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Non-targeted metabolomics analysis of cardiac Muscle Ring Finger-1 (MuRF1), MuRF2, and MuRF3 in vivo reveals novel and redundant metabolic changes

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Abstract

The muscle-specific ubiquitin ligases MuRF1, MuRF2, MuRF3 have been reported to have overlapping substrate specificities, interacting with each other as well as proteins involved in metabolism and cardiac function. In the heart, all three MuRF family proteins have proven critical to cardiac responses to ischemia and heart failure. The non-targeted metabolomics analysis of MuRF1^{-/-}, MuRF2^{-/-}, and MuRF3^{-/-} hearts was initiated to investigate the hypothesis that MuRF1, MuRF2, and MuRF3 have a similarly altered metabolome, representing alterations in overlapping metabolic processes. Ventricular tissue was flash frozen and quantitatively analyzed by GC/MS using a library built upon the Fiehn GC/MS Metabolomics RTL Library. Non-targeted metabolomic analysis identified significant differences (via VIP statistical analysis) in taurine, myoinositol, and stearic acid for the three MuRF^{-/-} phenotypes relative to their matched controls. Moreover, pathway enrichment analysis demonstrated that MuRF1^{-/-} had significant changes in metabolite(s) involved in taurine metabolism and primary acid biosynthesis while MuRF2^{-/-} had changes associated with ascorbic acid/aldarate metabolism (via VIP and t-test analysis vs. sibling-matched wildtype controls). By identifying the functional metabolic consequences of MuRF1, MuRF2, and MuRF3 in the intact heart, non-targeted metabolomics analysis discovered common pathways functionally affected by cardiac MuRF family proteins *in vivo*. These novel

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metabolomics findings will aid in guiding the molecular studies delineating the mechanisms that MuRF family proteins regulate metabolic pathways. Understanding these mechanism is an important key to understanding MuRF family proteins' protective effects on the heart during cardiac disease.

Keywords

cardiac; ubiquitin ligase; metabolomics; Muscle Ring Finger-1 (MuRF1); MuRF2; MuRF3

Introduction

Muscle Ring Finger-1 (MuRF1), a striated muscle specific protein, was initially identified as a critical ubiquitin ligase that mediated skeletal muscle and cardiac atrophy induced from multiple stimuli (Bodine, Latres, Baumhueter, Lai, Nunez, Clarke, Poueymirou et al. 2001; Willis, Rojas, Li, Selzman, Tang, Stansfield, Rodriguez et al. 2009). Given the importance of the specificity of ubiquitin ligases in directing ubiquitination and proteasome-dependent degradation of their substrates, the search for MuRF1 interacting proteins (substrates) began. In the first of these studies, MuRF1 was found to interact with two other highly homologous proteins, subsequently named MuRF2 and MuRF3 using yeast two hybrid assays (Centner, Yano, Kimura, McElhinny, Pelin, Witt, Bang et al. 2001). Subsequent studies found that both MuRF1 and MuRF2 interacted with specific sarcomere proteins (e.g. titin, troponin I, troponin T, myosin light chain 2), while MuRF3 did not (Witt, Witt, Lerche, Labeit, Back, Labeit 2008). Interestingly, MuRF1 was found to bind 11 enzymes required for ATP/energy production in muscle, including mitochondrial ATP synthase and creatine kinase (Witt, Witt, Lerche, Labeit, Back, Labeit 2008). However, the role of MuRF1, MuRF2, and MuRF3 proteins on mitochondrial function and metabolism has not been investigated.

The first clues that MuRF1 and MuRF2 have overlapping roles in vivo came from studies crossing MuRF1^{-/-} and MuRF2^{-/-} mice to create MuRF1^{-/-} /MuRF2^{-/-} double-deficient mice (dKO)(Willis, Wadosky, Rodriguez, Schisler, Lockyer, Hilliard, Glass et al. 2014; Witt, Witt, Lerche, Labeit, Back, Labeit 2008). These studies identified that MuRF1 and MuRF2 are redundant in the heart as only one of the four MuRF1 / MuRF2 alleles was needed to maintain normal cardiac phenotype and function (Willis, Wadosky, Rodriguez, Schisler, Lockyer, Hilliard, Glass et al. 2014), they identified the redundancy of their sarcomere specificity in vivo, along with specific defects in metabolism (Willis, Wadosky, Rodriguez, Schisler, Lockyer, Hilliard, Glass et al. 2014; Witt, Witt, Lerche, Labeit, Back, Labeit 2008). In skeletal muscle, MuRF1/MuRF2 dKO mice demonstrated a profound loss of type-II muscle fibers, consistent with a relative increase in mitochondria (type-II muscle fibers have relatively fewer mitochondria) in soleus muscle (Witt, Witt, Lerche, Labeit, Back, Labeit 2008). These studies illustrate how MuRF1 and MuRF2 redundancy may be linked to both sarcomere protein turnover as well as metabolism in vivo.

Evidence for redundancy between MuRF1 and MuRF3 has been shown studies creating mice lacking both MuRF1 and MuRF3 (MuRF1/MuRF3 dKO). MuRF1/MuRF3 dKO mice demonstrate an accumulation of myosin heavy chain in vivo, each having the ability to poly-

ubiquitinate MHCIIa in vitro (Fielitz, van Rooij, Spencer, Shelton, Latif, van der Nagel, Bezprozvannaya et al. 2007). Mice lacking MuRF3 undergo cardiac rupture after myocardial infarction, illustrating the importance of MuRF3 in regulating the maintenance of its substrates FHL2 and gamma-filamin to ensure cardiac integrity by degradation and subsequent turnover (Fielitz, Kim, Shelton, Latif, Spencer, Glass, Richardson et al. 2007). While MuRF3 has complementary roles in maintaining the sarcomere apparatus to MuRF1 and MuRF2, evidence for its role in metabolism has not been identified. In this study, we investigated the hypothesis that mice lacking cardiac MuRF1, MuRF2, or MuRF3 exhibited overlapping differences in metabolism evidenced by their metabolome.

Recent advancements in technology have afforded a more comprehensive analysis of a tissue's metabolome. Both targeted and non-targeted mass-spectrometry based analysis have become common, with the non-targeted approach enabling exploration of phenotypes across many types of metabolites (Bain, Stevens, Wenner, Ilkayeva, Muoio, Newgard 2009). Non-targeted technologies have been used to detect metabolic genetic diseases of lipid and amino acid metabolism (Frazier, Millington, McCandless, Koeberl, Weavil, Chaing, Muenzer 2006; Shekhawat, Matern, Strauss 2005) and are beginning to be applied more broadly to disease processes in identifying unique mechanisms and/or markers of common diseases (Bain, Stevens, Wenner, Ilkayeva, Muoio, Newgard 2009). In the present study, we performed non-targeted metabolomics analysis on the hearts of adult MuRF1^{-/-}, MuRF2^{-/-}, and MuRF3^{-/-} mice to identify novel metabolic changes compared to wildtype strain-matched sibling controls. While each MuRF family protein had unique signatures, mice lacking MuRF1 or MuRF2 appeared to have the most significant alterations in metabolites, several which appear to be overlapping.

Materials and Methods

Animals

MuRF1^{-/-} and MuRF2^{-/-} mice have been previously described and the absence of protein expression confirmed (Bodine, Latres, Baumhueter, Lai, Nunez, Clarke, Poueymirou et al. 2001; Willis, Ike, Li, Wang, Glass, Patterson 2007; Willis, Wadosky, Rodriguez, Schisler, Lockyer, Hilliard, Glass et al. 2014). The MuRF3^{-/-} mice have a with a LacZ cassette inserted in its place. Immunoblot analysis of MuRF3^{-/-} hearts demonstrate that MuRF1 and MuRF2 expression is increased (Supplemental Figure 1A), while MuRF3 protein is not present in MuRF3^{-/-} hearts (Supplemental Figure 1B). Quantitative analysis of MuRF1, MuRF2, and MuRF3 mRNA demonstrate the lack of MuRF3 resulted in no compensatory changes in MuRF1 and MuRF2 expression (Supplemental Figure 1C). Consistent with previously published models of MuRF3^{-/-} mice (Fielitz, Kim, Shelton, Latif, Spencer, Glass, Richardson et al. 2007; Fielitz, van Rooij, Spencer, Shelton, Latif, van der Nagel, Bezprozvannaya et al. 2007), conscious echocardiographic analysis of MuRF3^{-/-} mice revealed no differences from strain-matched wildtype mice, as has been reported for MuRF1^{-/-} and MuRF2^{-/-} mice (Willis, Ike, Li, Wang, Glass, Patterson 2007) (Supplemental Figure 1C, Supplemental Table 1). MuRF3^{-/-} mice are born in Mendelian ratios from heterozygous crosses and do not have obvious developmental defects as reported in other investigators MuRF3^{-/-} mouse lines created independently (Fielitz, Kim, Shelton, Latif,

Spencer, Glass, Richardson et al. 2007; Fielitz, van Rooij, Spencer, Shelton, Latif, van der Nagel, Bezprozvannaya et al. 2007). Hearts were harvested from eleven MuRF1^{-/-} and strain-matched MuRF1^{+/+} mice, twelve MuRF2^{-/-} and strain-matched MuRF2^{+/+}, and ten MuRF3^{-/-} and strain-matched MuRF3^{+/+} mice.

All mouse experiments were approved by the Institutional Animal Care and Use Committee review board at the University of North Carolina and were in compliance with the rules governing animal use as published by the National Institutes of Health.

RNA isolation, qPCR mRNA detection, and immunoblot analysis of MuRF3^{-/-} hearts

Total RNA was isolated by using TRIzol reagent according to the manufacturer's protocols (Ambion, Grand Island, NY). Briefly, 25 mg of ventricular tissue was homogenized in 1 ml TRIzol on ice (Fisher Scientific, Power Gen 125, setting 5). mRNA expression was determined using a two-step reaction: 1) cDNA was made using a iScriptTM Reverse Transcription Supermix for RT-qPCR kit (Cat.# 170-8841, BIO-RAD); 2) 2 µl cDNA was amplified on a Roche Lightcycler 480II system with exon-spanning Taqman Probes (Cat.# Mm01185221_m1, MuRF1/Trim63; Cat.# Mm01292963_g1, MuRF2/Trim55; Cat.# Mm00491308_m1, MuRF3/Trim 54; and Cat.# Hs99999901_s1, 18S, Applied Biosystems, Inc.) in 2× Lightcycler 480 Master Mix (Cat.# 04 707 494 001, Roche Diagnostics, Corp., Indianapolis, IN). Each reaction was run in triplicate and relative mRNA expression was determined using 18S as internal endogenous controls using CT analysis.

Immunoblot analysis of MuRF3^{-/-} hearts

MuRF3^{-/-} and wild type control hearts were flash frozen, homogenized, and resolved (30 µg) on a 10% BisTris NuPage gel and MOPS run buffer, transferred to PVDF, then incubated overnight with the following primary antibodies: 1) goat anti-MuRF1 (1:250, cat.#sc-27642, Santa Cruz Biotechnology, Inc., Dallas, TX); 2) goat anti-MuRF2 (1:5000, cat.#Ab-4387, AbCam, Cambridge, MA); 3) and goat anti-MuRF3 (1:200, cat.#sc-50252; Santa Cruz Biotechnology). Anti-goat secondary HRP-labeled antibody (1:10,000, cat.#A4174; Sigma Aldrich, Inc.) was added for 1 hour and developed using Amersham ECLSelect (Cat#RPN 2235) for chemiluminescence detection. Loading controls were performed with primary mouse anti-β-actin antibody (1:4,000, cat.#A2228; Sigma Aldrich, Inc., St. Louis, MO) and mouse anti-GAPDH (1:4,000, Millipore, Cat#MAB374), followed by secondary HRP-labeled anti-mouse (1:10,000, cat.#A9917; Sigma Aldrich, Inc.).

Tissue harvest and preparation

Mice were anesthetized with isoflurane, cervical spine severed, and the heart harvested and immediately weighed while still beating. The atria were removed, the ventricles flash frozen with a liquid nitrogen cooled Biosqueezer Snap Freeze press (cat.#1210, Biospec Products, Inc., Bartlesville, OK). For each 25 mg removed from the frozen heart (25 mg weighed in a cooled weigh boat), and 475 µl of freshly made buffer (50% acetonitrile, 50% water, 0.3% formic acid) was added. The tissue was then homogenized on ice for 15-30 seconds (cat.#14-261, Fisher Scientific, Power Gen 125, Setting 5.5, Pittsburgh, PA), aliquoted, and stored at -80C.

Metabolomics determination and instrumentation

The homogenized samples were then “crash” deprotonized by methanol precipitation and spiked with D27-deuterated myristic acid (D27-C14:0) as an internal standard for retention-time locking and dried. The retention time of the TMS-D27-C14:0 standard was set at ~16.727 min. Reactive carbonyls were stabilized at 50°C with methoxyamine hydrochloride in dry pyridine. Metabolites were made volatile with trimethylsilyl (TMS) groups using *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide or MSTFA with catalytic trimethylchlorosilane at 50°C. GC/MS methods generally follow those of Roessner *et al.* (Roessner, Wagner, Kopka, Trethewey, Willmitzer 2000), Fiehn *et al.* (Fiehn, Wohlgemuth, Scholz, Kind, Lee do, Lu, Moon et al. 2008), and Kind *et al.* (Kind, Wohlgemuth, Lee do, Lu, Palazoglu, Shahbaz, Fiehn 2009), and used a 6890N GC connected to a 5975 Inert single-quadrupole MS (Agilent Technologies, Santa Clara, CA). The two wall-coated, open-tubular (WCOT) GC columns connected in series are both from J&W/Agilent (part 122-5512), DB5-MS, 15 meters in length, 0.25 mm in diameter, with an 0.25- μ m luminal film. Positive ions generated with conventional electron-ionization (EI) at 70 eV are scanned broadly from 600 to 50 m/z in the detector throughout the 45 min cycle time.

Raw data from Agilent's ChemStation software environment were imported into Automatic Mass Spectral Deconvolution and Identification Software or AMDIS, freeware developed by Drs. Steve Stein, W. Gary Mallard, and their coworkers at National Institute of Standards and Technology or NIST (Mallard and Reed (Mallard 1997), Halket *et al.* (Halket, Przyborowska, Stein, Mallard, Down, Chalmers 1999), and Stein (Stein 1999)). Deconvoluted spectra are annotated as metabolites, to the extent possible, using an orthogonal approach that incorporates both retention time (RT) from GC and the fragmentation pattern observed in EI-MS. Peak annotation is based primarily on our own RT-locked spectral library of metabolites. The library is built upon the Fiehn GC/MS Metabolomics RTL Library (a gift from Agilent, part number G1676-90000; Kind *et al.* (Kind, Wohlgemuth, Lee do, Lu, Palazoglu, Shahbaz, Fiehn 2009), Golm Metabolome Library (courtesy of Dr. Joachim Kopka and coworkers at the Max Planck Institute of Molecular Plant Physiology, Golm, Germany (Kopka J. 2005) the Wiley 9th-NIST 2011 commercial library (Agilent G1730-64000), and other spectral libraries. Once annotation was complete, a cross-tabulated spreadsheet was created, listing the integrated peak area for each metabolite versus sample identity. This was accomplished using a custom Visual Basic program in Microsoft Excel that groups together peaks across samples based on identical metabolite annotation and retention time proximity. Peak alignment across samples was further confirmed using SpectConnect (Styczynski, Moxley, Tong, Walther, Jensen, Stephanopoulos 2007) to assess similarity in spectral fragmentation patterns, and by manual curation (Supplemental Table 2, Supplemental Table 3, Supplemental Table 4).

Metabolomic Analyses

Metabolite peaks areas (as representative of concentration) were analyzed using Metaboanalyst (v2.0) run on the statistical package R (v2.14.0) as recently described (Xia, Psychogios, Young, Wishart 2009); (Xia, Mandal, Sinelnikov, Broadhurst, Wishart 2012). These data underwent both an unsupervised evaluation using principal component analysis (PCA) identified the presence of the MuRF gene (wildtype) as the principal source of

variance, after which Partial Least Squares Discriminant Analysis (PLS-DA) was performed. Metabolites contributing most significantly to the differences between wildtype and MuRF^{-/-} hearts were then determined using the Variable Importance in Projection (VIP) analysis in the Metaboanalyst environment. The metabolites that best differentiated the groups were then individually tested using the Student's t-test (Microsoft Excel 2011, Seattle, WA). The t-test significant metabolites were matched to metabolomics pathways using the Pathway Analysis feature in Metaboanalyst 2.0. Heat maps of the metabolite data (individual and grouped) were generated using the GENE-E software (<http://www.broadinstitute.org/cancer/software/GENE-E/index.html>).

Statistical analysis

A Student's t-test was performed to determine differences between MuRF3^{-/-} and strain-matched control MuRF3^{+/+} using Sigma Plot 11 (Systat Software, Inc., San Jose, CA). Statistical significance was defined as $p < 0.05$.

Results & Discussion

At baseline, MuRF1^{-/-} mice do not exhibit a functional or histological cardiac phenotype (Willis, Ike, Li, Wang, Glass, Patterson 2007); (Willis, Rojas, Li, Selzman, Tang, Stansfield, Rodriguez et al. 2009). Compared to sibling wildtype mice, metabolomics non-targeted profiling identified distinct differences in MuRF1^{-/-} and MuRF1^{+/+} mice by both PCA and PLS-DA analysis (Figure 1A, 1B, respectively). In both analyses, the principal component 1 accounts for over 40% of the differences between the two genotypes. Further analysis of the top 15 metabolites using a Variable Importance in Projection (VIP) that differentiated MuRF1^{-/-} hearts include phosphoric acid, taurine, and glutamic acid (Figure 1C), which were quantitatively different compared to MuRF1^{+/+} from up to +3.3 fold (Figure 2A). The unsupervised heat maps illustrating the differences in metabolite quantities for each individual heart, ranging from -7.5 to +3.3 fold change difference, are shown in Figure 2A.

Like MuRF1^{-/-} mice, MuRF2^{-/-} mice do not exhibit a functional or histological cardiac phenotype (Willis, Ike, Li, Wang, Glass, Patterson 2007); (Willis, Rojas, Li, Selzman, Tang, Stansfield, Rodriguez et al. 2009). Compared to sibling wildtype mice, metabolomics non-targeted profiling identified distinct differences in MuRF2^{-/-} and MuRF2^{+/+} mice by both an unsupervised PCA and a supervised PLS-DA analysis (Figure 1D, 1E, respectively). Greater than 52% of the differences between MuRF2^{-/-} and MuRF2^{+/+} could be accounted for by the principal component 1, in both analyses. Using VIP analysis, the top 15 metabolites that could differentiate MuRF2^{-/-} from MuRF2^{+/+} hearts included phosphoric acid, taurine, aspartic acid, and d-malic acid, among others (Figure 2B). The unsupervised heat maps illustrating the differences in metabolite quantities for each individual heart, ranging from -2.2 to +1.3 fold, are shown in Figure 2B.

MuRF3^{-/-} mice do not have any functional deficits in heart function at baseline (Supplemental Table 1), like MuRF1^{-/-} and MuRF2^{-/-} mice (Willis, Ike, Li, Wang, Glass, Patterson 2007). In contrast to the MuRF1^{-/-} and MuRF2^{-/-} metabolic analysis, MuRF3^{-/-} heart metabolite differences did not separate distinctly from MuRF3^{+/+} hearts by PCA analysis (Figure 1G). In the unsupervised PCA analysis, the principal component 1

accounted for 65% of the differences (Figure 1H), although the separation by supervised PLS-DA revealed that only 14% of the differences between the two groups could be attributable to the principal component 1 (Figure 1H). VIP analysis revealed that taurine, α -monostearin, aldohexose1, and glutamic were some of the 15 top metabolites that were different between the two groups (Figure 2C). The unsupervised heat maps illustrating the differences in metabolites for each individual heart, ranging from -117 to +1.9 fold, are shown in Figure 2C.

Three metabolites were found to be VIP significant in MuRF1^{-/-}, MuRF2^{-/-}, and MuRF3^{-/-} hearts: taurine, myoinositol, and stearic acid. All three MuRF proteins appear necessary for the regulation of taurine, myoinositol, and stearic acid (Table 1). The physiological relevance of this in these mouse models is not clear, but the importance of each of these metabolites in cardiac (patho)physiology may offer insights for future investigations.

Taurine, a conditional and abundant amino acid in most body tissues including cardiac muscle (Ripps, Shen 2012), is essential for normal cardiac function. Taurine deficiency has been described in heart failure patients (Soukoulis, Dihu, Sole, Anker, Cleland, Fonarow, Metra et al. 2009). Animal models fed taurine-deficient diets as well as a taurine transporter knock-out rat model induce cardiomyopathy (Schaffer, Jong, Ramila, Azuma 2010). The mechanism for taurine deficiency-induced cardiomyopathy has not been fully elucidated but not surprising given taurine's role in calcium homeostasis regulation, enhanced calcium sensitivity of contractile proteins, osmotic stress, mitochondria protein production, anti-oxidant properties (Schaffer, Jong, Ramila, Azuma 2010), and blood pressure regulation (El Idrissi, Okeke, Yan, Sidime, Neuwirth 2013). Increased cardiac taurine has been reported in heart failure (increased here in MuRF1^{-/-} hearts), while decreased taurine (decreased here in MuRF2^{-/-} and MuRF3^{-/-} hearts) has been reported in ischemia-reperfusion injury (Crass, Lombardini 1977); (Huxtable, Bressler 1974). Taurine therapy is beneficial in several heart failure models (Azuma, Hasegawa, Sawamura, Awata, Harada, Ogura, Kishimoto 1982); (Azuma, Hasegawa, Sawamura, Awata, Ogura, Harada, Yamamura et al. 1983); (Azuma, Sawamura, Awata, Ohta, Hamaguchi, Harada, Takihara et al. 1985); (McBroom, Welty 1977); (Takihara, Azuma, Awata, Ohta, Hamaguchi, Sawamura, Tanaka et al. 1986); (Li, Cao, Zeng, Liu, Zhang, Dai, Hu et al. 2005) and taurine is an accepted therapy in Japan (Ito, Schaffer, Azuma 2014).

Myoinositol (increased in MuRF1^{-/-}, decreased in MuRF2^{-/-} hearts) is a component in inositol phosphate metabolism. The role of inositol phosphate in ischemia and reperfusion has been previously studied. Inositol phosphate and its metabolites coordinate numerous cellular responses, including contraction, secretion, and mitogenesis (Berridge 1993). Inositol phosphate production has been shown to cease at the onset of myocardial ischemia, but reperfusion causes a rapid and transient increase in inositol phosphate production in the window in which reperfusion causes arrhythmias to occur (Anderson, Dart, Woodcock 1995). Cardiac stearic acid levels are positively correlated with cardiac diameter in hypertensive rats with cardiac hypertrophy (Kim, Jung, Cho, Chung, Hwang, Shin 2013). While the relevance of MuRF1, MuRF2, and MuRF3 in cardiac homeostasis is not clear, their common regulation of taurine, myoinositol, and stearic acid may offer insight how they may act to offer resistance to cardiac stress.

MuRF1^{-/-} and MuRF2^{-/-} hearts had seven metabolites significantly different from wildtype hearts by both VIP and t-test ($p > 0.05$) statistical analysis (Table 2). Both MuRF1^{-/-} and MuRF2^{-/-} hearts had myoinositol (discussed above) and palmitic acid that were significantly different from sibling-matched wildtype hearts. MuRF1^{-/-} hearts had myoinositol than their wildtype controls; in contrast MuRF2^{-/-} hearts had higher levels of metabolites compared to their wildtype controls (Table 2). Fatty acid oxidation is an important source of energy for myocardiocytes, and is the predominant source of ATP production following post ischemic reperfusion (Saddik, Lopaschuk 1992). The utilization of fatty acid oxidation at the expense of glucose oxidation may contribute to contractile dysfunction during reperfusion (Lopaschuk, Wambolt, Barr 1993). Ischemia decreases fatty acid oxidation, and palmitic acid retention and clearance following ischemia may be helpful in identifying reversible tissue injury (Schwaiger, Schelbert, Keen, Vinten-Johansen, Hansen, Selin, Barrio et al. 1985). These studies may suggest a role of MuRF1 and MuRF2 in the regulation of fatty acid metabolism; however, additional studies are needed to identify the specific changes in the absence of MuRF1 and MuRF2 protein in the heart.

MuRF1^{-/-} and MuRF2^{-/-} hearts had four metabolites in common different from wild type hearts (significant by VIP) that were not identified in MuRF3^{-/-} hearts: 1) glycerol 1-phosphate, urea, and malic acid, and phosphoric acid (Table 1). The malate-aspartate shuttle is a system used during glycolysis in mitochondrial oxidative phosphorylation. Activation of the malate/aspartate and alpha-glycerophosphate shuttles, both NADH shuttles, have been identified in glycolytically active newborn heart and are reactivated during pathological cardiac hypertrophy (Rupert, Segar, Schutte, Scholz 2000). These shuttle systems are critical as NADH is impermeable to the mitochondrial inner membrane where ETC takes place. They appear to accommodate increased flux as tissues become more glycolytically active and have been associated with regulation by thyroid hormone (Scholz, TenEyck, Schutte 2000). No studies to date have linked MuRF1 and MuRF2 to these critical NADH shuttles, warranting future investigation since the malate aspartate shuttle has been suggestive of ischemic cardioprotection in heart failure (Gonzalez-Loyola, Barba 2010; Nielsen, Stottrup, Lofgren, Botker 2011; Stottrup, Lofgren, Birkler, Nielsen, Wang, Caldarone, Kristiansen et al. 2010).

The pathway enrichment analysis for MuRF1^{-/-} and MuRF2^{-/-} hearts identified 3 significant pathways affected ($p < 0.05$) (Table 2). In the MuRF1^{-/-} hearts, biosynthesis of unsaturated fatty acids/fatty acid synthesis was the most significant pathway affected, due to its significantly altered palmitic acid and stearic acid. Both MuRF1^{-/-} and MuRF2^{-/-} hearts had changes in ascorbate and aldarate metabolism identified (Table 2). Dehydroascorbic acid has antioxidant properties and is cytoprotective following stroke (Huang, Agus, Winfree, Kiss, Mack, McTaggart, Choudhri et al. 2001) and demonstrates cardioprotective properties following myocardial infarction (Guaiquil, Golde, Beckles, Mascareno, Siddiqui 2004; Sernov, Sokolova, Gatsura 1991). These studies suggest that hearts lacking MuRF1 and MuRF2 may have alterations that could contribute to their resiliency in the face of normal wear or pathological stress, while altering metabolic parameters including biosynthesis of unsaturated fatty acids/fatty acid synthesis and regulation of critical anti-oxidant (ascorbic acid and aldarate metabolism) systems.

Concluding Remarks

While previous studies have identified overlapping specificities of MuRF1, MuRF2, and MuRF3 in binding (Witt, Witt, Lerche, Labeit, Back, Labeit 2008) and function (Willis, Wadosky, Rodriguez, Schisler, Lockyer, Hilliard, Glass et al. 2014; Witt, Witt, Lerche, Labeit, Back, Labeit 2008), the present studies do not allow comparison as changes in metabolites are the end product of multiple, overlapping pathways. For example, while MuRF1^{-/-} and MuRF2^{-/-} hearts had differences in myoinositol and palmitic acid that were significantly different from sibling-matched wildtype hearts, they were different in opposite ways. MuRF1^{-/-} hearts had lower concentration of each, while MuRF2^{-/-} had higher levels (Table 2). This may be interpreted that MuRF1 and MuRF2 may have opposite functions on the same substrate(s) mediating the myoinositol and palmitic acid pathways, or that they regulate different intermediate(s). Alternatively, these changes may represent indirect effects of MuRF1 proteins primary effectors. Even as indirect effects, MuRF's ability to regulate taurine and myoinositol levels is significant as they are involved in the hearts ability to tolerate both ischemic and heart failure (Li, Du, Fan, Zhang, Liu, Li, Lockyer et al. 2011; Willis, Schisler, Li, Rodriguez, Hilliard, Charles, Patterson 2009; Willis, Wadosky, Rodriguez, Schisler, Lockyer, Hilliard, Glass et al. 2014). In the case of taurine, evidence for its role in cardiac physiology is exemplified by its development as a therapy for heart disease in Japan (Ito, Schaffer, Azuma 2014). Similarly, the role of MuRF1 and MuRF2 on fatty acid synthesis and ascorbic acid/aldarate metabolism is provocative given the primary role of fatty acids in cardiomyocyte energy production (Lopaschuk, Ussher, Folmes, Jaswal, Stanley 2010) and the production of antioxidants critical to the hearts resistance to ischemia (Rodrigo, Prieto, Castillo 2013), respectively. These novel metabolomics findings will aid in guiding the molecular studies delineating the mechanisms that MuRF family proteins regulate metabolic pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard abbreviations

E3	ubiquitin ligase
MuRF1(2,3)	muscle ring finger-1(2,3)
VIP	Variable Importance in Projection
PCA	principal components analysis
PLS-DA	Partial Least Squares Discriminant Analysis

Figure 1.

The metabolomic profile of MuRF1 $^{-/-}$, MuRF2 $^{-/-}$, and MuRF3 $^{-/-}$ hearts are significantly different from wildtype control hearts analyzed in parallel. Principle Components Analysis (PCA) illustrating the unsupervised relationship between **A.** MuRF1 $^{-/-}$ **B.** MuRF2 $^{-/-}$, and **C.** MuRF3 $^{-/-}$ vs. strain-matched wild type hearts. Data points that are closer together indicate higher degree of similarity than data points that are further apart. Partial Least Squares Discriminant Analysis (PLS-DA) score visualizes supervised (assigned group) clustering of **D.** MuRF1 $^{-/-}$ **E.** MuRF2 $^{-/-}$, and **F.** MuRF3 $^{-/-}$ vs. strain-matched wild type hearts. PLS-DA score plots for MuRF1 $^{-/-}$ and MuRF3 $^{-/-}$ heart metabolites (red) compared to metabolites identified in wildtype control hearts (green). Each point represents the combined metabolite profile in an individual mouse. Variable Importance in Projection (VIP) plot of VIP statistical analysis identifying the top 15 metabolites contributing to the differences between the **G.** MuRF1 $^{-/-}$ **H.** MuRF2 $^{-/-}$, and **I.** MuRF3 $^{-/-}$ vs. strain-matched wild type hearts (relative concentration to the right of the figure). N=5 (MuRF1 $+/+$), N=6 (MuRF1 $^{-/-}$), N=6 (MuRF2 $+/+$), N=6 (MuRF2 $^{-/-}$), N=5 (MuRF3 $+/+$), and N=5 (MuRF3 $^{-/-}$) hearts per group analyzed.

Figure 2. Unsupervised heat maps of all identified metabolites and their associated fold changes in **A.** MuRF1^{-/-}, **B.** MuRF2^{-/-}, and **C.** MuRF3^{-/-} hearts compared to strain-matched wildtype hearts. **D.** Identification of the VIP significant metabolites that were also significant by t-test (p<0.05). N=5 (MuRF1 +/+), N=6 (MuRF1^{-/-}), N=6 (MuRF2^{+/+}), N=6 (MuRF2^{-/-}), N=5 (MuRF3^{+/+}), and N=5 (MuRF3^{-/-}) hearts per group analyzed.

Figure 3. Metabolic pathways related to cardiac MuRF family protein activity *in vivo* determined by non-targeted metabolomics analysis of ventricular cardiac tissue (see Table 2 for analytic details). Metabolites identified by VIP and t-test significance are shown in red in the context of generalized **A.** Taurine and primary bile acid synthesis; **B.** Myo-inositol and ascorbate metabolism; and **C.** Citric acid cycle metabolic pathways (Torabinejad, Donahue, Guneseckera, Allen-Daniels, Gillaspay 2009; Ueki, Roman, Valli, Fieselmann, Lam, Peters, Hirschberger et al. 2011). N=5 (MuRF1 +/+), N=6 (MuRF1 -/-), N=6 (MuRF2+/+), N=6 (MuRF2-/-), N=5 (MuRF3+/+), and N=5 (MuRF3-/-) hearts per group analyzed.

Table 1

VIP significant metabolites in MuRF1^{-/-}, MuRF2^{-/-}, and MuRF3^{-/-} hearts vs. strain-matched wildtype control hearts. * indicates this metabolite was not found to be a VIP significant metabolite. The ranking (1-15) indicates the ranking given to the metabolites in their respective groups (MuRF1^{-/-}, MuRF2^{-/-}, and MuRF3^{-/-}).

MuRF1 ^{-/-} (VIP Rank)	MuRF2 ^{-/-} (VIP Rank)	MuRF3 ^{-/-} (VIP Rank)
Phosphoric acid (1)	Phosphoric acid (1)	*
Taurine (2)	Taurine (2)	Taurine (1)
Glutamic Acid 2 (3)	*	Glutamic acid (5)
Glutamic Acid 3 (4)	*	*
Glycerol 1-phosphate (5)	Glycerol 1-phosphate (11)	*
Myoinositol (6)	Myoinositol (9)	Myoinositol (15)
Stearic acid (7)	Stearic acid (8)	Stearic acid (12)
Aldohexose 1 (8)	*	Aldohexose 1 (4)
Cholesterol (9)	Cholesterol (5)	Cholesterol (11)
Palmitic Acid (10)	Palmitic acid (7)	Palmitic Acid (8)
Urea (11)	Urea (10)	*
Mannose-6-Phosphate (12)	*	*
Succinic Acid (13)	*	Succinic acid (10)
Aldohexose2 (14)	*	*
Malic Acid (15)	D-malic acid (4)	*
*	Aspartic Acid (3)	*
*	L-lactic Acid (6)	*
*	Dehydroascorbic Acid (12)	*
*	Glycine (13)	*
*	L-threonine (14)	Alpha-monostearin (2)
*	L-alanine (15)	
*	*	Unknown, similar to Tris (3)
*	*	2-Hydroxybutyric acid (6)
*	*	2-Aminoadipic acid (7)
*	*	Docosohexanoic Acid (9)
*	*	Linoleic Acid (13)
*	*	Phenol (14)

Table 2

VIP significant metabolites that were also t-test significant ($p < 0.05$) in MuRF1^{-/-} and MuRF2^{-/-} hearts. MuRF3^{-/-} hearts did not have any VIP significant metabolites that were also significant by t-test. Matched pathways from pathway enrichment analysis for MuRF1^{-/-}, MuRF2^{-/-}, and MuRF3^{-/-} hearts. “Hits” indicate number of metabolites from the data set that matched pathway. Since no VIP significant metabolites were found that were also t-test significant for MuRF 3, no pathways could be matched in Metaboanalyst. N/A=not applicable. VIP significance was based on the analysis of MuRF1^{+/+} (N=5), MuRF1^{-/-} (N=6), MuRF2^{+/+} (N=6), MuRF2^{-/-} (N=6), MuRF3^{+/+} (N=5), and MuRF3^{-/-} (N=5) hearts.

Metabolite	p-value	Pathway (Hits)	p-value
MuRF 1			
Taurine	0.02600	Taurine and hypotaurine metabolism (1)Primary bile acid biosynthesis (1)	0.00560.0325
MuRF 2			
Palmitic Acid	0.00002	Ascorbate and aldarate metabolism (1)	0.0375
Dehydroascorbic acid	0.01098		
Aspartic Acid	0.01300		
L-Threonine	0.01792		
D-malic acid	0.01792		
Myoinositol	0.02398		
MuRF 3			
None	N/A	None	N/A