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Cardiac oxidative stress in a mouse model of neutral lipid storage disease ☆

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ABSTRACT

Cardiac oxidative stress has been implicated in the pathogenesis of hypertrophy, cardiomyopathy and heart failure. Systemic deletion of the gene encoding adipose triglyceride lipase (ATGL), the enzyme that catalyzes the rate-limiting step of triglyceride lipolysis, results in a phenotype characterized by severe steatotic cardiac dysfunction. The objective of the present study was to investigate a potential role of oxidative stress in cardiac ATGL deficiency. Hearts of mice with global ATGL knockout were compared to those of mice with cardiomyocyte-restricted overexpression of ATGL and to those of wildtype littermates. Our results demonstrate that oxidative stress, measured as lucigenin chemiluminescence, was increased ~6-fold in ATGL-deficient hearts. In parallel, cytosolic NADPH oxidase subunits p67phox and p47phox were upregulated 4–5-fold at the protein level. Moreover, a prominent upregulation of different inflammatory markers (tumor necrosis factor α, monocyte chemotactant protein-1, interleukin 6, and galectin-3) was observed in those hearts. Both the oxidative and inflammatory responses were abolished upon cardiomyocyte-restricted overexpression of ATGL. Investigating the effect of oxidative and inflammatory stress on nitric oxide/cGMP signal transduction we observed a ~2.5-fold upregulation of soluble guanylate cyclase activity and a ~2-fold increase in cardiac tetrahydrobiopterin levels. Systemic treatment of ATGL-deficient mice with the superoxide dismutase mimetic Mn(III)tetrakis (4-benzoic acid) porphyrin did not ameliorate but rather aggravated cardiac oxidative stress. Our data suggest that oxidative and inflammatory stress seems involved in lipotoxic heart disease. Upregulation of soluble guanylate cyclase and cardiac tetrahydrobiopterin might be regarded as counterregulatory mechanisms in cardiac ATGL deficiency.

1. Introduction

Cardiac lipotoxicity is regarded as sum of various cell-damaging processes: Besides disruption of organelle membranes and activation of the apoptotic machinery, ER stress and oxidative stress have been identified as crucial components of the lipotoxic response [1]. Different enzymatic sources of oxidative stress including NADPH oxidase (for review, see [2]), uncoupled nitric oxide synthase (NOS; [3]), and xanthine oxidase [4], have been implicated in the development of cardiac dysfunction. Recent evidence points to an essential role of NADPH oxidases (NAD(P)H:oxygen oxidoreductases; EC 1.6.3.1) as mediators of lipid-driven oxidative stress. Thus, diet-induced obesity in rats resulted in ectopic fat deposition and concomitant upregulation of the NADPH oxidase 2 (NOX2) system in liver and heart [5]. Similarly, a previous study on Lep/Lep mice, a genetic model of obesity, suggested that cardiac contractile dysfunction is related to enhanced NOX2-dependent oxidative stress [6]. Besides NOX2, NOX4 has been described as the second important NADPH oxidase isoform in the heart. However, its distinct role in cardiac pathophysiology is unclear yet, since both

Abbreviations: ATGL, adipose triglyceride lipase; ATGL(−/−), adipose triglyceride lipase knockout; BH_2, dihydrobiopterin, [2-amino-6-(1,2-dihydroxypropyl)-7,8-dihydro-1H-pteridin-4-one]; BH_4, tetrahydrobiopterin, [(6R)-2-amino-6-[(1R,2S)-1,2-dihydroxypropyl]-5,6,7,8-tetrahydropteridin-4(1H)-one]; DEA/NO, 2,2-diethyl-1-nitroso-oxyhydrazine; DAG, diacylglycerol; eNOS, endothelial nitric oxide synthase; FFA, free fatty acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; (s)GC, (soluble) guanylate cyclase; IL-6, interleukin 6; Mac-2, galectin-3; MCP-1, monocyte chemotactic protein-1; MnTBAP, Mn(III) tetrakis (4-benzoic acid) porphyrin chloride; NADPH, nicotinamide adenine dinucleotide phosphate; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOX, NADPH oxidase; $ONOO^-$, peroxynitrite; PBS, phosphate-buffered saline; PKC, protein kinase C; PPARα, peroxisome proliferator receptor α; SOD, superoxide dismutase; TG, triacylglycerol; TNFα, tumor necrosis factor α; VASP, vasodilator-stimulated phosphoprotein; pVASP, phosphorylated vasodilator-stimulated phosphoprotein

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beneficial and harmful effects have been demonstrated in different animal models of NOX4 overexpression or knockout [7,8].

With the recent discovery of adipose triglyceride lipase (ATGL) a key enzyme of triglyceride catabolism was identified [9]. ATGL catalyzes the initial step of the triglyceride lipolysis cascade *i.e.* the hydrolytic cleavage of triacylglycerols (TGs) into free fatty acids (FFAs) and diacylglycerols (DAGs). The enzyme is predominantly expressed in adipose tissue, but it is also present to a lesser extent in cardiac muscle, skeletal muscle, and other tissues. Systemic ablation of ATGL in mice (ATGL(−/−)) yielded a phenotype with increased whole body fat mass and neutral lipids accumulating in adipose and non-adipose tissues [10]. In cardiac muscle, ATGL deficiency caused an age-dependent increase of myocyte lipid droplets in number and size. In parallel, echocardiographic analysis demonstrated the progressive development of ventricular hypertrophy with abnormal myocardial texture and systolic dysfunction [10]. Using Langendorff perfusion of isolated hearts we have recently demonstrated that the contractile and microvascular response to ß-adrenergic stimulation was drastically reduced in ATGL deficiency [11]. Contrariwise, transgenic mice with supraphysiological protein expression of ATGL in cardiomyocytes (WT/MHC-A35) showed reduced triglyceride levels in the heart as well as improved basal pump function and protection against experimentally induced pathophysiological stress [12].

Concerning the prevalence of obesity- and diabetes-related human cardiomyopathy that is characterized by myocardial fat deposition and compromised contractile function, ATGL-deficient and overexpressing mice are invaluable tools to study lipotoxic heart disease. Using these animal models we aimed to investigate the potential role of oxidative stress in the development and progression of lipid-driven cardiomyopathy with special emphasis on the specific role of cardiac NADPH oxidases.

2. Theory

ATGL(−/−) mice suffer from severe cardiac function due to abnormal accumulation of triglycerides within cardiomyocytes. Since oxidative stress has been identified as crucial component of cardiac lipotoxicity in experimental animals [5,6] we hypothesized that a similar mechanism might be operative in ATGL-deficient hearts. The theory will be tested on the one hand by identifying potential sources of oxidative stress and on the other hand by chronic treatment of animals with a superoxide dismutase mimetic.

3. Materials and methods

3.1. Materials

2,2-Diethyl-1-nitroso-oxyhydrazine (DEA/NO), 1-benzyl-3-(5-hydroxymethylfur-2-yl) indazole (YC-1) and Mn(III)tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP) (Enzo Life Sciences AG, Lausen, Switzerland) were purchased through Eubio (Vienna, Austria). Antibodies were purchased as indicated: NOX2 and $p67^{phox}$ (BD Transduction Laboratories, Heidelberg, Germany); $p47^{phox}$ (Novus Biologicals, Cambridge, UK); VASP (Cell Signaling through New England Biolabs, Frankfurt, Germany); NOX4 (Abcam, Cambridge, UK); pVASP (Calbiochem through VWR, Vienna, Austria); Mac-2 (galectin-3; Cedarlane through Szabo-Scandic, Vienna, Austria); phosphorylated protein kinase C (PKC; pan; Thr 410 of human PKCζ; Cell Signaling); GAPDH (Sigma, Vienna, Austria). [^{3}H]L-Arginine (40 Ci/mmol) and [α-^{32}P]GTP (400 Ci/mmol) were obtained from PerkinElmer (Vienna, Austria); gp91ds-*tat* was synthesized by piChem (Graz, Austria). ChemiGlow™ was obtained from Biozym (Vienna, Austria). Protease Inhibitor Cocktail tablets (Complete™) were from Roche (Vienna, Austria). Materials for real-time PCR experiments were from Applied Biosystems (Vienna, Austria). GenElute™ Mammalian Total RNA Miniprep Kit, RIPA buffer, and all other chemicals were from Sigma (Vienna, Austria).

3.2. Animals

Homozygous ATGL(−/−) mice [10] and corresponding wild-type (WT) littermates were used for this study. In addition, WT and ATGL(−/−) mice with cardiomyocyte-specific overexpression of ATGL termed WT/MHC-A35 mice and ATGL(−/−)/MHC-A35 mice, respectively [12,13], were used for most experiments. After weaning, animals received standard laboratory mouse chow and water *ad libitum*. Mice were housed in approved cages and kept on a regular 12 h dark/light cycle. Animals were sacrificed at 9 weeks of age. Hearts were dissected, cleaned, and frozen for further biochemical analysis. All animals received care in accordance with the Austrian law on experimentation with laboratory animals (last amendment, 2012), which is based on the US National Institutes of Health guidelines.

3.3. MnTBAP treatment

WT and ATGL(−/−) mice (aged 6 weeks) were treated with MnTBAP ($5\ mg \times kg^{-1} \times d^{-1}$; i.p.) dissolved in phosphate-buffered saline (PBS) as vehicle once per day for 3 weeks. Application of vehicle to WT and ATGL(−/−) mice had no effect. After sacrifice of the animals, hearts were excised and plasma was collected and frozen for biochemical analysis. Cardiac and plasma MnTBAP levels were measured by HPLC as described [14]. The protocol was approved by the Austrian Federal Ministry for Science and Research (BMWF-66.007/0002-II/3b/2012).

3.4. NADPH oxidase activity

Hearts were homogenized in 10 volumes of PBS containing Complete™ using a glass potter Elvehjem homogenizer. Total homogenates (50–100 μg of protein) were incubated in PBS containing diethylenetriamine pentaacetic acid (100 μM) at 37 °C for 30 min in the absence or presence of the NADPH oxidase inhibitor gp91ds-*tat* (50 μM) [15], the SOD mimetic MnTBAP (10 μM) or Cu, Zn SOD (500 U/ml). Thereafter, NADPH (300 μM) was added to activate NADPH oxidases, followed by addition of lucigenin at a non-redox cycling concentration of 5 μM [16]. Lucigenin-derived chemiluminescence was measured every 10 s for 3 min using a TriCarb® 2100TR Liquid Scintillation Counter (PerkinElmer, Vienna, Austria). Results were corrected for protein-deficient blanks and expressed as cpm per μg protein.

3.5. Determination of NOS activity

Hearts were homogenized in 10 volumes of a 50 mM triethanolamine/HCl buffer (pH 7.4) containing 1% (v/v) ß-mercaptoethanol, 10 mM 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate, 0.5 mM EDTA and Complete™ with a glass potter Elvehjem homogenizer. Homogenates were centrifuged at 10,000 *g* for 10 min at 4 °C. Supernatants were assayed for NOS activity as conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline as previously described [17]. Results were corrected for enzyme-deficient blanks and recovery of L-citrulline.

3.6. Real-time PCR

Total RNA was isolated from homogenized hearts using the GenElute™ Mammalian Total RNA Miniprep Kit including DNAse treatment of samples. RNA was transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit. Real-time PCR analysis was performed with ~25 ng cDNA using TaqMan® Universal PCR Master Mix and pre-designed TaqMan® Gene Expression Assays for NOX2 (Mm 00432775_m1), NOX4 (Mm 00479246_m1), tumor necrosis factor α (TNFα; Mm 00443258_m1), monocyte chemotactic protein-1 (MCP-1; Mm 00441243_g1), and interleukin 6 (IL-6; Mm 00446190_m1). Reactions were carried out on a 7300 Real-Time