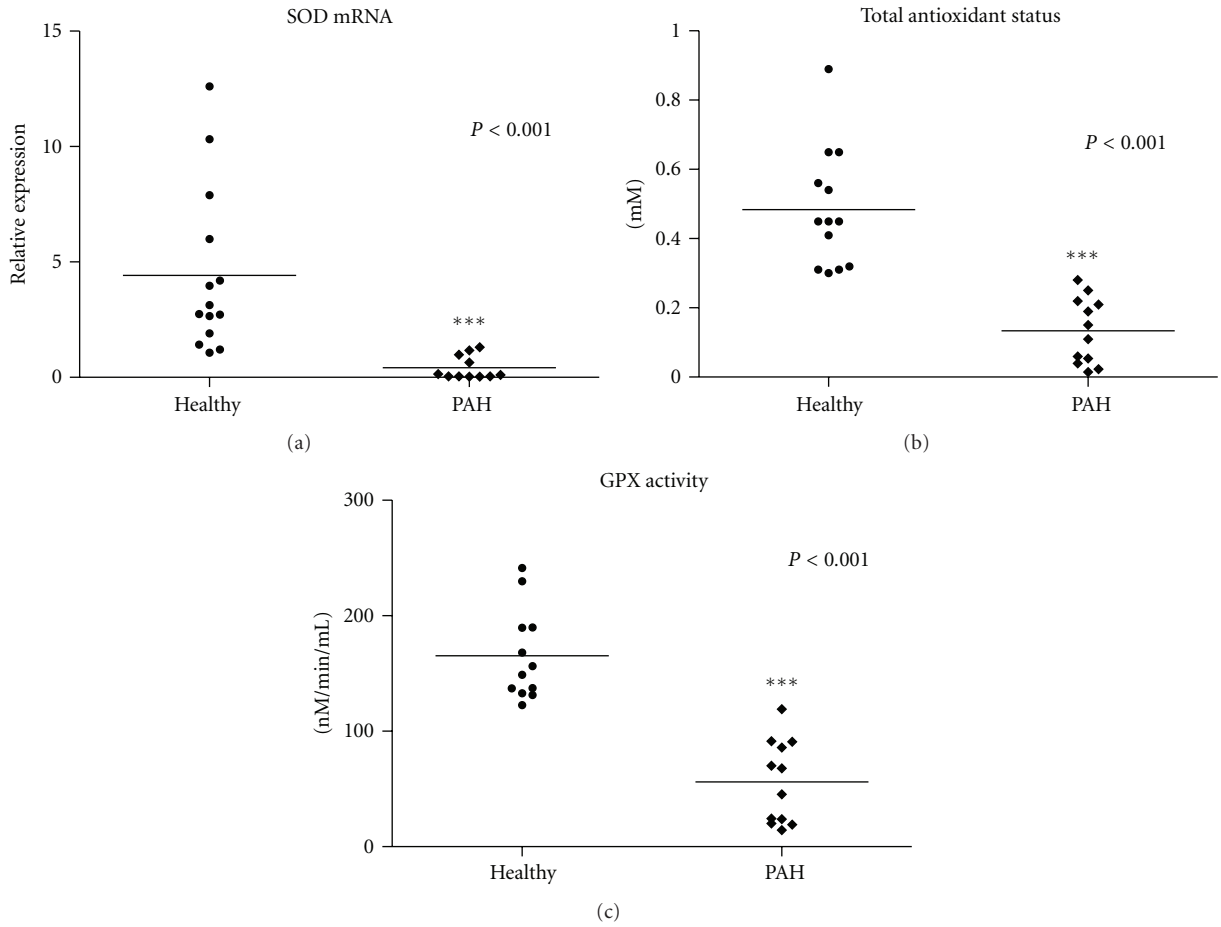

Figure 1: Oxidative status of idiopathic pulmonary hypertension patients (IPAH). Relative expression of SOD (a), TAS (b), and glutathione peroxidase (GPX) activity were evaluated in peripheral blood from 12 IPAH patients and 15 healthy donors. The nonparametric Mann-Whitney test was used to analyze data. Significance was accepted when $P < 0.05$.

3. Results

3.1. PGC-1α CYTC Are Expressed in PAH Blood Samples. Our first objective in this study was to evaluate if the expression levels of PGC-1α and CYTC mRNA were differentially expressed in blood of PAH-diagnosed patients compared to healthy volunteers. Gene expression analysis was undertaken and results indicated that neither of the genes were expressed in healthy donors, contrary to what happened in PAH patients, in which the expression levels of both genes were clearly detected. In order to obtain comparative data, the average ΔC_t of PAH group was calculated and expression levels of each patient were estimated. Results are represented in Table 1.

3.2. Superoxide Dismutase (SOD) Expression Is Reduced in PAH Patients. SOD expression levels were measured in PAH patients. Our results indicate that the expression of this enzyme is lower in PAH patients compared to healthy volunteers (1.01- ± 0.61- and 3.93- ± 0.89-fold change, resp.) as it is represented in Figure 1(a). Next we calculated the relative expression of this gene in a similar way to PGC-1α and CYTC in order to correlate its expression levels with indicators of progression of the diseases. The results obtained are shown in Table 1.

3.3. Total Antioxidant Status Is Reduced in PAH Patients. Hypoxia is one of the most important factors affecting PAH patients. Due to the relevance of PGC-1α induction in the responsiveness against the oxidant injury, we decided to analyze the TAS in PAH patients included in this study. On one hand, we found that TAS is lower in PAH patients compared to healthy donors (0.13 ± 0.027 and 0.484 ± 0.048 mM, resp.), as it is shown in Figure 1(b). On the other hand, we found a clear correlation of TAS levels and the relative expression levels of PGC-1α as described in point 3.5. TAS levels of PAH patients are summarized in Table 1.

3.4. PAH Patients Activity of Glutathione Peroxidase (GPX) Is Decreased. The activity of GPX enzyme in plasma samples of PAH patients and healthy donors was done. Results obtained are represented in Figure 1(c) and clearly demonstrate that the activity of this enzyme is reduced in PAH patients

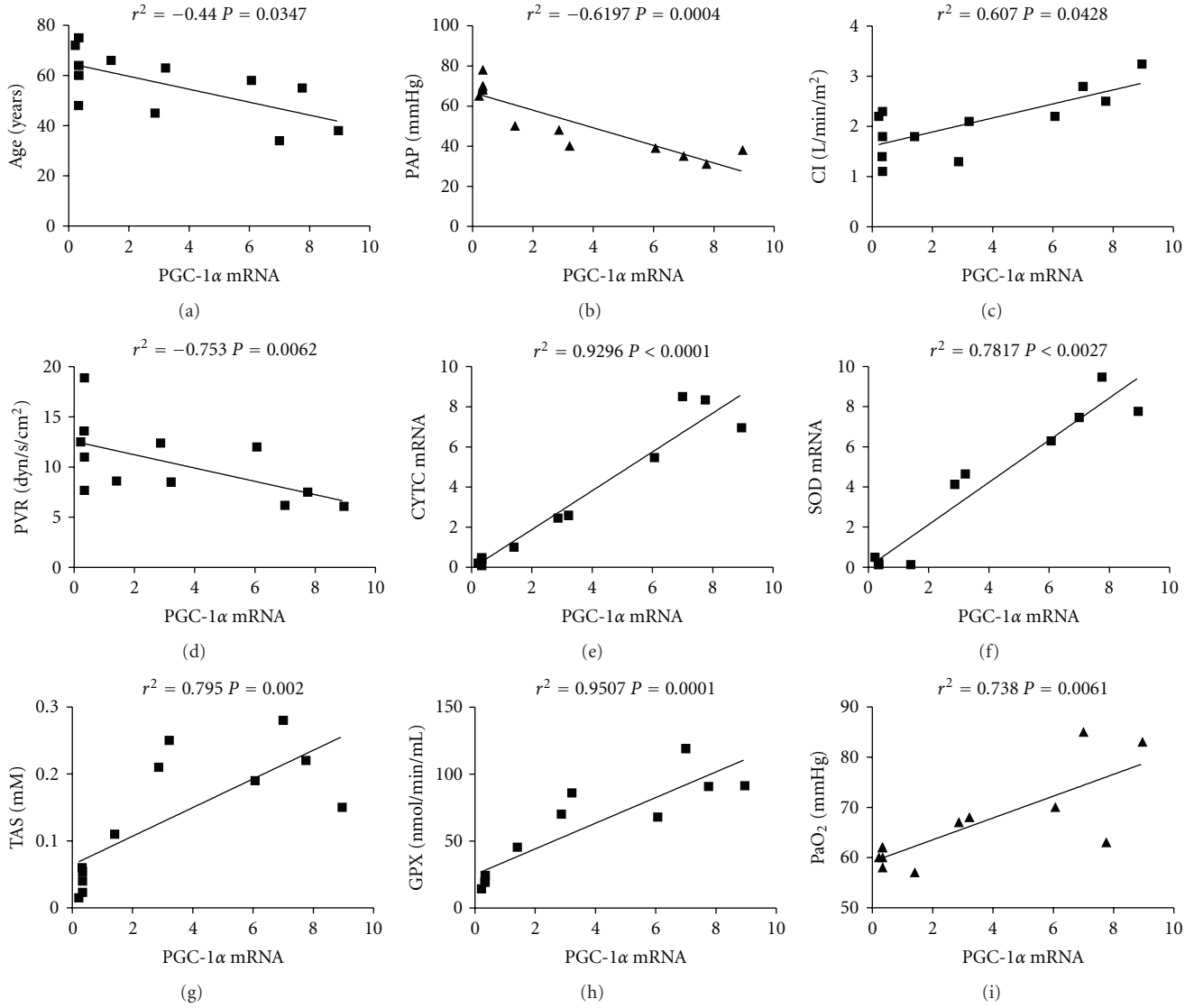

Figure 2: Correlation of PGC-1α with clinical, molecular, and biochemical features. 12 IPAH patients and 15 healthy donors were included in the analysis. The nonparametric Spearman test was used to analyze data. Significance was accepted when $P < 0.05$.

compared to healthy volunteers (56.034 ± 10.37 and 165.46 ± 11.38 nM/min/mL, resp.). GPX activity is summarized in Table 1.

3.5. Multiple Regression Analysis of Clinical, Molecular, and Biochemical Features of IPAH Patients. Finally, multiple-regression analysis of clinical, molecular, and biochemical data was done using the nonparametric Spearman test. The correlation matrix is shown in Table 2. Concerning PGC-1α, results are summarized in Figure 2. We found a negative correlation between age, PAP, and PVR (Figures 2(a), 2(b), and 2(d)). This correlation was significant for PAP and PVR ($P = 0.0001$ and $P = 0.0426$, resp.) but not for the age ($P = 0.108$). On the contrary, the correlation was positive for CI, PaO_2, CYTC and SOD relative expression, TAS, and GPX activity (Figures 2(c), 2(e), 2(f), 2(g), 2(h), and 2(i)). In all cases the change was considered significant. No correlation was found between PGC-1α levels and 6 MWT, which was only significantly correlated with age of the patients.

4. Discussion

In this study we analyzed the expression levels of PGC-1α, CYTC and SOD mRNA as well as TAS and GPX activity in peripheral blood of 12 well-characterized IPAH-diagnosed patients and 15 healthy donors. Our results demonstrate that mRNA of PGC-1α and CYTC can be detected by real time RT-PCR in PAH patients, but not in healthy volunteers. On the other hand, relative expression levels of SOD are decreased in these patients, compared to controls, as well as TAS and GPX activity. Correlation studies carried out indicate that there is a clear correlation between PGC-1α levels and the clinical parameters included in this study, indicating the progression of the disease and the oxidative

Table 2: Multiple-regression analysis of clinical, molecular, and biochemical features of IPAH patients.

	PGC-1α	6 MWT	Age	PAP	CI	PVR	CYTC	SOD	TAS	GPX	PaO_2	
PGC-1α		0.15585	0.03470	0.00041	0.04289	0.00625	0.00012	0.00277	0.00206	0.00010	0.00618	
6 MWT	0.15585		0.00451	0.07089	0.70377	0.55674	0.06251	0.12445	0.01532	0.09516	0.01279	
Age	0.03470	0.00451		0.04786	0.35841	0.31914	0.03581	0.04786	0.03317	0.01683	0.01097	
PAP	0.00041	0.07089	0.04786		0.00791	0.00824	0.00000	0.00033	0.00136	0.00095	0.00705	
CI	0.04289	0.70377	0.35841	0.00791		0.00053	0.01155	0.01412	0.26858	0.10484	0.06642	
PVR	0.00625	0.55674	0.31914	0.00824	0.00053		0.00136	0.04461	0.05484	0.01025	0.07415	*P* values
CYTC	0.00012	0.06251	0.03581	0.00000	0.01155	0.00136		0.00136	0.00015	0.00008	0.00209	
SOD	0.00277	0.12445	0.04786	0.00033	0.01412	0.04461	0.00136		0.01391	0.00736	0.00038	
TAS	0.00206	0.01532	0.03317	0.00136	0.26858	0.05484	0.00015	0.01391		0.00033	0.00401	
GPX	0.00010	0.09516	0.01683	0.00095	0.10484	0.01025	0.00008	0.00736	0.00033		0.00554	
PaO_2	0.00618	0.01279	0.01097	0.00705	0.06642	0.07415	0.00209	0.00038	0.00401	0.00554		
PGC-1α		0.43663	−0.61973	−0.87326	0.60071	−0.75354	0.92960	0.78171	0.79579	0.95073	0.73852	
6 MWT	0.43663		−0.75524	−0.53846	0.12281	−0.18881	0.55245	0.46853	0.67832	0.50350	0.69123	
Age	−0.61973	−0.75524		0.58042	−0.29123	0.31469	−0.60839	−0.58042	−0.61538	−0.67133	−0.70176	
PAP	−0.87326	−0.53846	0.58042		−0.72281	0.72028	−0.94406	−0.86014	−0.81119	−0.82517	−0.72983	
CI	0.60071	0.12281	−0.29123	−0.72281		−0.84562	0.69825	0.68421	0.34737	0.49123	0.54577	
PVR	−0.75354	−0.18881	0.31469	0.72028	−0.84562		−0.81119	−0.58741	−0.56643	−0.70629	−0.53334	Correlation coefficients
CYTC	0.92960	0.55245	−0.60839	−0.94406	0.69825	−0.81119		0.81119	0.88112	0.89510	0.79299	
SOD	0.78171	0.46853	−0.58042	−0.86014	0.68421	−0.58741	0.81119		0.68531	0.72727	0.85615	
TAS	0.79579	0.67832	−0.61538	−0.81119	0.34737	−0.56643	0.88112	0.68531		0.86014	0.76141	
GPX	0.95073	0.50350	−0.67133	−0.82517	0.49123	−0.70629	0.89510	0.72727	0.86014		0.74386	
PaO_2	0.73852	0.69123	−0.70176	−0.72983	0.54577	−0.53334	0.79299	0.85615	0.76141	0.74386		

PaO_2: partial pressure of oxygen in arterial blood, 6 MWT: 6-minute walk test, PAP: pulmonary arterial pressure, CI: cardiac index, PVR: pulmonary vascular resistance, VR: vasoreactivity, PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1-α, RE: relative mRNA expression, CYTC: cytochrome c, SOD: superoxide dismutase, TAS: total antioxidant status, GPX: glutathione peroxidase.

status of patients. We found a negative correlation between expression levels of PGC-1α and age, PAP and PVR, as well as a positive correlation with CI, indicating that those patients with higher levels of PGC-1α have an improvement of lung and heart functions. As pointed out in the results section, no correlation was found between 6 MWT and PGC-1α levels. A possible explanation is that this parameter is affected by other factors like the age of the patients [24].

PGC-1α is a transcriptional coactivator which has been shown to activate a broad range of transcription factors and to regulate genes encoding mitochondrial proteins including CYTC under hypoxemia, as it has been shown in animal models [25–27]. Results presented here demonstrate a good correlation between expression levels of PGC-1α and CYTC in circulating blood of IPHA patients, supporting these findings.

IPAH is characterized by a sustained hypoxia situation that leads to cellular damage and contributes to RV failure [9, 10]. TAS determines the response capacity of a biological system to oxidative-mediated events. This status represents the balance between oxidant and antioxidant molecules. We found a significant decrease of TAS in IPAH patients compared to healthy donors. Despite the coherence of the results obtained, under our knowledge, this is the first study demonstrating this decrease in IPAH patients.

One of the most important antioxidant enzymes is SOD and its expression is reduced under chronic hypoxia [28]. These findings are consistent with the decrease of SOD observed in PAH patients compared to healthy volunteers. SOD over expression prevents the development of PH and ameliorates established PH in hypoxia-induced pulmonary hypertension in mice and primary human endothelial cells [29]. We observed a good correlation between PGC-1α and SOD expression levels, which could support the implication of this antioxidant enzyme in the pathogenesis of IPAH.

Another key enzyme controlling oxidative damage is GPX as it has been observed in lung of IPAH patients [30]. Similar to what happens with SOD, we observed a significant decrease of the activity of this enzyme in IPAH patients compared to healthy donors and a positive correlation with the expression levels of PGC-1α, which is coherent with the antioxidant role of this transcriptional coactivator in oxidative enzymopathies [16].

All patients included in this study are being treated with different combinations of drugs including calcium channel blockers, endothelin receptor antagonists, PDE5 inhibitors and synthetic analogues of prostacyclin. Although the effects of the treatment attenuating the oxidant level are well known [31–33], heterogeneity on treatments linked to the limited number of patients included in this study make difficult to reach a conclusion about the effect of medication on altering the antioxidant/oxidant status. However, regardless of the treatment, all patients considered in this study are under mild/moderate hypoxemia. More studies including the novo patients and comparing the situation before and after medication are needed to understand the effects of treatment on hypoxemia status and the relation with mechanisms controlled by PGC-1α.

The principal limitation of this study is the number of patients included, however IPAH is a rare disease with a prevalence of 2-3 per million per year [34]. Data presented here indicate the possible role of PGC-1α in controlling the progression of the disease and the oxidative status of IPAH patients. This study suggests that the monitoring of circulating PGC-1α mRNA levels could be indicative of the progression of the disease, providing valuable information of parameters like the treatment efficacy and the severity of the progression of the disease. Another important aspect is that the monitoring of circulating PGC-1α involves noninvasive procedures without risk for the patient and that it could be carried out in a fast and economical way. For these reasons we proposed PGC-1α as a potential new biomarker of the progression of PAH.

Acknowledgments

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