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Article

Lysophospholipids, Lysophosphatidic Acids and Monoacyl-Glycerols: New Therapeutic Targets in Cardiovascular Diseases?

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Abstract: Cardiovascular diseases (CVD) are the leading cause of premature death and disability in humans. Increasing data suggest that CVD is closely related to lipid metabolism and signaling. This study aimed to assess whether circulating lysophospholipids (LPL), lysophosphatidic acids (LPA) and monoacylglycerols (MAG) may be considered as biomarkers of CVD. For this objective, the evolution of the plasma levels of 22 compounds (13 LPL, 6 LPA and 3 MAG) was monitored by liquid chromatography coupled with tandem mass spectrometry (HPLC/MS²) in different rat models of CVD, *i.e.* angiotensin-II-induced hypertension (HTN), ischemic chronic heart failure (CHF) and sugen/hypoxia(SuHx)-induced pulmonary hypertension (PH). On one hand, there was modest changes on the monitored compounds in HTN (LPA 16:0, 18:1 and 20:4 and LPC 16:1) and CHF (LPA 16:0, LPC 18:1 and LPE 16:0 and 18:0) models compared to control rats but these changes were no longer significant after correction for multiple testing. On the other hand, PH was associated with important changes in plasma LPA with a significant increase in the 16:0, 18:1, 18:2, 20:4 and 22:6 species. A deleterious impact of LPA was confirmed on isolated human pulmonary smooth muscle cells with an increase in their proliferation. This study demonstrates that circulating LPA species are increased in rats with PH and may contribute to the pathophysiology of this disease. Additional experiments are needed to assess whether the modulation of LPA signaling in PH may be of interest.

Keywords: lysophospholipids; lysophosphatidic acids; cardiovascular diseases; HPLC-MS/MS; rodent models, pulmonary hypertension, chronic heart failure, hypertension

1. Introduction

Cardiovascular diseases (CVD) are the leading cause of mortality and a major contributor to disability. In 2019, 523 million cases and 18.6 millions of deaths were reported corresponding to a 100% and a 50% increase since 1990 respectively. Because CVD remain the leading cause of disease burden in the world and still rise in all countries, the need to

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find new therapeutic target is of utmost importance [1]. Atherosclerosis, a common denominator of CVD, is characterized by an increase in oxidative stress and chronic inflammatory condition linked to lipid abnormality [2]. The effectiveness of therapeutic strategies based on circulating lipids reduction such as statins or PCSK-9 inhibitors confirms the close link between atherosclerosis and lipid metabolism and it is now well admitted that those strategies significantly reduce long-term mortality and cardiovascular morbidity, especially in the elderly [3-5]. Most lipids circulate through bloodstream as lipoproteins that are complex particles with a central hydrophobic core containing non-polar lipids such as triglycerides and cholesterol esters surrounded by a hydrophilic layer composed of phospholipids, free cholesterol and apolipoproteins. Oxidative stress and chronic inflammation trigger the production of oxidized low density lipoprotein (ox-LDL) and oxidized lipoprotein(a) (oxLp(a)) which become highly enriched in oxidized phospholipids (ox-PL) displaying pro-atherogenic properties [6-8]. Ox-PL are especially prone to hydrolysis by the lipoprotein associated phospholipase A_2 (Lp-PLA_2), also known as serum platelet-activating factor-acetyl hydrolase (PAF-AH), an inflammatory marker of cardiovascular disease, leading to the release of both oxidized fatty acids and lysophospholipids (LPL) [9,10]. These compounds exert deleterious biological effects due to their interaction with nucleic acids, phospholipids and proteins promoting atherosclerosis and cardiovascular events [11]. Interestingly, bioactive LPL and in particular lysophosphatidic acid (LPA) have potent effects on vascular cells, promoting vacoconstrictor, pro-inflammatory and proliferating effects that are known to contribute to atherosclerosis development but that could also participate to the pathophysiology of many CVD [12-17]. LPA metabolism is complex with various anabolic and catabolic pathways. There are four major enzymatic pathways for LPA production: the LPL-autotaxin (LPL-ATX) pathway (1), the phosphatidic acid-phospholipase pathway (PA-PLA_1/PLA_2) (2), the monoacylglycerol kinase (MAGK) pathway (3) and the *de novo* glycerophosphate acyltransferase (GPAT) pathway (4). Amongst these pathways responsible for LPA production, the last three are reversible with the intervention of lysophosphatidic acid acyltransferases (LPAAT), lipid phosphate phosphatases (LPP) and lysophospholipases (lysoPL) for pathways (2), (3) and (4) respectively (Figure 1) [18].

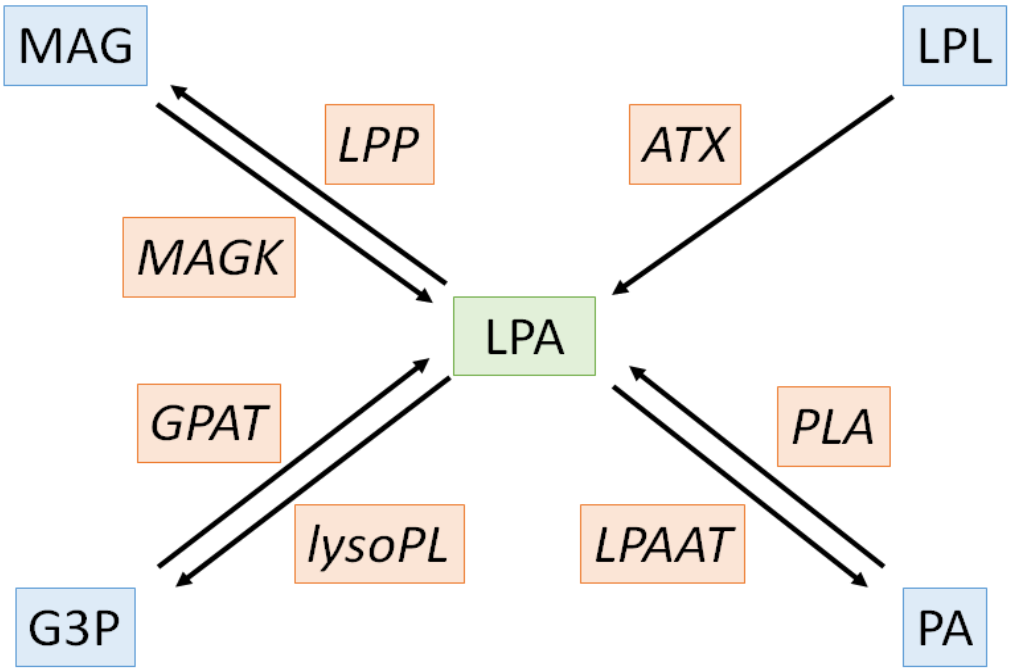

Figure 1. Biochemical pathways of lysophosphatidic acid metabolism. LPA, substrates/products and enzymes are depicted in green, blue and orange respectively. ATX: autotaxin, G3P: glycero-3-phosphate, GPAT: glycerophosphate acyltransferase, LPA: lysophosphatidic acid LPAAT: lyso-

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phosphatidic acid acyltransferases, LPL: lysophospholipids, LPP: lysophosphatidic acid phosphatase, lysoPL: lysophospholipase, MAG: monoacylglycerol, MAGK: monoacylglycerol kinase, PA: phosphatidic acid, PLA: phospholipase

Thus, this study aims to evaluate the evolution of circulating LPL, LPA and MAG levels in different rat models of CVD, *i.e.*, hypertension (HTN), heart failure (HF) and pulmonary hypertension (PH) in order to provide new insights to the interest of targeting LPA metabolism with pharmacological compounds.

2. Results

2.1. Angiotensin-II induced hypertension (Ang-II HTN)

As expected, administration of Ang-II during 28 days induced an increase in aldosterone level (242 ± 108 vs. 1030 ± 633 pM, p=0.004; Figure 2A) and in systolic blood pressure (SBP) (117 ± 4 vs. 176 ± 11 mm Hg, p<0.001; Figure 2B) compared to control rats. Ang-II HTN also led to a decrease in left ventricular fractional shortening (LVFS) (45 ± 3 vs. 26 ± 3 %, p<0.001; Figure 2C) and an increase in heart weight (1.54 ± 0.13 vs. 1.76 ± 0.13 g, p=0.004, Figure 2D), showing the development of cardiac dysfunction and hypertrophy.

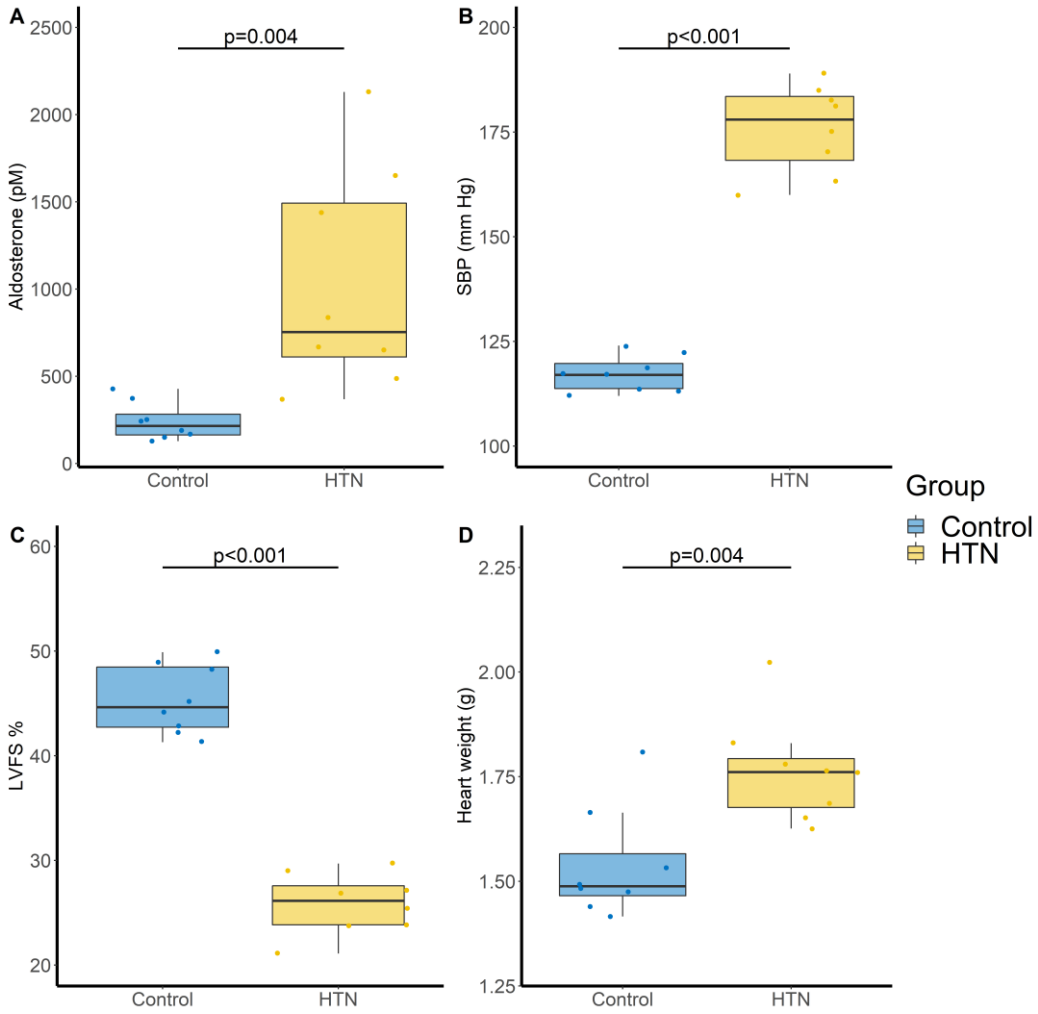

Figure 2. Evaluation of cardiovascular parameters in control and hypertensive (HTN) rats. (A) Aldoste-rone levels (pM); (B) Systolic blood pressure (SBP, mmHg); (C) Left ventricular fractional shortening (LVFS, %) and (D) Heart weight (g).

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Amongst the 22 monitored compounds, four LPA species exhibited significant increase in the HTN group compared with control group (LPA(16:0), LPA(16:1), LPA(18:1) and LPA(20:4)). However, these increases were no longer statistically significant after Benjamini–Hochberg (BH) correction to control the false discovery rate (Table 1) [19].

Table 1. Impact of Ang-II HTN on LPA, LPL and MAG levels

Analyte	Control group	HTN group	p-value	Adjusted p-value[a]
LPA(16:0)	0.11 [0.11-0.12]	0.15 [0.13-0.22]	**0.0229**	0.241
LPA(18:0)[b]	0.09 [0.08-0.10]	0.11 [0.10-0.14]	*0.0832*	0.341
LPA(18:1)	0.09 [0.09-0.10]	0.13 [0.10-0.14]	**0.0438**	0.241
LPA(18:2)[b]	1.78 [1.57-1.97]	2.20 [1.56-2.46]	0.163	0.398
LPA(20:4)[b]	1.91 [1.50-2.13]	2.56 [2.14-2.92]	**0.0308**	0.241
LPA(22:6)[b]	0.10 [0.08-0.11]	0.14 [0.11-0.17]	*0.0931*	0.341
LPC(16:0)	16.7 [16.2-17.4]	17.3 [15.0-18.0]	0.568	0.781
LPC(16:1)[b]	1.24 [1.10-1.40]	1.58 [1.40-1.77]	**0.0438**	0.241
LPC(18:0)[b]	29.7 [27.3-32.9]	30.4 [28.7-31.7]	0.914	0.914
LPC(18:1)	7.24 [6.36-7.53]	7.22 [6.83-8.14]	0.343	0.629
LPC(18:2)[b]	31.5 [28.5-32.6]	30.9 [28.2-32.0]	0.749	0.867
LPC(20:4)[b]	11.1 [10.3-12.1]	12.6 [11.6-13.7]	0.148	0.398
LPC(22:6)[b]	0.34 [0.31-0.43]	0.43 [0.38-0.46]	0.269	0.538
LPE(16:0)	0.54 [0.47-0.61]	0.62 [0.58-0.68]	0.118	0.371
LPE(18:0)[b]	0.80 [0.76-0.93]	0.95 [0.89-1.00]	0.205	0.451
LPE(18:1)[b]	0.22 [0.21-0.27]	0.25 [0.22-0.27]	0.376	0.636
LPE(18:2)[b]	0.45 [0.44-0.52]	0.46 [0.41-0.49]	0.439	0.690
LPE(20:4)[b]	0.16 [0.15-0.17]	0.17 [0.16-0.20]	0.836	0.876
LPE(22:6)[b]	0.11 [0.09-0.12]	0.12 [0.10-0.15]	0.612	0.792
MAG(18:1)	0.13 [0.10-0.16]	0.12 [0.11-0.15]	0.805	0.876
MAG(18:2)[b]	0.67 [0.57-1.00]	0.64 [0.58-0.79]	0.673	0.823
MAG(20:4)[b]	0.02 [0.02-0.03]	0.02 [0.01-0.02]	0.508	0.745

[a]p-values adjusted according to Benjamini & Hochberg. [b]Analytes without analytical standard. HTN: hypertension, LPA: lysophosphatidic acid, LPC: lysophosphatidylcholine, LPE: lysophosphatidylethanolamine, MAG: monoacylglycerol.

2.2. Chronic heart failure (CHF)

Definitive coronary artery ligation-induced CHF was demonstrated by the decrease in LVFS (54 ± 4 vs. 26 ± 5 % for control and CHF groups respectively, $p<0.001$, Figure 3A), the increase in heart weight (1.48 ± 0.12 vs. 1.68 ± 0.15 g, $p=0.008$, Figure 3B), which concerned both the left (1.13 ± 0.09 vs. 1.27 ± 0.13 g, $p=0.016$, Figure 3C) and right (0.24 ± 0.04 vs. 0.30 ± 0.05 g, $p=0.009$, Figure 3D) ventricles but no change in mean pulmonary artery pressure (17.1 ± 1.1 vs. 17.0 ± 1.4 mm Hg, $p=0.861$).

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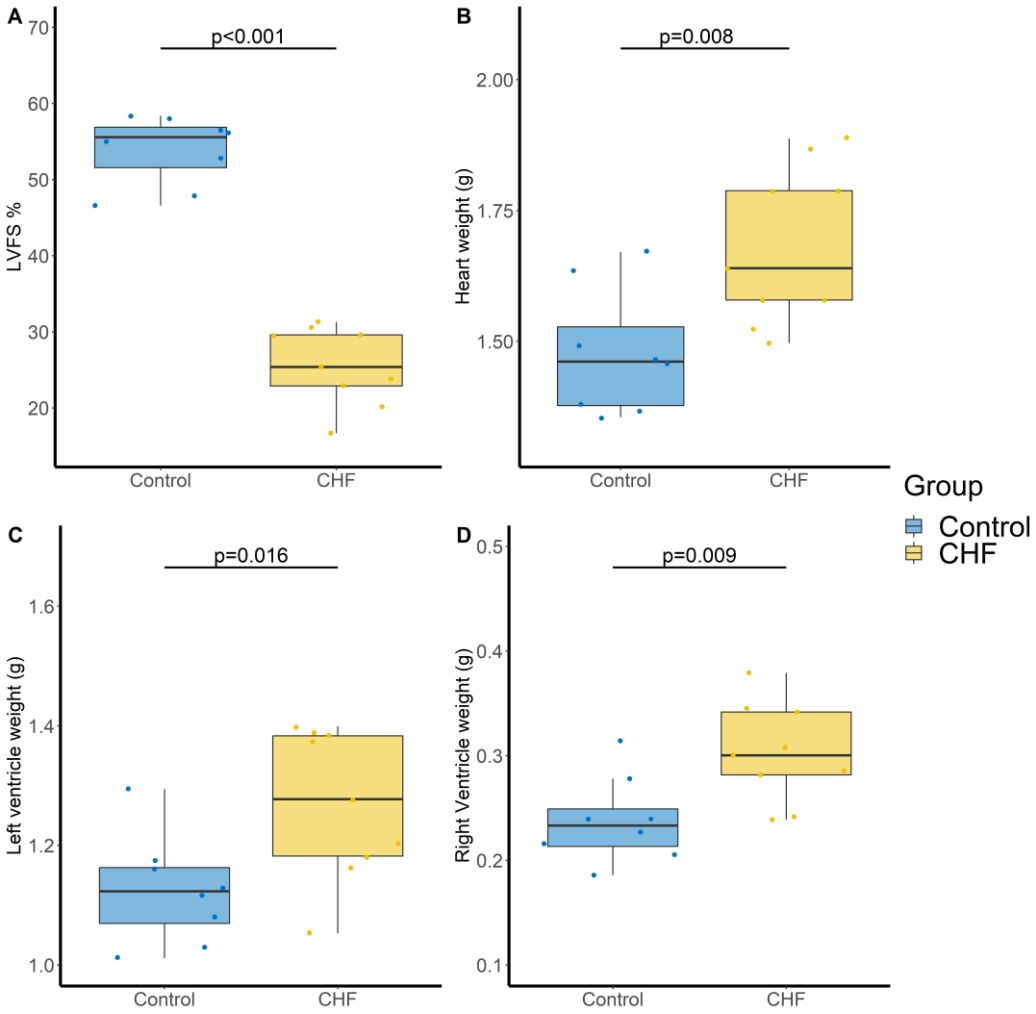

Figure 3. Evaluation of cardiac parameters in control and chronic heart failure (CHF) rats. (A) Left ventricular fractional shortening (LVFS, %), (B) Heart weight (g), (C) Left ventricle weight (g) and (D) Right ventricle weight (g).

Regarding LPA metabolism, LPA (16:0), LPE(16:0) and LPE(18:0) were increase in CHF rats compared to control rats, whereas LPC(18:1) was decrease. However, these differences were no longer significant after BH correction (Table 2).

2.3. Pulmonary hypertension (PH) induced by sugen/hypoxia (SuHx)

Administration of the VEGF-receptor antagonist SU5416 associated with hypoxia was used to induce severe PH and is a well-recognized animal model of severe PH (Group 1 PH). In this model, mean arterial pulmonary pressure (mPAP) was markedly increased compared with the control group (48 ± 7 vs. 16 ± 1 mm Hg, p<0.001, Figure 4A). Heart weight was also increased (2.3 ± 0.3 vs. 1.4 ± 0.1 g, p<0.001, Figure 4B), which concerned both the left (1.68 ± 0.09 vs. 1.04 ± 0.11 g, p<0.001, Figure 4C) and right (0.38 ± 0.05 vs. 0.23 ± 0.02 g, p<0.001, Figure 4D) ventricles. Moreover, cardiac output was decreased in the PH group compared with the control group (53 ± 9 vs. 98 ± 4 ml/min, p<0.001, Figure 4E).

Table 2. Impact of CHF on LPA, LPL and MAG levels

Analyte	Control group	CHF group	p-value	Adjusted p-value[a]
LPA(16:0)	0.058 [0.046-0.060]	0.082 [0.056-0.103]	**0.025**	0.186
LPA(18:0)[b]	0.050 [0.048-0.057]	0.070 [0.065-0.080]	0.336	0.615

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LPA(18:1)	0.069 [0.063-0.076]	0.067 [0.054-0.075]	0.757	0.779
LPA(18:2)[b]	0.87 [0.77-0.95]	1.00 [0.88-1.06]	0.193	0.499
LPA(20:4)[b]	1.24 [1.04-1.31]	1.21 [1.14-1.56]	0.204	0.499
LPA(22:6)[b]	0.066 [0.062-0.070]	0.068 [0.061-0.084]	0.447	0.615
LPC(16:0)	13.0 [11.3-14.2]	12.3 [11.1-13.2]	0.302	0.615
LPC(16:1)[b]	1.06 [0.98-1.37]	0.93 [0.71-1.60]	0.750	0.779
LPC(18:0)[b]	19.4 [15.7-20.5]	18.7 [17.1-21.4]	0.608	0.743
LPC(18:1)	5.02 [4.61-5.64]	3.97 [3.3-4.48]	**0.016**	0.186
LPC(18:2)[b]	19.7 [18.7-21.6]	19.4 [17.1-20.5]	0.430	0.615
LPC(20:4)[b]	8.97 [8.27-10.63]	8.4 [8.16-8.81]	0.350	0.615
LPC(22:6)[b]	0.386 [0.331-0.393]	0.325 [0.274-0.362]	0.196	0.499
LPE(16:0)	0.55 [0.52-0.58]	0.70 [0.61-0.72]	**0.017**	0.186
LPE(18:0)[b]	0.74 [0.63-0.79]	0.82 [0.78-0.88]	**0.046**	0.250
LPE(18:1)[b]	0.25 [0.23-0.27]	0.27 [0.25-0.30]	0.425	0.615
LPE(18:2)[b]	0.38 [0.37-0.42]	0.44 [0.37-0.48]	0.383	0.615
LPE(20:4)[b]	0.158 [0.143-0.169]	0.168 [0.167-0.201]	0.089	0.390
LPE(22:6)[b]	0.12 [0.11-0.14]	0.14 [0.12-0.15]	0.478	0.619
MAG(18:1)	0.07 [0.06-0.09]	0.11 [0.10-0.12]	0.126	0.462
MAG(18:2)[b]	0.18 [0.15-0.21]	0.17 [0.11-0.26]	0.756	0.779
MAG(20:4)[b]	0.0061 [0.0049-0.0084]	0.0068 [0.0045-0.0094]	0.779	0.779

[a]p-values adjusted according to Benjamini & Hochberg. [b]Analytes without analytical standard. HTN: hypertension, LPA: lysophosphatidic acid, LPC: lysophosphatidylcholine, LPE: lysophosphatidylethanolamine, MAG: monoacylglycerol.

2.3. Pulmonary hypertension (PH) induced by sugen/hypoxia (SuHx)

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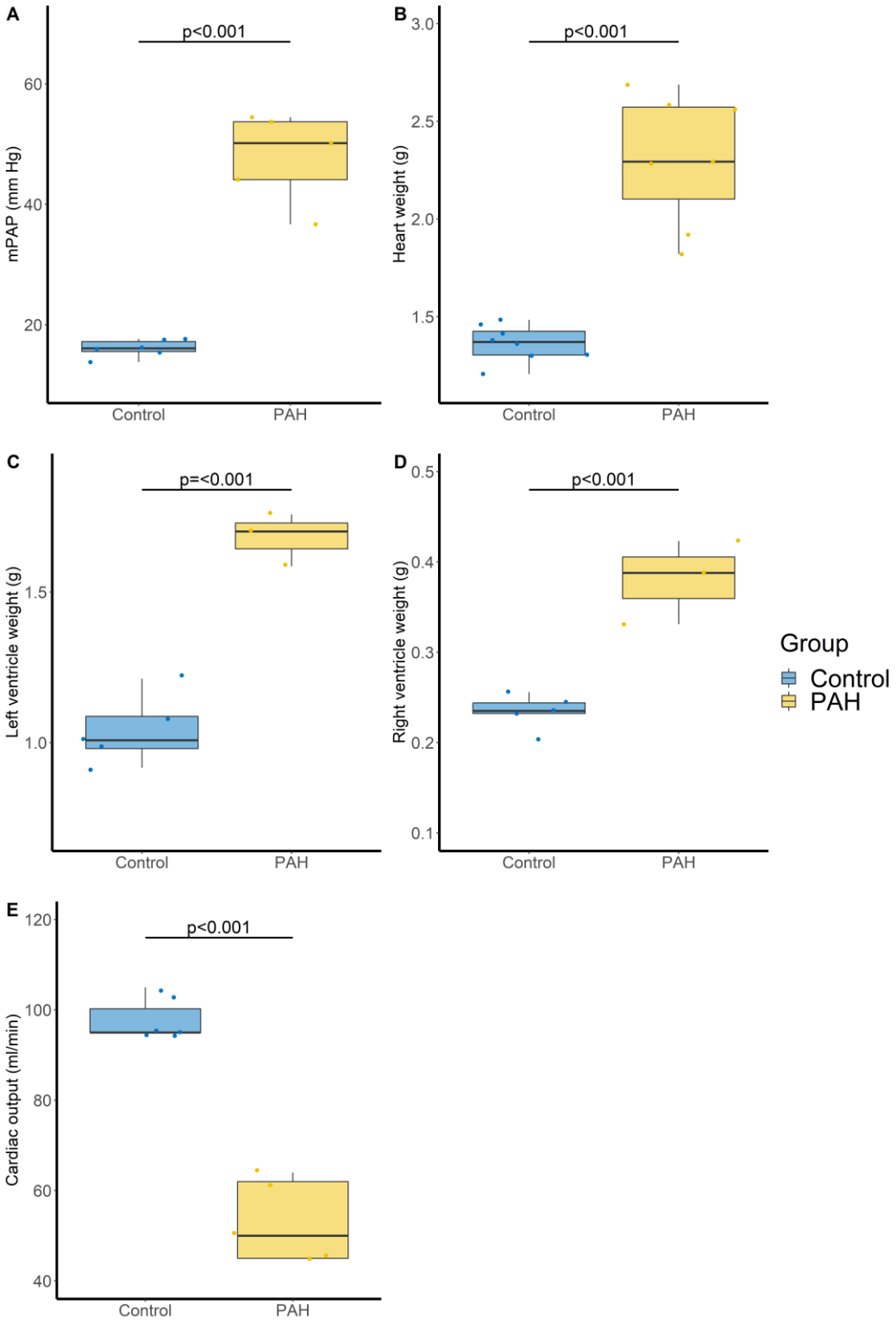

Figure 4. Evaluation of pulmonary and cardiac parameters in control and PH rats. (A) Mean pulmonary arterial pressure (mPAP, mm Hg), (B) Heart weight (g), (C) Left ventricle weight (g) (D) Right ventricle weight (g) and (E) cardiact output (ml/min). PH: pulmonary hypertension.

PH was associated with an increase in all monitored LPA that remains significant after BH correction except for LPA (18:0), and a trend tower an increase in LPE(16:0) with an adjusted p-value of 0.056 (Table 3 and Figure 5).

Table 3. Impact of PH induced by SuHx on LPA, LPL and MAG levels

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Analyte	Control group	PH group	p-value	Adjusted p-value[a]
LPA(16:0)	0.091 [0.080-0.12]	0.21 [0.19-0.26]	**<0.001**	**0.003**
LPA(18:0)[b]	0.13 [0.11-0.17]	0.19 [0.17-0.25]	0.080	0.219
LPA(18:1)	0.062 [0.056-0.085]	0.156 [0.125-0.182]	**<0.001**	**0.001**
LPA(18:2)[b]	0.99 [0.80-1.29]	2.31 [2.03-4.00]	**<0.001**	**0.001**
LPA(20:4)[b]	1.22 [1.05-1.51]	2.18 [1.99-2.76]	**<0.001**	**0.004**
LPA(22:6)[b]	0.064 [0.057-0.082]	0.137 [0.119-0.160]	**<0.001**	**<0.001**
LPC(16:0)	16.8 [16.0-17.3]	16.7 [14.9-17.2]	0.357	0.604
LPC(16:1)[b]	1.96 [1.625-2.07]	1.99 [1.585-2.86]	0.348	0.604
LPC(18:0)[b]	32.8 [31.3-35.9]	33.7 [30.7-36.0]	0.749	0.827
LPC(18:1)	7.01 [6.66-7.32]	6.80 [6.11-7.31]	0.485	0.686
LPC(18:2)[b]	28.7 [26.3-30.2]	28.1 [26.8-30.0]	0.669	0.827
LPC(20:4)[b]	11.1 [10.3-11.8]	9.3 [7.6-10.4]	0.231	0.462
LPC(22:6)[b]	0.36 [0.32-0.39]	0.36 [0.33-0.39]	0.693	0.827
LPE(16:0)	0.78 [0.73-0.79]	0.81 [0.80-1.03]	**0.015**	0.056
LPE(18:0)[b]	1.14 [1.05-1.28]	1.39 [1.23-1.48]	0.053	0.168
LPE(18:1)[b]	0.29 [0.27-0.34]	0.38 [0.335-0.42]	0.149	0.328
LPE(18:2)[b]	0.53 [0.47-0.59]	0.62 [0.53-0.66]	0.457	0.686
LPE(20:4)[b]	0.23 [0.21-0.25]	0.22 [0.18-0.24]	0.131	0.320
LPE(22:6)[b]	0.158 [0.143-0.167]	0.176 [0.146-0.179]	0.752	0.827
MAG(18:1)	0.095 [0.066-0.156]	0.089 [0.058-0.113]	0.499	0.686
MAG(18:2)[b]	0.70 [0.40-1.10]	0.60 [0.41-0.98]	0.860	0.860
MAG(20:4)[b]	0.014 [0.011-0.018]	0.015 [0.006-0.023]	0.806	0.844

[a]p-values adjusted according to Benjamini & Hochberg. [b]Analytes without analytical standard. HTN: hypertension, LPA: lysophosphatidic acid, LPC: lysophosphatidylcholine, LPE: lysophosphatidylethanolamine, MAG: monoacylglycerol.

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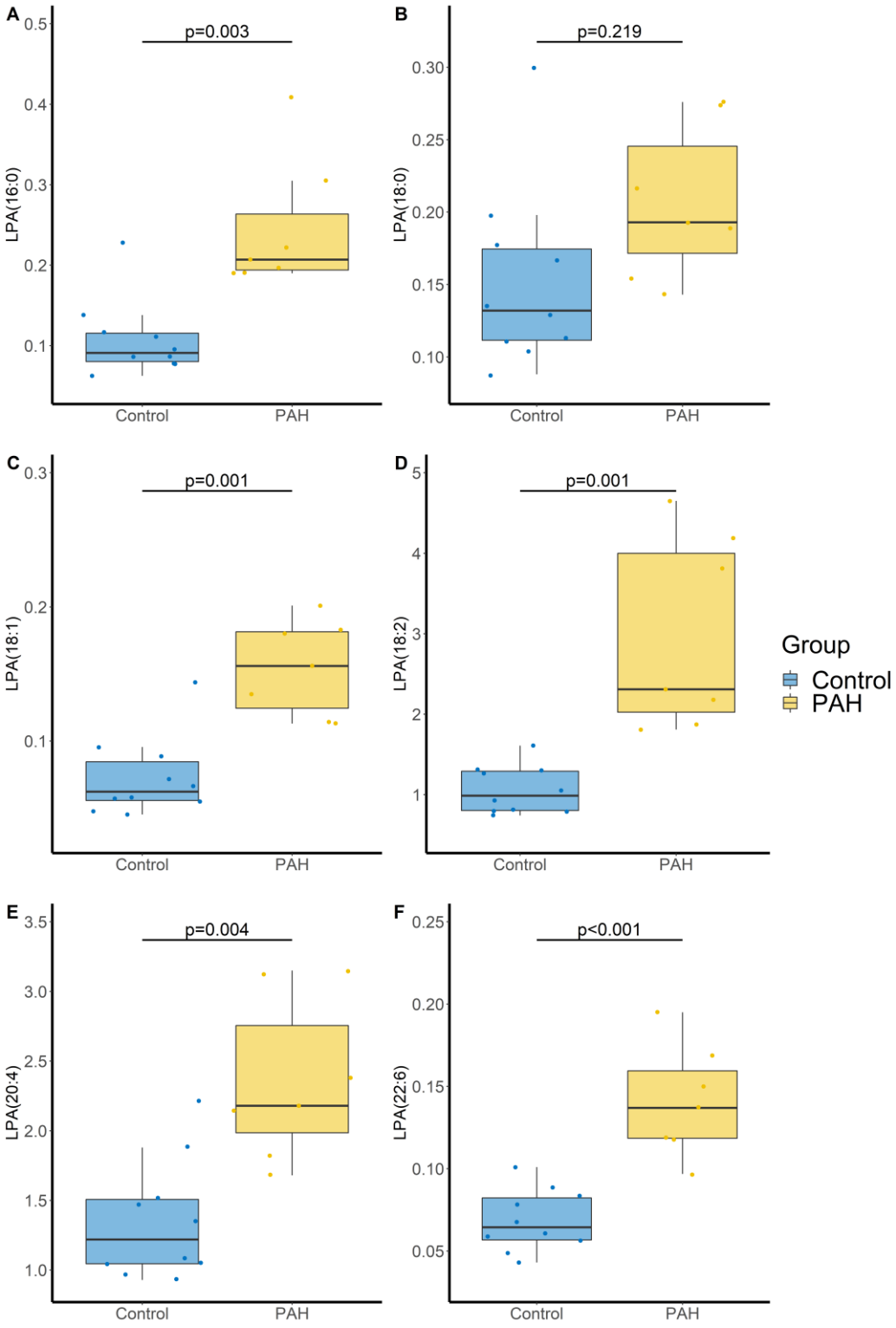

Figure 5. Impact of PH on lysophosphatidic acid (LPA) species (A) LPA(16:0), (B) LPA(18 :0); (C) LPA(18:1), (D) LPA(18:2), (E) LPA(20:4), (F) LPA(22:6)

2.4. Impact of LPA on pulmonary artery smooth muscle cell (PA-SMC) proliferation

To better evaluate whether LPA species may be involved in the pathophysiology of PH, we investigated their impact on the proliferation of human pulmonary artery smooth muscle cells (SMC) assessed using 5-bromo-2-deoxyuridine (BrdU) incorporation. Of interest, the conducted ANOVA was significant (p=0.004) and all tested LPA species (LPA(18:1), LPA(18:2) and LPA(20:4)) induced an increase SMC proliferation (Figure 6).

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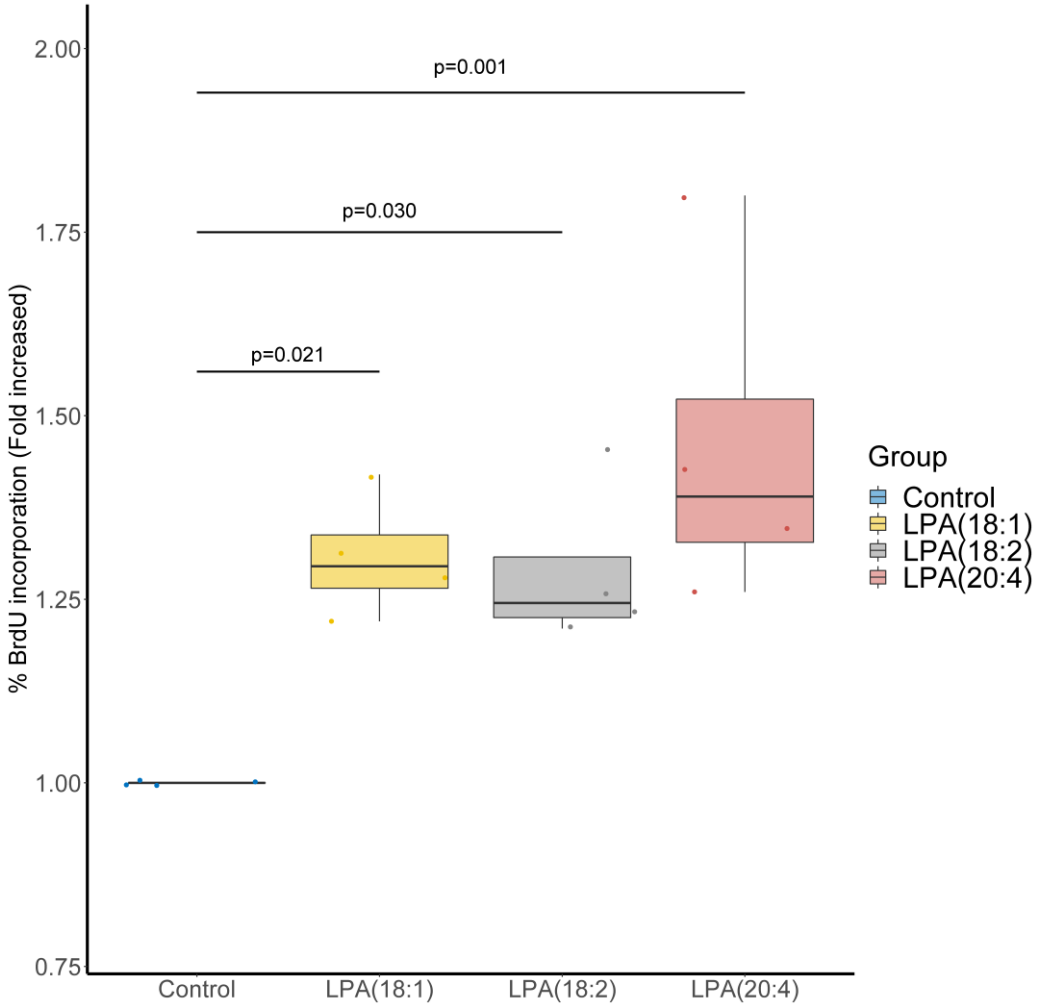

Figure 6. *Impact of LPA species on pulmonary artery smooth muscle cell proliferation assessed using 5-bromo-2-deoxyuridine (BrdU) incorporation. Technical replicates with PA-SMC derived from 4 patients.*

3. Discussion

The major finding of the present work is that amongst the three well-characterized models of CVD, induction of PH by SuHx strongly increased plasma LPA and that these lipid mediators potentiated the proliferation of isolated human PA- SMC.

3.1. LPL, LPA and MAG quantitation analytical method

The present analytical method aimed to monitor 22 compounds. The choice has been made to investigate compounds with and without analytical standards to have the widest possible overview of LPA metabolism based on known fragmentation patterns for each class. This leads to the limit that results are expressed as the compound-to-17:1-LPA area under the curve ratio. Furthermore, since we did not have specific internal standard for each class, we cannot be sure of the reliability of the correction of matrix effects for LPL and MAG. However, for each model, all samples were prepared and analyzed uninterruptedly resulting in the absence of inter-batch variability [20]. Furthermore, the use of 17:1-LPA, even if not being the best-suited internal standard for all compounds, allowed to decrease within-batch variability normalizing area under the curve of the monitored compounds. Finally, the low dispersibility of the intra-group values allows us to be sufficiently confident about the robustness and reliability of the results obtained.

Precautions should be taken in the preparation and analysis of these compounds. First of all, since LPA are highly acidic compounds, it is strongly recommended to perform an acidic extraction since extractions at non-acidic pH will result in poor recovery. This

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could be done using hydrochloric or formic acid. Here, we used formic acid to avoid production of LPA by LPL hydrolysis that could be induced by too acidic solutions. Furthermore, it is mandatory to chromatographically resolved LPA and LPL with the same esterified fatty acid since collision-induced dissociation may artificially produce LPA. The natural abundance of LPA being a tiny fraction of LPL, hydrolysis of only a minor part of LPL to LPA, either during extraction of analysis by the MS, could dramatically increase the concentration of these compounds [21].

3.2. CVD models

All evaluated models have been well characterized with an increase in SBP, a decrease in LVFS and an increase in mPAP for the HTN, CHF and PH model respectively. As observed in humans, the HTN and PH models were also associated with the development of LV hypertrophy and dysfunction.

The development of HTN induced a significant increase of 3 LPA species under unadjusted assumption. This may be justified by the fact that Ang-II modulates LPA1 receptor function [22] and induces LPA production by phospholipases activation as well as the production of phosphatidic acids [23-24].

CHF rats exhibited an increase in LPA (16:0) and LPE(16:0-, 18:0-) associated with a decrease in LPC(18:1). Interestingly, all increased compounds possess an esterified saturated fatty acid (SFA) suggesting a link between CHF-mediated oxidative stress and desaturase activity [25-26]. Furthermore, it is now well known that reducing intake of saturated fatty acids may reduce LDL-c and CVD risks [27-28]. SFA possessed numerous deleterious effects such as inflammation, apoptosis, mitochondrial dysfunction and oxidative stress [29] but their impact has mainly been evaluated as non-esterified fatty acids [30]. Thus, the role of esterified SFAin LPA and LPL esterified needs further investigations.

In the SuHx rat model of severe PH, LPA metabolism and activity seem to be a promising pathway to target. Indeed, 80% of the monitored LPA exhibited a 2- to 3- fold increase when compared with the control group. Furthermore, addition of LPA species in a cell media of isolated human PA- SMCs at a concentration of 1µM, which is relevant when compared with circulating LPA levels [31], promoted an increased proliferation. In fact, accumulation of PA-SMCs within the pulmonary arterial walls are one of the most prominent features of PH, which can lead to the narrowing or occlusion of pulmonary vessels and therefore plays an important role in the occurrence and development of PH [32-35]. At this time, the role of LPA remains unclear even if their increase in hypoxic pulmonary vascular remodeling has already been described [36]. Of note, LPA are able to regulate the hypoxia-inducible factor 1α (HIF-1α) [37], that can interact with enzymes and other transcription factors in order to control vascularization and tissue growth in response to hypoxic conditions [38].

3.3. LPA signaling

Since LPA metabolism is complex with distinct anabolic and catabolic pathways, it may be easier to specifically act on LPA receptors rather than try to modulate enzymes involved in LPA metabolism regulation. However, it is now well admitted that LPA activates at least 6 specific G protein-coupled receptor named as $LPAR_{1-6}$. The downstream signals derived from activation of LPAR involve Rho, phospholipase C, phosphatidylinositol 3-kinase and adenylate cyclase intracellular pathways, thus producing diverse physio(patho)logical effects [18]. Recently, $LPAR_4$ was found to contribute to elevation of blood pressure with the more potent effect for LPA 18:1 and 20:4 species [39]. Interestingly, LPAR activation exhibited different reactivity according to the acyl chain (carbon length and number of insaturations) and the esterification position (sn-1 or sn-2) of the LPA [40]. Another key point is related to the relative expression of those receptors in lung tissue where PH takes place. All $LPAR_{1-6}$ were expressed in lung tissue according to https://www.genecards.org/ and to date, it has been demonstrated that $LPAR_1$ and $LPAR_2$ knock-down protects against pulmonary remodeling, lung inflammation and lung injury [41-43].

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4. Materials and Methods

4.1. Experimentation.

4.1.1. Chemicals

Methanol (MeOH), Dichloromethan (DCM) and water of HPLC grade were purchased from Carlo Erba (Fontenayaux-Roses, France). Formic acid was purchased from VWR chemicals (Leuven, Belgium). LPA(16:0), LPA(17:1), LPA(18:1), LPC(16:0), LPC(18:1), LPE(16:0) LPS(16:0) and MAG(18:1) were purchased from Avanti Polar Lipids (Interchim Montluçon, France). Ammonium acetate and formic acid were purchased from Carlo Erba (Fontenay-aux-Roses, France). Chromatographic Accucore XL C18 4 µm (150 x 3 mm) column was purchased from ThermoFisher Scientific (Illkirch, France)

4.1.2. Animals

All the animal care and procedures were approved by French Animal Experimentation Ethics Committees and performed in accordance with the guidelines from the French National Research Council for the Care and Use of Laboratory Animals (Permit Numbers: Apafis #24107 approved on February 12, 2020 and #11484 approved on April 6, 2018). All experiments were performed in 10-weeks-old male wild-type Sprague-Dawley (SD/Crl) (Charles-River) rats.

4.2. Murine models of cardiovascular diseases (CVD)

4.2.1. Angiotensin-II induced hypertension (HTN)

Systemic arterial hypertension was induced using 4-week ang-II infusion with osmotic pumps (0.25 µg/kg/day), implanted subcutaneously in isoflurane-anesthetized rats. Non-implanted rats served as controls. Tail-cuff plethysmography was used in trained conscious animals to confirm the development of systemic hypertension.

4.2.2. Chronic heart failure (CHF) induced by coronary artery ligation

For ischemic HF, myocardial infarction was induced by definitive left coronary artery ligation as previously described (J Mol Cell Cardiol. 2012 Mar;52(3):660-6.). Briefly, rats were anesthetized using methohexithal (50 mg/kg i.p.), intubated and ventilated at 60 cycles/min (tidal volume 1 ml/100 g of body weight). A left thoracotomy was performed, and the heart exposed. A 6/0 polypropylene suture was passed around the proximal left coronary artery, which was tied in order to induce myocardial ischemia. Ten minutes after coronary artery ligation, the chest was closed, the pneumothorax was evacuated, and the animals were allowed to recover. Sham-operated rats, subjected to the same protocol except that the coronary artery was not occluded, served as controls. Twelve weeks after surgery, the development of HF was confirmed using transthoracic echocardiography performed in rats anesthetized with isoflurane, using a Vivid 7 ultrasound echograph (GE Healthcare, Buc, France). A two-dimensional short axis view of the left ventricle was obtained at the level of the papillary muscle, in order to record M-mode tracings. Left ventricular end-diastolic (LVEDD) and systolic diameters (LVESD), allowing the determination of LV fractional shortening (FS) as FS (%)=((LVEDD-LVESD)/LVEDD)×100.

4.2.3. Pulmonary hypertension (PH) induced by sugen hypoxia (SuHx)

For PH, the SuHx model, in which occlusive neointimal lesions are observed, was used as previously described [33]. Briefly, rats were injected subcutaneously with SU5416 (a VEGF-receptor antagonist; 20 mg/kg) and exposed to hypoxia (10% FiO_2) for 3 weeks. Then, these rats returned to normoxia (21% FiO_2) for additional 5 weeks before evaluation. Control rats were not injected and remained under normoxia for 8 weeks.

To confirm the development of PH, right ventricular hemodynamic measurements were performed in anesthetized rats using a polyvinyl catheter introduced into the right external jugular vein, advanced in the right ventricle, and further, in the pulmonary artery, allowing the measurement of mean pulmonary arterial pressure.

4.3. Human PA-SMC proliferation

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Lung specimens were obtained during lobectomy or pneumonectomy for localized lung cancer. The lung specimens were collected at a distance from the tumor foci. This study was approved by the local ethics committee (CPP Est-III: N°ID RCB: 2018-A01252-53, N° CPP: 18.06.06) and all patients gave informed consent before the study. Human PA-SMC were isolated and cultured as previously described (J Clin Invest. 2001 Oct;108(8):1141-50; Cardiovasc Res. 2020 Mar 1;116(3):686-697.) PA-SMC proliferation was measured by BrdU incorporation and by cell counting. BrdU staining was measured by the DELFIA® Cell proliferation kit (PerkinElmer, Courtaboeuf, France) and Time-resolved fluorometer EnVisionTM Multilabel Reader (PerkinElmer) 24h of culture without and with LPA(18:1), LPA (18:2) and LPA (20:4) at 1µM.

4.4. LPL, LPA and MAG quantitation

4.4.1. Blood sampling

At the time of sacrifice, blood samples were drawn in the aorta of rats anesthetized with isoflurane using 2-mL syringes. Blood was transferred on a prechilled ethylenediaminetetraacetic acid (EDTA) tubes and immediately centrifuged 5 min at 4500g (4°C). Then, the plasma was frozen in liquid nitrogen and stored at -80°C until analysis.

4.4.2. High performance liquid chromatography (HPLC)

HPLC was carried out using a Prominence Shimadzu UFLC system consisting of a DGU-20A3 degasser, a LC-20AB pump, a SIL-20ACHT autosampler and a CTO20AC oven (Shimadzu, Prominence, Kyoto, Japan). Chromatographic separation was performed on an Accucore XL C18 column (4-µm particle size, 150-mm length x 3-mm inner diameter). The autosampler temperature was set at +8°C, the column oven at 50°C, the injected volume was 10µL, and the flow rate was 500 µL/min. The method had the following gradient conditions using 1% CH_3COOH and 10mM ammonium acetate in MeOH (solvent A) and 1% CH_3COOH and 10mM ammonium acetate in water (solvent B): 0 - 0.5 min, 70% A, 0.5 - 5.5 min, 70% - 95% A, 5.5 - 10 min, 95% A, 10 - 10.5 min, 95% - 70% A, 10.5 – 12 min, 70% A.

4.4.3. Tandem mass spectrometry (MS^2)

MS^2 was performed using a 4500QTRAP operating in the positive/negative electrospray ionization (ESI) switching mode (Sciex, Toronto, Canada). Instrument control and data acquisition were performed with Analyst 1.6.3 software. The source parameters were optimized as follow; ion spray voltage: -4500V and +4500V for negative and positive ionization mode respectively; nebulisation gas: 60 psi; desolvatation gas: 50 psi; curtain gas: 30 psi; source temperature: 500°C, entrance potential: -10V and +10V for negative and positive ionization mode respectively, collision activation dissociation: medium. Relative quantitation was done using multiple reaction mode (MRM) to monitor transition from precursor to product ions. MS^2 parameters were optimized by direct infusion of LPA(16:0), LPA(17:1), LPA(18:1), LPC(16:0), LPC(18:1), LPE(16:0) and MAG(18:1) in pure MeOH at a concentration of 1 µg/mL. For monitored compounds without analytical standards (LPA(18:0-; 18:2-; 20:4-; 22:6-); LPC(16:1-; 18:0-; 18:2-; 20:4-; 22:6-); LPE(18:0-; 18:1-; 18:2-; 20:4-; 22:6-) and MAG(18:2-; 20:4-), transitions used were obtained from the LIPID MAPS tools "Predict MS/MS spectrum for a glycero(phospho)lipid" (https://www.lipidmaps.org/tools/). According to typical ionization and fragmentation patterns of each LPL class, MS1 were $[M-H]^-$ adduct and MS2 was set to 152.9 for LPA (Glycerol-3-phosphate ion with loss of H_2O), MS1 were $[M-CH_3]^-$ adduct and MS2 was set to the carboxylate ion of the corresponding fatty acid (sn1 acyl chain $[RCOO]^-$) for LPC, MS1 were $[M-H]^-$ adduct and MS2 was set to 196.0 (neutral loss of fatty acid) for LPE and MS1 were $[M+H]^+$ adduct and MS2 was set to the acylium form of the corresponding fatty acid (sn1 acyl chain ($[RC=O]^+$)) for MAG (Table 4).

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Table 4. MS parameters for studied analytes

Analyte	Mass transition		MS parameters			Adduct
	m/z (MS1)	m/z (MS2)	DP (V)	CE (eV)	CXP (V)	
LPA(16:0)	409.5	152.9	-50	-40	-10	$[M-H]^-$
LPA(17:1)[a]	421.1	152.9	-50	-40	-10	$[M-H]^-$
LPA(18:0)[b]	437.3	152.9	-50	-40	-10	$[M-H]^-$
LPA(18:1)	435.2	152.9	-50	-40	-10	$[M-H]^-$
LPA(18:2)[b]	433.2	152.9	-50	-40	-10	$[M-H]^-$
LPA(20:4)[b]	457.2	152.9	-50	-40	-10	$[M-H]^-$
LPA(22:6)[b]	481.2	152.9	-50	-40	-10	$[M-H]^-$
LPC(16:0)	480.6	255.4	-80	-40	-10	$[M-CH_3]$
LPC(16:1)[b]	478.6	253.4	-100	-40	-10	$[M-CH_3]$
LPC(18:0)[b]	508.4	283.5	-100	-40	-10	$[M-CH_3]$
LPC(18:1)	506.4	281.5	-80	-40	-10	$[M-CH_3]$
LPC(18:2)[b]	504.4	279.5	-100	-40	-10	$[M-CH_3]$
LPC(20:4)[b]	528.4	303.4	-100	-40	-10	$[M-CH_3]$
LPC(22:6)[b]	552.4	327.4	-100	-40	-10	$[M-CH_3]$
LPE(16:0)	452.4	196.0	-50	-40	-10	$[M-H]^-$
LPE(18:0)[b]	480.3	196.0	-50	-40	-10	$[M-H]^-$
LPE(18:1)[b]	478.3	196.0	-50	-40	-10	$[M-H]^-$
LPE(18:2)[b]	476.3	196.0	-50	-40	-10	$[M-H]^-$
LPE(20:4)[b]	500.6	196.0	-50	-40	-10	$[M-H]^-$
LPE(22:6)[b]	524.3	196.0	-50	-40	-10	$[M-H]^-$
MAG(18:1)	357.2	265.0	50	17	16	$[M+H]^+$
MAG(18:2)[b]	355.2	263.0	50	17	16	$[M+H]^+$
MAG(20:4)[b]	379.3	287.1	50	17	16	$[M+H]^+$

[a] Analyte used as internal standard for area under the curve normalization. [b]Analytes without analytical standard. CE: collision energy, CXP: collision cell exit potential, DP: declustering potential, LPA: lysophosphatidic acid, LPC: lysophosphatidylcholine, LPE: lysophosphatidylethanolamine, MAG: monoacylglycerol

4.5. Data and statistical analysis

Data, statistical analysis and captions were performed using *R* v4.1.0 software [44] and *DescTools* package [45]. For relative quantitation of LPL, data were expressed as median (interquartile range or IQR) and p-values were computed by t.test followed by Benjamini & Hochberg correction [19]. Captions were performed using *ggplot2, ggsci* and *ggpubr* packages [46-48]. For cell culture data analysis, ANOVA was conducted first then p-values were computed using Dunnett's post-hoc test. For LPL, LPA and MAG quantitation, due to the lack of analytical standard for several compounds, 17:1-LPA served as internal standard (IS) and data are expressed as compound-to-IS area under the curve (AUC) ratio which is unit-free. R code for statistical analysis, rat models' data and cell culture data are available in Supplementary File S1, Supplementary File S2 and Supplementary File S3 respectively.

5. Conclusions

This study pointed out the fact that LPA metabolism and signaling represent promising therapeutic targets that may decrease occurrence and/or improve the outcome of CVD. Modulation of LPA metabolism and signaling using specific inhibitors, antagonists or gene deletions may be of particular interest to deal with PH, where a significant increase in plasmatic LPA species was observed; the latter being associated with an increase of pulmonary artery smooth muscle proliferation, which plays a central role in the physiopathology of this disease.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, File S1: R code for statistical analysis, File S2: raw data.

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Informed Consent Statement: Not applicable

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