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Suppression of cardiac phosphatidate phosphohydrolase 1 activity and lipin mRNA expression in Zucker diabetic fatty rats and humans with type 2 diabetes mellitus

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ABSTRACT

Lipin functions in mammalian phospholipid biosynthesis through its phosphatidate phosphohydrolase 1 (PAP_1) activity. Here, we studied cardiac PAP_1 activity and lipin expression *ex vivo* in 8-month-old Zucker diabetic fatty (ZDF) rats and humans with type 2 diabetes mellitus undergoing open heart surgery for coronary bypass grafting. Compared to non-diabetic littermates (ZDF-*fa/+*), left ventricular PAP_1 activity was 29% lower in diabetic ZDF-*fa/fa* rats. Left ventricular PAP_1 activities were 2.1-fold (ZDF-*fa/fa*) and 3.6-fold (ZDF-*fa/+*) higher than the respective atrial activities, indicating marked differences in cardiac distribution of PAP_1. PAP_1 activity was highly related with cardiac lipin-1 and lipin-3 mRNA expression in ZDF rats ($r = 0.99$ and 0.96). Consistent with the findings in experimental animals, human atrial tissue displayed PAP_1 activity that was 33% lower in those having diabetes than in non-diabetic controls. Accordingly, atrial lipin-1 and lipin-3 mRNA expression in diabetic patients was 50% and 59% lower as in non-diabetic patients, respectively. Insulin therapy increased both PAP_1 activity and lipin mRNA expression in diabetic patients. We conclude that suppression of cardiac PAP_1 activity/lipin expression may contribute to metabolic dysfunction of the diabetic heart.

Introduction

Phosphatidate phosphohydrolase 1 (PAP_1) is a key enzyme in mammalian lipid biosynthesis [1]. PAP_1 catalyzes the conversion of phosphatidate (PA) to diacylglycerol which serves as precursor for the *de novo* synthesis of the phospholipids phosphatidylcholine, phosphatidylethanolamine, and triacylglycerol (TAG). PAP_1 is regulated by translocation from the cytosol to the endoplasmic reticulum; its catalytic activity is Mg^{2+}-dependent and sensitive to sulfhydryl-reactive reagents such as *N*-ethylmaleimide. Only recently, it has been found that mammalian PAP_1 activity is determined by the lipin family of proteins (lipin-1, -2, -3) that exhibit unique, but overlapping, tissue distributions [2–4]. A second class of PAP activity (lipid phosphate phosphatase [LPP], basically termed PAP_2) was characterized in mammalian cells based upon a lack of requirement for bivalent cations and insensitivity to inhibition of *N*-ethylmaleimide [5]. In contrast to the substrate specificity of PAP_1, LPPs hydrolyze several other lipids in addition to PA and are localized to the exterior surface of plasma membranes or on the luminal surface of internal membranes. Biologically, LPPs are normally considered to regulate cell signaling by the phospholipase D pathway [6].

Although altered lipid homeostasis has emerged as an important predictor of metabolic cardiomyopathy and atherosclerotic heart disease in diabetes mellitus [7], surprisingly little information is available concerning the functional status of PAP_1 and lipins in diabetic myocardium. In rats with streptozotocin-induced acute diabetes, it has been found that total myocardial PAP activity (i.e. PAP_1 and LPP activity) was either unaffected or slightly increased [8–10]. In male JCR:LA corpulent rats (which are hyperphagic, hypertriglyceridaemic and insulin resistant), activity of PAP_1 in the heart was lower in corpulent than in lean control animals [11]. Phan and Reue [12] have demonstrated recently that muscle-specific lipin-1 transgenic mice on high-fat diet exhibit moderate obesity, hyperglycemia, hyperinsulinemia, and insulin resistance in a pattern very similar to type 2 diabetes mellitus. In humans, *LPIN1* and *LPIN2* gene polymorphisms have significant effects in metabolic traits such as insulin level, glucose metabolism, body mass index, and fat distribution [13,14]. So far, however,

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nothing is known about putative alterations of cardiac PAP_1 activity or lipin expression in humans suffering from type 2 diabetes.

Therefore, the purpose of this study was to investigate the enzymatic status of PAP_1 as well as lipin expression in myocardium of (1) genetically type 2 diabetic Zucker diabetic fatty (ZDF) rats and (2) type 2 diabetic patients with atherosclerotic heart disease.

Materials and methods

The present study has been carried out in accordance with *The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans and animals*. Animal and human study protocols were approved by an institutional ethics committee and all patients gave written informed consent.

Preparation of ZDF hearts. Fifteen homozygous (*fa/fa*) 6-week-old male ZDF rats and 15 heterozygous (*fa/+*) male littermates were obtained from Charles River (Sulzfeld, Germany). Animals were maintained on Purina 5008 diet (International Product Supplies, London, England) and water *ad libitum* at a 12-h light/dark cycle. At a mean age of 8 months, fed animals were anesthetized with 150 mg/kg thiopental sodium (Byk Gulden, Konstanz, Germany) intraperitoneally. After medial laparotomy, blood was taken from the inferior vena cava for estimation of blood glucose (Ascensia Elite, Bayer Diagnostics, Dublin, Ireland). The thorax was opened, the heart rapidly removed and weighed.

Of the 15 ZDF-*fa/+* and 15 ZDF-*fa/fa* hearts, 6 hearts each were instrumented for determination of left ventricular (LV) function applying a working heart apparatus from Hugo Sachs Elektronik (March-Hugstetten, Germany) as described previously in detail [15]. In the remaining 9 ZDF-*fa/+* and 9 ZDF-*fa/fa* hearts, the left ventricle (including the interventricular septum) and atria were dissected. Tissue samples (1.0–1.5 mm) from the middle of the left ventricle were fixed in 4.5% formalin solution and processed for paraffin embedding. The remaining LV tissue and the pooled right and left atrial tissue were frozen in liquid nitrogen, pulverized and homogenized in ice-cold lysis buffer (5 mM Tris, 2 mM EDTA, pH 7.5). After centrifugation at 1000*g* for 10 min and determination of protein concentration in the supernatant [16], small aliquots of LV and atrial proteins were frozen in liquid nitrogen and stored at −80 °C prior to PAP_1 and lipin assay.

Preparation of human atrial tissue. Atrial tissue was obtained from 15 patients with type 2 diabetes mellitus (mean duration 5.6 ± 1.2 years) and 21 non-diabetic patients who underwent open heart surgery for coronary bypass grafting. Each diabetic patient received a glucose-reduced diet, 7 patients were treated additionally with insulin. Specimens (mean weight 97 ± 8 mg) were obtained from the right atrial appendage during venous cannulation for extracorporal circulation. Immediately after excision, samples were dissected, snap-frozen in liquid nitrogen and stored at −80 °C until further analysis of PAP_1 activity, lipin expression and collagen content. For determination of PAP_1 activity and lipin expression, the tissue was processed as described above.

Quantification of preoperative non-fasting plasma glucose, plasma triglycerides, serum creatinine, and hemoglobin A_{1C} levels was performed using standard laboratory methods. Plasma glucose and serum creatinine of each patient was calculated from 1 to 4 measurements and considered as $n = 1$. Preoperative cardiac performance was assessed by left ventriculography/multi-plane coronary angiography and transthoracic echocardiography utilizing standard techniques. Mean arterial blood pressure and heart rate (taken from resting electrocardiogram) was determined immediately prior to surgery.

PAP_1 assay. PAP_1 activity was determined by a slightly modified nonradioactive assay developed recently in our laboratory [17]. An aliquot of the fluorescent derivative of PA 1-hexanoyl-2-[6-[(7-nitro-2–1,3-benzoxadiazol-4-yl)amino]hexanoyl]-*sn*-glycero-3-phosphate (NBD-PA, Avanti Polar Lipids, Alabaster, AL, USA) was dissolved in reaction buffer (20 mM Tris, 5 mM $MgCl_2$, 2 mM EDTA, 1 mM EGTA, pH 7.5) giving the concentration of 1 mM NBD-PA (starting reaction). The assay (for determination of total PAP activity) was initiated by addition of 5 μl starting reagent to 45 μl reaction buffer containing 1 μg of myocardial proteins from ZDF rats or humans. In a parallel assay, LPP activity was assayed in the absence of $MgCl_2$ in the reaction buffer containing 5 mM *N*-ethylmaleimide (Sigma–Aldrich, München, Germany). Incubations were allowed to proceed for 30 min at 37 °C in a thermomixer at 1000 rpm. PAP_1 activity was then calculated by subtracting LPP activity from total PAP activity. All experiments were performed in the presence of the phospholipase A_2 inhibitor methyl arachidonyl fluorophosphonate (1 μM, Merck Biosciences, Darmstadt, Germany).

Lipid extraction and separation by HPLC. Total lipids were extracted by a slightly modified method of Bligh and Dyer [18]. In brief, incubations were terminated by the addition of 300 μl ice-cold methanol and 150 μl chloroform containing 0.005% (w/v) butylated hydroxytoluene. After a cooling period of 10 min on ice, another 150 μl of chloroform and 220 μl 150 mM KCl were added and the lipids extracted into the chloroform phase by shaking for 2 min. For complete phase separation, samples were centrifuged for 3 min at 13,000*g* and the lower phase was evaporated to dryness under N_2 stream. Extracted lipids were separated by normal-phase HPLC on a LiChrosphere 100 Diol (5 μm) 250 × 4 mm column (Merck) with a binary gradient of hexane–isopropanol and isopropanol–water in the presence of 1% (v/v) acetic acid and 0.08% (v/v) triethylamine. The HPLC system was interfaced with a fluorescence detector (SFM 25, Kontron Instruments, Neufahrn, Germany) that was set at an excitation and emission wavelength of 460 and 534 nm (NBD), respectively. The detector signal was recorded and integrated with Clarity software from Techlab (Erkerode, Germany).

RNA extraction and quantitative RT-PCR. Total RNA from atrial and LV myocardial tissue of ZDF rats and human atrial tissue was isolated using NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) following the manufacturers instructions. For quantitative RT-PCR analysis, cDNA was synthesized using First Strand Synthesis kit (Invitrogen, Karlsruhe, Germany). Reverse-transcribed cDNA was subsequently amplified with Platinum SYBR Green qPCR Super Mix with Rox kit (Invitrogen) using 2 μl cDNA and 0.75 μl of appropriate lipin-specific upstream and downstream primers (Invitrogen) (Table 1). Each assay also included a standard curve of 4 serial dilution points of control cDNA and a no-template control. The cDNA was analyzed by RT-PCR during 40 cycles on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Relative concentrations of mRNA encoding for lipins were determined by plotting the threshold cycle vs. the log of the serial dilution points. The level of transcripts encoding for the constitutive housekeeping gene GAPDH was used for data normalization.

Sirius red staining. Deparaffinized LV circumferential sections (2 μm) from ZDF rats and frozen human atrial sections (4 μm) (embedded in Tissue Freezing Medium, Jung, Nussloch, Germany) were incubated in 0.1% Sirius red in a saturated solution of picric acid for 60 min (rat tissue) and 10 min (human tissue), respectively. Unbound Sirius red was removed with distilled water and samples were placed in Weigert's hematoxylin solution (Sigma–Aldrich) for 2 min. After dehydration with increasing concentrations of ethanol (70%, 96%, and 100%), samples were embedded in Eukitt (Kindler, Freiburg, Germany) and examined and photographed at a magnification of 10× with an Olympus IX70 microscope (Hamburg, Germany). Five fields located in the middle third of the myocardium (covering the majority of the myocardium) were

Table 1
Lipin-specific primers with corresponding accession numbers.

Primer	ZDF rat	Human
Lipin-1	NM_001012111	NM_145693
Forward	5′-CTTCCAGACCATTCACAGCGA-3′	5′-CAGAGTTGTTGCCTCCGTTGT-3′
Reverse	5′-TTTCCGTCTGAGCCTTGCTCT-3′	5′-AGAAATCTGCGATCGATGGC-3′
Lipin-2	NM_001108236	NM_014646
Forward	5′-ACCGAATGCCTCTCGGATT-3′	5′-GGATCACAGGAGCTCGAAGAA-3′
Reverse	5′-CGTGGTACGTGATGATGTGCT-3′	5′-TTTGCGATCTGGTCTGAGGAG-3′
Lipin-3	NM_001014184	NM_022896
Forward	5′-TTGAAACACAAATGGCCGTG-3′	5′-GCCCTGAGGAACATGCATTT-3′
Reverse	5′-ATTCGGTGCCATCTCTGTCAG-3′	5′-TTAACACCCCTGCAAAACCC-3′

evaluated. Analysis and quantification of total interstitial fibrillar collagen content were performed with digital image software cell^{F} (Soft Imaging System, Münster, Germany). Data are presented as fractional area of collagen content in % of myocardial tissue.

Statistical analysis. All data are given as means ± standard error of the mean. Statistical analysis was performed by two-tailed unpaired Student's *t*-test when two groups/parameters were compared. One-way analysis of variance combined with Bonferroni's *post hoc* test was applied to assess differences between three or more groups/parameters. Categorical variables were analyzed using the χ^2-test. Correlation coefficients (r) were determined and evaluated by the Pearson test. A value of $p < 0.05$ was considered statistically significant. All analyses were performed using GraphPad Prism software (Version 4.00, San Diego, CA, USA).

Results

ZDF rats

Baseline and hemodynamic characteristics at 8 months of age are summarized in Table 2. Non-fasting blood glucose was 4.6-fold higher in ZDF-*fa/fa* rats compared to ZDF-*fa/+* littermates. ZDF-*fa/fa* rats weighed markedly less than ZDF-*fa/+* rats reflecting the natural course of severe, uncontrolled diabetes in ZDF rats [19]. The heart weight as well as the heart weight to body weight ratio did not differ significantly between both rat strains. Mean heart rate, LV developed pressure (LV systolic pressure – LV diastolic pressure), and LV contraction and relaxation were 14–34% lower in isolated working hearts from ZDF-*fa/fa* rats thus indicating the presence of cardiac dysfunction. Coronary flow and LV collagen content was found to be nearly identical in both groups (Table 2).

PAP_1 activity in the left ventricle was 29% lower in diabetic than in non-diabetic ZDF rats (Fig. 1). No such difference of enzyme activity was found in atrial myocardium. In ZDF-*fa/fa* and ZDF-*fa/*

Table 2
Baseline parameters of heterozygous (*fa/+*) and homozygous (*fa/fa*) Zucker diabetic fatty (ZDF) rats.

	ZDF-*fa/+* (n = 15)	ZDF-*fa/fa* (n = 15)
Blood glucose (mg/dl)	109 ± 4	498 ± 29**
BW (g)	430 ± 11	363 ± 15**
HW (g)	1.79 ± 0.08	1.67 ± 0.07
HW/BW (g/kg)	4.19 ± 0.18	4.68 ± 0.21
Heart rate (beats per min)[a]	171 ± 12	133 ± 11*
LVDP (mm Hg)[a]	138 ± 2	119 ± 5**
LV dP/dt_{max} (mm Hg/s)[a]	5371 ± 266	3549 ± 241**
LV dP/dt_{min} (mm Hg/s)[a]	−4608 ± 106	−3256 ± 279**
Coronary flow (ml/min)[a]	11.71 ± 1.61	11.65 ± 1.24
LV collagen content (%)[b]	13 ± 2	15 ± 2

BW, body weight; HW, heart weight; LV, left ventricular; LVDP, left ventricular developed pressure; LV dP/dt_{max} and LV dP/dt_{min}, maximal rates of LV pressure rise and fall.

[a] Hemodynamic parameters were obtained from 6 ZDF-*fa/+* and 6 ZDF-*fa/fa* isolated working hearts.

[b] Collagen content was determined in 9 untreated ZDF-*fa/+* and 9 untreated ZDF-*fa/fa* hearts.

* $p < 0.05$ vs. ZDF-*fa/+*.

** $p < 0.01$ vs. ZDF-*fa/+*.

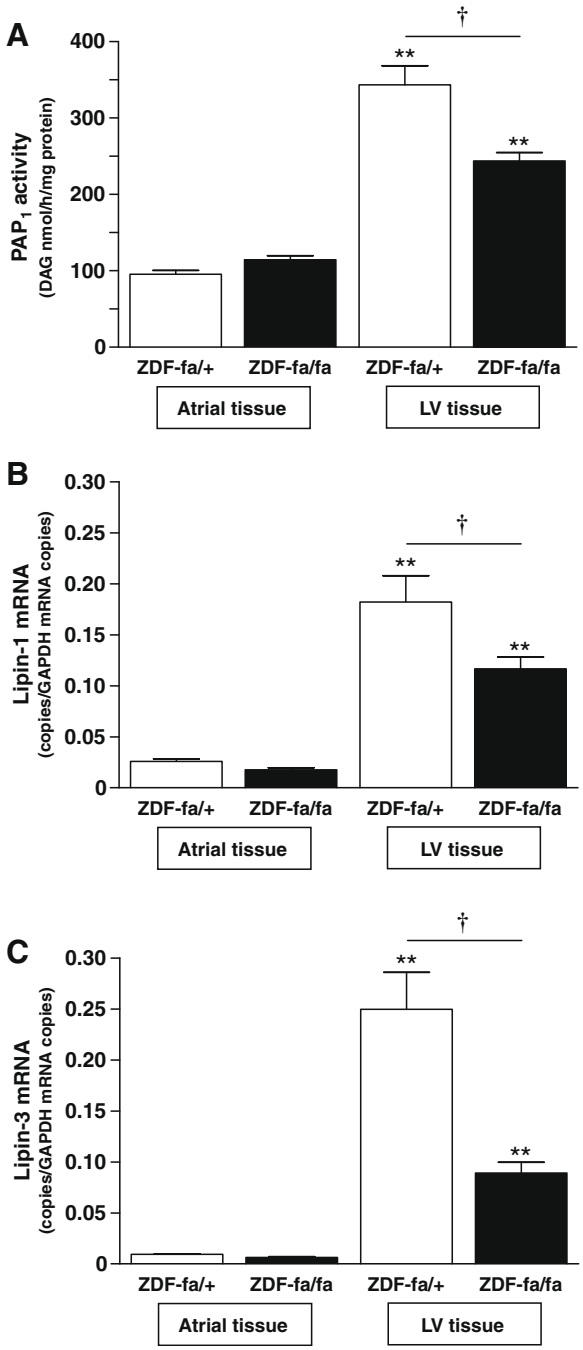

Fig. 1. Activity of phosphatidate phosphohydrolase 1 (PAP_1) (A) and mRNA expression of lipin-1 (B) and lipin-3 (C) in atrial and left ventricular (LV) tissue of 8-month-old heterozygous (*fa/+*) and homozygous (*fa/fa*) Zucker diabetic fatty (ZDF) rats (n = 9 each). DAG, diacylglycerol. **$p < 0.01$ vs. respective atrial enzyme activity/lipin expression, †$p < 0.01$.

+ rats, LV PAP_1 activities were 2.1- and 3.6-fold higher than the respective atrial activities, indicating marked differences in cardiac distribution of PAP_1 (Fig. 1). Accordingly to the reduced LV PAP_1 activity but unaffected atrial activity in ZDF-*fa/fa* rats, LV lipin-1 and lipin-3 mRNA expression was 36% and 64% lower in ZDF-*fa/fa* rats whereas atrial lipin-1 and lipin-3 mRNA expression was not affected compared to ZDF-*fa/+* littermates (Fig. 1). Furthermore, in accordance to the regional distribution of myocardial PAP_1, LV lipin-1 and lipin-3 expression was 7- to 27-fold higher than respective atrial mRNA expression (Fig. 1). Lipin-2 was not detectable in hearts of ZDF rats. Correlation analysis revealed a strong relation between PAP_1 activity and lipin-1 and lipin-3 expression in myocardial tissue from ZDF rats (Fig. 2) further supporting that lipin codes for PAP activity.

Human atrial tissue

Clinical characteristics of the non-diabetic and diabetic cohorts are summarized in Table S1. Significant differences were found with respect to the preoperative patient age and plasma glucose and triglyceride levels. The haemoglobin A_{1C} concentration was moderately increased in diabetic patients. All other baseline characteristics including gender, body mass index, serum creatinine, heart rate, mean arterial blood pressure, and cardiac performance were comparable between the groups (Table S1).

Compared to non-diabetic patients, atrial PAP_1 activity was reduced by 33% in diabetic patients treated solely with a glucose-reduced diet whereas no significant difference of atrial PAP_1 activity was observed between diabetic patients with insulin therapy and non-diabetic patients (Fig. 3). Accordingly, atrial lipin-1 and lipin-3 mRNA expression in diet-treated diabetic patients was 50% and 59% lower as in non-diabetic patients, respectively (Fig. 3). Insulin therapy increased both lipin-1 and lipin-3 mRNA expression. Lipin-2 at levels of mRNA was not detectable in human atrial tissue. Atrial collagen content as assessed by Sirius red staining was not significantly different between the groups (non-diabetic patients 12 ± 1%, diet-treated diabetic patients 12 ± 2%, insulin-treated diabetic patients 15 ± 3%).

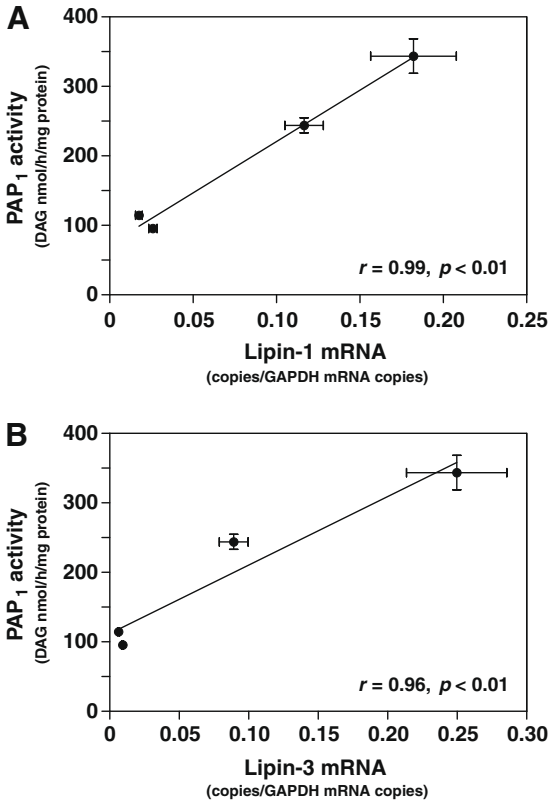

Fig. 2. Correlation analyses between phosphatidate phosphohydrolase 1 (PAP_1) activity and lipin-1 (A) and lipin-3 (B) mRNA expression in Zucker diabetic fatty rats. Correlation coefficients (r) were analyzed by the Pearson test. DAG, diacylglycerol.

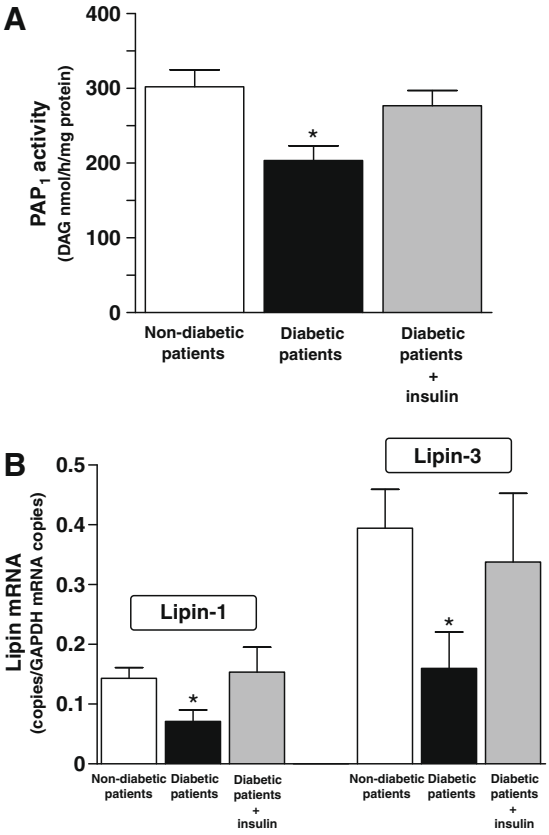

Fig. 3. Activity of phosphatidate phosphohydrolase 1 (PAP_1) (A) and mRNA expression of lipin-1 and lipin-3 (B) in atrial tissue from non-diabetic patients ($n = 21$), diet-treated diabetic patients ($n = 8$), and insulin-treated diabetic patients ($n = 7$) undergoing open heart surgery for coronary bypass grafting. DAG = diacylglycerol. $^*p < 0.05$ vs. non-diabetic patients.

Discussion

It is well known that PAP_1 is regulated in response to altered metabolic conditions such as obesity or diabetes mellitus. For example, in experimental models of type 1 diabetes, suppression of enzymatic activity was found in kidney and adipose tissue whereas in the liver and the heart, activities were either unaffected or increased [8–10,20–23]. In insulin-resistant humans, expression levels of lipin-1 (which biochemically acts as PAP_1 enzyme) are markedly attenuated in liver and adipose tissue [24–26]. Our present results in diabetic ZDF rats are in line with the latter studies supporting the finding that reduction of LV PAP_1 activity results from a lack of endogenous lipin-1 and lipin-3 expression. Previously, transfection of each of the lipin protein family members in cultured 293T cells revealed that every member display PAP_1 activity and that in contrast to LPPs, this activity is specific for PA [4]. In the same study, it was demonstrated also that lipin-1, lipin-2, and lipin-3 mRNA is expressed in considerable amounts in the human heart. Although we found that atrial PAP_1 activity and lipin-1/lipin-3 mRNA were consensual reduced in diabetic patients, atrial PAP_1 activity/lipin mRNA expression was not affected in diabetic ZDF

rats. The precise cause for these distinct findings is not known. However, we believe that species disparities, difference in duration of diabetes and/or absence of pharmacological interventions in ZDF rats might had fundamental influences. On the other hand, the regional difference of PAP_1 activity and expression of lipin in ZDF hearts indicate that metabolically more active tissue (such as the left ventricle) have a significantly higher enzyme/mRNA abundance. Donkor and colleagues [4] have noticed that human lipin-1 mRNA was most abundant in adipose tissue followed by skeletal muscle whereas lipin-2 was expressed at substantial levels in liver, brain and also adipose tissue. Notably, lipin-3 displayed a very distinct pattern with significant expression mainly in the digestive tract. It may be regarded as a limitation of our study that regional differences of PAP_1 activity and lipin levels were presently not investigated in the human heart. However, as the disposability of human LV samples is very limited for obvious reasons, we used human atrial tissue to study cardiac PAP_1 activity and lipin mRNA expression. In contrast to the study of Donkor et al. [4], we could not detect lipin-2 mRNA in the examined human tissue specimens. Unfortunately, in the mentioned study it was not stated where in the heart the samples were obtained from. Thus, it might be possible that the discrepancy arises from different cardiac tissues used. To our best knowledge, we provided first evidence of regional differences in activities and distribution pattern of PAP_1 and LPP in human myocardial samples [17]. The present paper further demonstrates that PAP_1 activity and lipin expression in the human heart is differentially regulated in response to type 2 diabetes mellitus.

The diabetic heart is usually characterized by high levels of TAG and thus, a reduction in the enzymatic activity of PAP_1 was virtually unexpected. Although this was not the focus of our study, our findings may be explained by reduced endogenous insulin levels that could have affected PAP_1 activity and/or lipin expression. Plasma insulin levels in diabetic ZDF rats markedly decreased at 28 weeks of age, which is the same time point that these rats began to lose weight [19]. We observed that atrial PAP_1 activity as well as expression of lipins were higher in insulin-treated than in diet-treated diabetic patients. Harris and colleagues [27] have shown in 3T3-L1 adipocytes that the alterations in lipin phosphorylation induced by exogenously applied insulin were associated with a redistribution of lipin between soluble proteins and microsomal membranes. In addition, changes in PAP activity in the two fractions exactly matched the changes in lipin protein. It was further proposed that under conditions of insulin resistance, increased action of glucocorticoids relative to insulin stimulates hepatic *LPIN1* transcription and leads to an increase in lipin-1 protein amounts and PAP_1 activity [28]. However, we are aware that the exact underlying pathophysiological mechanisms for our findings remain speculative since other important components of phospholipid biosynthesis such as fatty acid uptake/β-oxidation rates or phospholipid concentrations were presently not determined. Moreover, it is well known that PAP_1/lipin activity appears to be regulated at several other levels including transcription, posttranslational modification, and subcellular localization [3]. Underlying structural alterations which could have modulated PAP_1 activity and/or lipin expression appear nevertheless unlikely since myocardial fibrosis, LV hypertrophy, or atrial dilatation was not markedly augmented in diabetic ZDF rats or humans, respectively.

Conclusion

This study provides the first evidence that the activity of PAP_1 and expression of lipin-1 and lipin-3 mRNA is significantly reduced in failing LV myocardium of type 2 diabetic ZDF rats and in atrial tissue from patients with type 2 diabetes mellitus. Suppression of PAP_1/lipin may contribute to metabolic cardiomyopathy which is frequently seen in rodents and humans with type 2 diabetes mellitus.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.09.108.

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