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Original Article

Fenofibrate unexpectedly induces cardiac hypertrophy in mice lacking MuRF1

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ABSTRACT

The muscle-specific ubiquitin ligase muscle ring finger-1 (MuRF1) is critical in regulating both pathological and physiological cardiac hypertrophy in vivo. Previous work from our group has identified MuRF1's ability to inhibit serum response factor and insulin-like growth factor-1 signaling pathways (via targeted inhibition of cJun as underlying mechanisms). More recently, we have identified that MuRF1 inhibits fatty acid metabolism by targeting peroxisome proliferator-activated receptor alpha (PPARα) for nuclear export via mono-ubiquitination. Since MuRF1 −/− mice have an estimated fivefold increase in PPARα activity, we sought to determine how challenge with the PPARα agonist fenofibrate, a PPARα ligand, would affect the heart physiologically. In as little as 3 weeks, feeding with fenofibrate/chow (0.05% wt/wt) induced unexpected pathological cardiac hypertrophy not present in age-matched sibling wild-type (MuRF1 +/+) mice, identified by echocardiography, cardiomyocyte cross-sectional area, and increased beta-myosin heavy chain, brain natriuretic peptide, and skeletal muscle α-actin mRNA. In addition to pathological hypertrophy, MuRF1 −/− mice had an unexpected differential expression in genes associated with the pleiotropic effects of fenofibrate involved in the extracellular matrix, protease inhibition, hemostasis, and the sarcomere. At both 3 and 8 weeks of fenofibrate treatment, the differentially expressed MuRF1 −/− genes most commonly had SREBP-1 and E2F1/E2F promoter regions by TRANSFAC analysis (54 and 50 genes, respectively, of the 111 of the genes >4 and <−4 log fold change; $P \leq .0004$). These studies identify MuRF1's unexpected regulation of fenofibrate's pleiotropic effects and bridges, for the first time, MuRF1's regulation of PPARα, cardiac hypertrophy, and hemostasis.

Abbreviations: ANP, atrial natriuretic peptide; apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-I; APOH, apolipoprotein H; βMHC, beta-myosin heavy chain; MYH7, myosin heavy chain 7; BNP, brain natriuretic peptide; cJun, Jun proto-oncogene; cMyBP-c, cardiac myosin binding protein-c; Col1A, collagen, type I, alpha I; cpt1, carnitine palmitoyltransferase 1A; cTnI, cardiac troponin I; CSTA, cystatin A; DAVID, Database for Annotation, Visualization and Integrated Discovery; E2F-1/E2F1, E2F transcription factor (1); FA, fatty acid; FABP1, fatty acid binding protein 1; FABP3, fatty acid binding protein 3; FABP4, fatty acid binding protein 4; FATP1, fatty acid transporter, aka SLC27A1 (solute carrier family 27 member 1); H19, imprinted maternally expressed transcript (nonprotein coding); KNG1, kininogen 1; IGF-1, insulin-like growth factor-1; lpl, lipoprotein lipase; mt-C01, mitochondrial cytochrome *c* oxidase subunit 1; mt-Cytb, mitochondrial cytochrome *b*; mt-ND1, mitochondrial NADH dehydrogenase 1; MuRF1, muscle ring finger-1; Myl-2, myosin light chain-2; Myl-3, myosin light chain-3; NKX2.5, NK2 homeobox 5; PGC-1, peroxisome proliferator-activated receptor gamma, coactivator 1 alpha; PPARα, peroxisome proliferator-activated receptor alpha; PDK4, pyruvate dehydrogenase kinase 4; PLN, phospholamban; PKP2, plakophilin 2; SRF, serum response factor; PRKAA2, protein kinase, AMP-activated alpha2 catalytic subunit; SREBP-1, sterol regulatory element binding transcription factor 1; TnnI3, troponin I; Serpin A1B, serpin peptidase inhibitor.

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1. Introduction

The muscle-specific ubiquitin ligase muscle ring finger-1 (MuRF1) plays a critical role in the regulation of both pathological and physiological cardiac hypertrophy in vivo. Mice globally lacking the striated muscle-specific MuRF1 (MuRF1 −/−) exhibit an exaggerated physiological hypertrophy in response to exercise and an exaggerated pathological hypertrophy in response to pressure overload. This is attributed to MuRF1's ability to inhibit serum response factor (SRF) and insulin-like growth factor-1 (IGF-1) signaling pathways, in part, through its targeted inhibition of cJun) [1–3]. MuRF1 also has a more direct role in regulating cardiac muscle mass by targeting proteasome dependent degradation of sarcomere proteins [4–6]. MuRF1 interacts directly with cardiac troponin I, cardiac myosin binding protein-c (cMyBP-c), and myosin heavy chain, to direct their subsequent poly-ubiquitination and proteasome-dependent degradation [4,6,7]. This regulation of sarcomere degradation explains why MuRF1 −/− mice have resistance to cardiac atrophy and a limited ability to regress upon unloading after the induction of pressure overload-induced cardiac hypertrophy [8]. By regulating both the indirect signaling processes that activate cardiac hypertrophy and directly targeting sarcomere proteins for degradation, MuRF1 regulates the heart's response to external stress that lead to disease.

Increasing cardiomyocyte MuRF1 inhibits fatty acid (FA) oxidation, which has been found to be mediated, in part, by MuRF1's inhibition of the FA metabolism [9]. Increasing MuRF1 inhibits peroxisome proliferator-activated receptor alpha (PPARα) though mono-ubiquitination, which targets the nuclear export of PPARα resulting in activity inhibition [9]. Conversely, MuRF1 −/− hearts have a 500% increase in PPARα activity, while demonstrating no significant differences in cardiac PPARδ/β and PPARγ activities in vivo [9]. Enhanced PPARα activity has been attributed to the pathogenesis of diabetic heart disease, where excess dietary fat acts as a ligand to drive FA oxidation, lipid accumulation, reduced glucose utilization, and a characteristic cardiomyopathy, clinically [10]. In mice with cardiomyocyte overexpression of PPARα, this diabetic cardiomyopathy is recapitulated, demonstrating its potential role in disease [10].

Since MuRF1 −/− mice exhibit a fivefold increase in PPARα activity, we sought to determine how challenge with fenofibrate, a PPARα ligand, would affect the heart physiologically. In as little as 3 weeks, fenofibrate treatment induced an unexpected cardiac phenotype in MuRF1 −/−, but not age-matched sibling wild-type (MuRF1 +/+) mice. Moreover, MuRF1's regulation of fenofibrate's pleiotropic effects was identified for the first time, connecting MuRF1's regulation of PPARα, cardiac hypertrophy, and hemostasis for the first time.

2. Materials and methods

2.1. Animals

Twelve- to 16-week-old MuRF1 −/− mice [2] and age-matched MuRF1 +/+ controls (n=21; 50% male/50% female) underwent conscious echocardiography using a Vevo 770 ultrasound biomicroscopy system (VisualSonics, Inc., Toronto, Canada) as previously described [8,11,12]. Animal use was approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill.

2.2. Fenofibrate feeding protocols

Mice were randomized to receive either standard mouse chow (n=10; Prolab RMH 3000; Purina LabDiet, Oxford, NC, USA) or standard mouse chow containing fenofibrate (n=11; 0.05% wt/wt, F6020; Sigma, St. Louis, MO, USA). Standard mouse chow and fenofibrate were sent to TestDiet (St. Louis, MO, USA) and Granville Milling (Creedmoor, NC, USA) milled the experimental fenofibrate chow. Mouse chow (fenofibrate and standard sham chow) were administered ad libitum starting on day 1 of the protocol and commencing at the end of the 3- or 8-week protocol.

2.3. Morphological analysis of the heart by histology and transmission electron microscopy (TEM)

Cardiac tissue was fixed via perfusion for use in histological analysis, as described previously [2,3]. Fixed heart tissues were paraffin embedded, sectioned, and stained with H&E or Masson's trichrome staining. Imaging of stained sections was obtained using Aperio Scanscope and Aperio Imagescope software (version 10.0.36.1805, Aperio Technologies, Inc., Vista, CA). Heart apices were fixed in preparation for transmission electron microscopy (TEM), as described previously [3], or stained with *Triticum vulgaris* lectin TRITC conjugate as previously described [2]. Myocyte area was determined using NIH ImageJ (version 1.38X) based on photomicrographs of a standard graticule ruler. Fibrosis was determined using the Aperio Imagescope's Positive Pixel Count Algorithm to analyze Masson's trichrome-stained four-chamber sections (n=3/mouse), hue value=0.66 (blue), and hue width=0.1 (detection threshold above background white). The pen tool was used to isolated tissue sections to analyze, and the % fibrosis was expressed as the weighted average % of the n positive (collagen blue)/n total (tissue, defined by the nonwhite area).

2.4. RNA isolation from cardiac tissue

Cardiac tissues were homogenized using a TissueLyser LT (Cat. #69980; Qiagen N.V., Venlo, the Netherlands) according to the manufacturer's protocols. Approximately 20–40 mg of apical ventricle was homogenized in 1 ml of Trizol (Cat. #15596-026; Life Technologies, Inc., Carlsbad, CA, USA) using a 5-mm stainless steel bead (Cat. #69989; Qiagen, N.V.). Chloroform (200 μl) was added and centrifuged at 12,000g (15 min at 4°C), isopropanol (0.5 ml) was added to the aqueous phase and centrifuged at 12,000g (10 min at 4°C), and the resulting RNA pellet was washed with 1 ml of 75% ethanol, dried, and resuspended in RNAse-free water. RNA concentration was then determined by UV spectroscopy (absorbance of 260–280 nm).

2.5. Real-time polymerase chain reaction and statistical analysis

RNA (500 ng) was reverse-transcribed using iScript reverse transcription supermix (Cat. #170-8841; Bio-Rad, Laboratories, Inc., Hercules, CA, USA). Gene expression assays were performed using Taqman Gene Expression Assays (Life Technologies) and Universal Taqman Master mix (Life Technologies, Cat. #4304437). Cardiac hypertrophy fetal gene expression was monitored using probes for beta-myosin heavy chain (*β-MHC*; Mm00600555_m1), skeletal muscle α-actin (Mm00808218_g1), and brain natriuretic peptide (*BNP*; Mm00435304_g1) mRNA. MuRF1's regulation of PPAR-associated genes was monitored using probes for *CD36* (Mm00432403_m1), *CPT-1* (Mm00487200_m1), *PGC-1* (Mm00447183_m1), *PDK4* (Mm00443325_m1), *FATP1* (Mm0449811_m1), *PRKAA2* (Mm01264791_g1), *PPARα* (Mm00440939_m1), *PPARβ* (Mm01305434_m1), *Acox1* (Mm00443579_m1), *FABP3* (Mm00445880_1), *FABP4* (mm0232494_m1), *LPL* (Mm00434770_m1), and reference 18S (Hs99999901_s1). Mitochondrial number was quantified by quantitative polymerase chain reaction (qPCR), and DNA was isolated from 50 μl whole-heart homogenates using the DNAeasy Blood and Tissue Kit (Qiagen; Cat. #69506). Isolated DNA and oligomer primers for mitochondrial cytochrome *c* oxidase subunit 1 (CO1; aka mt-CO1), cytochrome *b* (Cyt-b; aka mt-Cyb), and NADH dehydrogenase 1 (ND1; aka mt-nd1) DNA normalized to nuclear H19 (imprinted maternally expressed transcript, nonprotein coding) DNA were run in SYBR green mastermix by qPCR including melting curves as previously detailed [13]. Select genes differentially expressed by microarray (*cMyBP-C*, *MuRF1*, *TNNI3*, *FABP3*, *COL1A*, *PLN*, *NKX2.5*, and *β-actin* were analyzed by RT-qPCR as previously detailed [14]. PCR primers and fluorogenic probes [reporter dye, FAM (F); quencher dye, TAMRA (Q)] were created using Primer Express (Table 1) and quantified using the ABI Prism 7700 sequence detector (PE Biosystems, Foster City, CA, USA). GraphPad Prism 6 (GraphPad Prism Software Inc., La Jolla, CA, USA) was used to determine significant statistical difference by one-way analysis of variance followed