Inhibition of Class I Histone Deacetylases Unveils a Mitochondrial Signature and Enhances Oxidative Metabolism in Skeletal Muscle and Adipose Tissue

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Chromatin modifications are sensitive to environmental and nutritional stimuli. Abnormalities in epigenetic regulation are associated with metabolic disorders such as obesity and diabetes that are often linked with defects in oxidative metabolism. Here, we evaluated the potential of class-specific synthetic inhibitors of histone deacetylases (HDACs), central chromatin-remodeling enzymes, to ameliorate metabolic dysfunction. Cultured myotubes and primary brown adipocytes treated with a class I-specific HDAC inhibitor showed higher expression of Pgc-1α, increased mitochondrial biogenesis, and augmented oxygen consumption. Treatment of obese diabetic mice with a class I– selective HDAC inhibitor enhanced oxidative metabolism in skeletal muscle and adipose tissue and promoted energy expenditure, thus reducing body weight and glucose and insulin levels. These effects can be ascribed to increased Pgc-1α action in skeletal muscle and enhanced PPARγ/PGC-1α signaling in adipose tissue. In vivo ChIP experiments indicated that inhibition of HDAC3 may account for the beneficial effect of the class I–selective HDAC inhibitor. These results suggest that class I HDAC inhibitors may provide a pharmacologic approach to treating type 2 diabetes.

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nother example of a publication that explores the role of class I HDAC inhibitors in diabetes and associated metabolic disorders is the study by Galmozzi et al. (2012). The authors examined the effects of a class I HDAC inhibitor on metabolic parameters in obese diabetic mice. They found that treating these animals with the inhibitor led to improvements in body weight, glucose, and insulin levels, as well as increased expression of Pgc-1α in skeletal muscle and adipose tissue. These findings suggest that class I HDAC inhibitors may be useful in treating type 2 diabetes and other metabolic disorders associated with defects in oxidative metabolism.
complexes (MitoProfile Total OXPHOS Rodent WB Antibody Cocktail) were from Mitosciences.

**Cell culture.** C2C12 cells were maintained in Dulbecco’s modified Eagle’s medium—10% FBS and differentiated in Dulbecco’s modified Eagle’s medium—2% horse serum treated with 1% penicillin, 1% streptomycin, 5 mM L-glutamine, 5 mM sodium pyruvate, and 10% FBS and differentiated in Dulbecco’s modified Eagle’s medium—2% horse serum treated with 1% penicillin, 1% streptomycin, 5 mM L-glutamine, 5 mM sodium pyruvate, and 10% FBS. The slice immediately frontal to bladder inclusive. The T1-weighted slices were placed in the abdominal region spanning from kidneys to bladder inclusive. The field of view was 30 × 30 mm² with a matrix of 128 × 128 pixels. Four averages of a spin echo sequence with time to echo 10 ms and time of repetition 400 ms were acquired in 3°. The slice immediately frontal with respect to the ilium bone was chosen for visceral fat estimation and was computed as follows: (fat area)/(slice area). Areas were measured with Photoshop (Adobe Systems).

**RESULTS**

**Inhibition of class I HDACs promotes mitochondrial biogenesis.** For evaluation of whether synthetic class I or class II HDAC inhibitors can enhance mitochondrial function, C2C12 myotubes were treated with the HDAC pan-inhibitor SAHA, a class I HDAC–selective inhibitor (MS275), or a class II HDAC–selective inhibitor (MC1568). Concentrations were chosen based on dose-response curves (0.5–50 μmol/L) used to determine an effective, nontoxic, and selective concentration for each inhibitor based on the hyperacetylated state of histone H3 (a class I HDAC substrate) and α-tubulin (a class II HDAC substrate) (Supplementary Fig. 1A). After 60 h of treatment, global or class I HDAC inhibition resulted in increased mitochondrial density and activity, while inhibition of class II HDACs had no effect on these parameters (Fig. 1A and Supplementary Fig. 1B). These increases were accompanied by robust increases in mitochondrial DNA (Fig. 1A). Transmission electron microscopy confirmed that SAHA and MS275 induced mitochondrial biogenesis. Treatment with SAHA or MS275 resulted in an increase in mitochondrial density and greater electron opacity of the matrix typical of metabolically active cells. Cells treated with the class II HDAC inhibitor MC1568 showed mitochondrial similar to those of controls (Fig. 1B).

**Transcriptome analysis.** Transcriptome analysis revealed that global or class I–selective HDAC inhibition increased expression of several key mitochondria-related transcription factors, such as Ptf1m, Tfb1m, and the coactivator Pgc-1α (Fig. 1C and D), as well as the levels of multiple genes involved in glucose and lipid metabolism (Supplementary Fig. 1D). These changes in gene expression and mitochondrial density translated to differences in oxidative metabolism, as global and class I–selective HDAC inhibitors induced a 20% increase in basal respiration (Fig. 1E). In the presence of oligomycin, only cells treated with SAHA showed a small but consistent increase (~15%) in oxygen consumption, while MS275 treatment increased maximal respiratory capacity by ~30%. SAHA treatment showed a tendency to increase maximal respiratory capacity but to
These changes were accompanied by corresponding increases in mitochondrial complex proteins (Fig. 1F). No differences were seen with the class II HDAC inhibitor. These results indicate that inhibition of class I HDACs reprograms myotubes toward a more oxidative state.

Class I–selective HDAC inhibitors ameliorate obesity and diabetes. Next, we tested the physiologic relevance of HDAC inhibition in a model of obesity and diabetes, the db/db mouse. At the doses used (25 mg/kg SAHA, 10 mg/kg MS275, and 6.5 mg/kg MC1568 administered every other day for a 23-day period), the compounds reached skeletal muscle and retained their class-selective inhibitory activity (Supplementary Fig. 2A). Mice treated with MS275 showed a significant reduction of body weight (Fig. 2A and Supplementary Fig. 2B), in spite of similar food intake (Supplementary Fig. 2B). Interestingly, we observed a dramatic reduction of fasting glycemia, of circulating insulin, and of the homeostasis model assessment of insulin resistance index in animals treated with SAHA or MS275 but not in those treated with the class II HDAC inhibitor (Fig. 2B and C and Supplementary Fig. 2C). Moreover, global or class I–selective HDAC inhibition improved glucose clearance during glucose tolerance tests (Fig. 2D). Circulating triglycerides and nonesterified fatty acids were also decreased in SAHA and MS275 groups (Fig. 2E). MS275 completely cleared the lipids that accumulate in the liver of db/db mice, while SAHA had a significant but milder effect (Fig. 2F). The reduced hepatic steatosis was mirrored by decreased plasma transaminases, confirming that no toxic effects were observed with these compounds (Supplementary Fig. 2D and E). Interestingly, we did not observe any significant differences in hepatic gene expression or mitochondrial content, which suggests that the lack of hepatic steatosis is likely a reflection of the effect of MS275 in tissues other

FIG. 1. Inhibition of class I histone deacetylases promotes mitochondrial biogenesis and oxidative metabolism in C2C12 myotubes. A: Quantification of mitochondrial density, activity, and mitochondrial DNA (mtDNA) in C2C12 myotubes after treatment with 5 μmol/L SAHA, 5 μmol/L MS275, 5 μmol/L MC1568, or vehicle. Fluorescence intensity (FI) of mitochondrial probes was normalized to a nuclear stain (Hoechst 33258). B: Representative electron microphotographs of ultrathin sections of C2C12 monolayers. The ultrastructural appearance of mitochondria in vehicle-treated cells was characterized by a dense matrix and well-organized cristae with dilated intracristae spaces in the typical condensed conformation of metabolically active cells (44). Treatment with SAHA or MS275 resulted in an increase in mitochondrial density and greater electron opacity of the matrix to the detriment of the development and organization of cristae. Cells treated with the class II HDAC inhibitor MC1568 showed rod-like mitochondria similar to those of controls (bars = 500 nm). C: Expression of genes associated with mitochondrial biogenesis (24 h) and Western blot analysis of mitochondrial transcription factor A (Tfam) in C2C12 myotubes treated for 48 h with HDAC inhibitors (D) and the electron transfer chain in C2C12 myotubes treated with HDAC inhibitors for 48 h. Data are presented as means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control. C and CTRL, control; MC, MC1568; MS, MS275; S, SAHA.
than liver (Supplementary Fig. 2F and G). MC1568 had no effect on any parameter, indicating that inhibition of class I HDACs underlies the observed improvements in metabolic profile.

**Class I HDAC inhibitors induce oxidative metabolism in skeletal muscle.** To explore the molecular basis of the beneficial effects of SAHA and MS275 on metabolic parameters, we measured the expression of metabolic genes in skeletal muscle. In gastrocnemius, SAHA and MS275 increased expression of transcription factors and cofactors that regulate mitochondrial function (e.g., Pgc-1α, Pgc-1β, Tfam, and Tfb1m) (Fig. 3A) and of genes involved in glucose (Glut4 and Pk) and lipid metabolism (Acadl, TCA cycle (Idh3a and Suclg1), and oxidative phosphorylation (CytC, Cox6a1, and Etfdh) (Fig. 3B). Similar effects were observed in the vastus lateralis and soleus (Supplementary Fig. 3A and D). Changes in gene expression translated to differences in protein levels (e.g., Tfam, Acadl [Fig. 3C]). Electron microscopy provided suggestions of differences in mitochondrial content, but these were not conclusive (Fig. 3E). No changes were detected in ectopic lipid levels (Supplementary Fig. 3C), perhaps because the extreme obesity of db/db mice did not allow detection of modest changes. Absence of toxicity was confirmed by lack of increased alkaline phosphatase or esterase staining (Supplementary Fig. 4A).

Notably, while Tnn1 mRNA levels increased in soleus, no concomitant changes in mRNA levels of contractile proteins characteristic of type I and II myofibers occurred in gastrocnemius or vastus lateralis (Supplementary Fig. 4A). These findings indicate that inhibition of class I HDACs in skeletal muscle contributes to ameliorating the phenotype of db/db mice at least in part by increasing expression of genes involved in fatty acid oxidation and glucose clearance.

**Inhibition of class I HDACs promotes energy expenditure in db/db mice.** Since the oxidative pattern of gene expression induced by global and class I–selective HDAC inhibitors has been associated with increased energy expenditure, we assessed energy balance in db/db mice treated with SAHA or MS275. Animals treated for 15 days with the class I–selective HDAC inhibitor showed increased oxygen consumption and carbon dioxide release.
during the dark cycle, while only a trend for increased oxygen consumption was observed during the light cycle (Fig. 4A and B and Supplementary Fig. 5A). Nonetheless, these changes were sufficient to reduce the respiratory exchange ratio (RER) during both light and dark cycles, an indication that these mice use lipids preferentially as fuel (Fig. 4C). Heat production was also 12% greater in the MS275-treated group (Fig. 4D), while locomotor activity did not change (Supplementary Fig. 5B). SAHA had no significant effect on oxygen consumption, but treated mice showed a mild but significant reduction of RER during the day (Fig. 4A–D).

**HDAC3 regulates oxidative metabolism in a Pgc-1α-dependent manner.** To explore the mechanism whereby class I HDAC inhibition promotes mitochondrial biogenesis, oxidative metabolism, and increased energy expenditure, we evaluated the contribution of Pgc-1α to these effects. C2C12 myotubes infected with shRNA against Pgc-1α lost the ability to increase oxidative gene expression upon SAHA or MS275 exposure (Fig. 5A), indicating that Pgc-1α is a primary mediator of the effect of class I HDAC inhibition on oxidative gene expression. Absence of Rip140 did not abolish the response to treatment with SAHA or MS275 (Supplementary Fig. 6A and B), suggesting that Rip140 is not a central mediator of the effect of these compounds.

Next, we sought to understand how class I HDAC inhibition enhances Pgc-1α expression. Two class I HDACs are recruited onto two different regions of the Pgc-1α promoter: Hdac1 represses Creb-mediated Pgc-1α transcription (23), while Hdac3, together with Hdac4, Hdac5, and the nuclear corepressor NCoR, is recruited onto members of the Mef2 family to repress transcriptional activation of Pgc-1α (24,25) (Fig. 5B). We found that a 70% reduction of Hdac3 (Fig. 5C) is sufficient to mimic the effect of class I HDAC inhibitors on the expression of Pgc-1α, Glut4, Tfam, and Idh3a (Fig. 5D). In contrast, similar silencing of Hdac1 had no effect on expression of these genes (Supplementary Fig. 6C and D). ChIP showed that treatment with SAHA or MS275 reduced Hdac3 recruitment onto the Mef-binding site in the Pgc-1α promoter (Fig. 5E). The presence of Hdac3 in the cAMP-responsive element region was barely detectable and was unaffected by compound treatment. Similar results were obtained

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**FIG. 3.** Inhibition of class I HDACs promotes oxidative metabolism in skeletal muscle. mRNA expression levels of mitochondrial biogenesis-associated (A) and metabolic pathway (B) genes in skeletal muscle of db/db mice treated with HDAC inhibitors. C: Western blot analysis of Acadl and Tfam in skeletal muscle of db/db mice after treatment with HDAC inhibitors. D: Succinate dehydrogenase staining and quantification of dark fibers (arrows) in gastrocnemius sections (bars = 100 μm). E: Representative electron microphotographs of ultrathin sections of gastrocnemius from mice treated with vehicle or MS275 (magnification: upper panels, ×15,000; lower panels, ×8,000). Data are presented as means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control. C, control; MS, MS275; S, SAHA. (A high-quality color representation of this figure is available in the online issue.)

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when ChIP was performed in skeletal muscle extracts derived from db/db mice treated with SAHA or MS275: both compounds decreased Hdac3 recruitment on the Pyc-1a promoter in vivo, though MS275 had a greater effect (Fig. 5F). These results suggest that the beneficial effects of class I HDAC inhibition in muscle are primarily due to Hdac3 inhibition that results in increased Pyc-1a expression.

Inhibition of class I HDACs promotes uncoupled metabolism in brown adipose tissue. As observed for skeletal muscle, the compounds retained their class-selective inhibitory activity in the brown adipose tissue (BAT) of treated animals (Fig. 6E). BAT of obese diabetic animals treated with SAHA or MS275 showed a significant reduction in cell size (Fig. 6A). MRI analysis showed a 13.6 and 15.7% increase of interscapular BAT in SAHA- and MS275-treated animals, respectively (Fig. 6B). db/db mice treated with MS275, but not SAHA, maintained body temperature better during an acute cold challenge, indicating that the increased BAT mass is functional (Fig. 6C). This observation suggests that Hdac3 inhibition, and its consequent dissociation from the promoter, may be primarily responsible for Pyc-1a induction in mice treated with MS275.

WAT treated with a class I HDAC inhibitor acquires brown fat features. White adipocyte size was reduced in db/db mice treated with the class I-selective HDAC inhibitor (Fig. 7A and Supplementary Fig. 7A), and MRI analysis also showed an 18% reduction of WAT in these animals (Supplementary Fig. 7B). These findings were associated with increased expression of genes that regulate lipid metabolism (Fig. 7G) and increased mitochondrial DNA content (Fig. 6H). As a consequence, treated primary brown adipocytes showed higher basal, uncoupled, and maximal respiratory capacity (Fig. 6J).

In analogy to skeletal muscle, we performed ChIP in BAT and found significantly diminished amounts of Hdac3 associated with the region containing the PPAR-responsive element of the Pyc-1a promoter in animals treated with MS275 (Fig. 6J). This observation suggests that Hdac3 inhibition, and its consequent dissociation from the promoter, may be primarily responsible for Pyc-1a induction in mice treated with MS275.
Surprisingly, inhibition of class I HDACs also resulted in a dramatic increase in the expression of genes normally associated with brown fat (Fig. 7 and Supplementary Table 1). Ucp1 (~50-fold increase), Cidea, Dio2, Adrb3, and other markers characteristic of brown adipocytes were robustly induced in WAT treated with MS275 and, to a lesser extent, with SAHA (Fig. 7A and G). As reported in other cases of “browning” of white adipose depots (26–29), the class I inhibitor appears to transcriptionally reprogram WAT toward a more oxidative phenotype characterized by a strong upregulation of Ucp1 expression.

**DISCUSSION**

In spite of provocative genetics data that demonstrate a role for class I and II HDACs in muscle physiology, significantly less is known about the ability of modulators of these HDAC classes to regulate systemic metabolism. Recent studies used the natural short-chain fatty acid sodium butyrate to show that this dual class I/II HDAC inhibitor increases insulin sensitivity and energy expenditure in high-fat–fed mice (12,30,31). However, sodium butyrate has a cornucopia of cellular effects, many of which are independent of its ability to block HDAC function (32). Conclusive association between chemical inhibition of specific HDAC classes and systemic energy metabolism has thus been lacking. In this study, we have used a pan-inhibitor and class I– or class II–selective synthetic HDAC inhibitors to establish the contribution of specific HDACs to whole-body metabolism.

In vitro, class I HDAC inhibition enhanced expression of critical mitochondrial regulators resulting in increased mitochondrial biogenesis and greater oxygen consumption in muscle cells and primary brown adipocytes. The lack of an effect of the class II HDAC inhibitor was surprising; however, class II HDACs are thought to have minimal deacetylase activity and to behave primarily as bridging molecules that recruit catalytically active HDAC complexes and other corepressors (33). Hence, it is possible that chemical inhibition of class II HDACs is not sufficient to interfere with assembly of silencing complexes. Pgc-1α mediates the effects of class I HDAC inhibition, as silencing of Pgc-1α abolishes the effect of these compounds on oxidative gene expression and genetic knockdown of Hdac3 recapitulated the effects seen with the chemical inhibitor of class I HDACs on Pgc-1α expression. We have shown in vitro and in vivo that the class I–selective inhibitor induces Pgc-1α transcription by blunting Hdac3 recruitment onto the Pgc-1α promoter, thus driving oxidative gene expression in skeletal muscle and BAT and browning of WAT.

FIG. 5. HDAC3 ablation mimics the effect of class I HDAC inhibitors in a PGC-1α–dependent manner. A: Pgc-1α, Glut4, Tfam, and Idh3α expression in C2C12 myotubes infected with adenoviruses expressing shRNA against Pgc-1α (■) or scramble control (□). Note that the effect of HDAC inhibitors is lost in the absence of Pgc-1α. B: Schematic representation of the HDACs known to be present on the Pgc-1α promoter (prom). C: Hdac3 protein levels in C2C12 myoblasts transfected with small interfering RNA against Hdac3 or control. D: Gene expression profile after silencing Hdac3 in C2C12 myoblasts. E and F: Hdac3 ChIP of C2C12 myotubes or skeletal muscle (SKM) of db/db mice treated with HDAC inhibitors. Bars represent presence of Hdac3 on the Pgc-1α promoter within the Mef2 or the cAMP-responsive element regions shown in B. A distal region was used as a negative control. Data are presented as means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control. C, control; MC, MC1568; MS, MS275; Rel., relative; S, SAHA. (A high-quality color representation of this figure is available in the online issue.)
Treatment of db/db mice with SAHA or MS275 resulted in enhanced glucose tolerance and insulin sensitivity, clearance of liver lipids, and decreased plasma triglycerides and free fatty acids. Given the large mass of skeletal muscle, its contribution to glucose clearance is likely to be comparable in mice treated with either SAHA or MS275, perhaps explaining why glucose and insulin levels were similarly decreased in these two groups. The reduction in hepatic steatosis contrasts with the accumulation of liver lipids seen in liver-specific Hdac3-null mice (34). This difference may be ascribed to the effect of global versus local HDAC3 inhibition: in diabetic mice, systemic HDAC3 inhibition (i.e., MS275 treatment) increases peripheral oxidative metabolism and energy expenditure, thus preventing hepatic lipid buildup. The increased PPARγ activity that we observed in WAT of MS275-treated mice also likely means that the capacity of this tissue to remove from the circulation and store excess fatty acids is enhanced, further preventing ectopic lipid deposition. The greater effect of the class I HDAC inhibitor relative to SAHA may be due to the fact that MS275 treatment resulted in significantly more dramatic changes in gene expression in adipose tissue. This could be a reflection of lower adipose tissue exposure to SAHA, perhaps a result of its pharmacokinetic profile (the in vivo half-life of SAHA is significantly shorter than that of MS275: 2 vs. 80 h) (35). This result suggests that robust suppression of class I HDACs in both skeletal muscle and adipose tissue is necessary to obtain the full metabolic benefit of inhibition of class I HDACs.

MS275 potentiates BAT function by increasing expression of markers of oxidative and uncoupled metabolism. These changes underlie the increased heat production and may contribute to the improvement of circulating lipid levels, as BAT plays a major role in triglyceride clearance.
In vivo ChIP experiments indicate that inhibition of Hdac3 may be responsible for the beneficial effects of MS275 treatment in BAT. MS275 treatment suppressed inflammatory markers and macrophage infiltration into white adipose, yet its most dramatic effect in WAT was its ability to induce robust expression of markers of BAT. This browning occurred in the absence of a change in expression of Prdm16, a major determinant of interscapular brown fat development (38). Consistent with work showing that chronic treatment of primary white adipocytes with Pparγ ligands results in the acquisition of brown adipocyte features in the absence of Prdm16 expression (26), we found that treatment with the class I HDAC inhibitor increased Pparγ expression and that of its targets in WAT. Increased Pparγ signaling in WAT could be a major determinant of the improvement of metabolic parameters seen in MS275-treated mice, for increased expression or enhanced activity of Pparγ exclusively in WAT has profound effects on systemic insulin sensitivity and lipid homeostasis (19,39). In analogy with our results in muscle and BAT, it is likely that enhanced Pparγ activity in WAT upon MS275 treatment is due to inhibition of Hdac3, as this HDAC has been shown to associate with Pparγ to block its function, though we cannot exclude the possibility of contribution of other class I HDACs to this effect (40).

Mice lacking the nuclear corepressor NCoR1 in either skeletal muscle or adipose tissue exhibit a phenotype similar to animals treated with MS275 (41,42). NCoR1 participates in transcriptional repression together with silencing mediator for retinoid and thyroid hormone receptors (SMRTs) and Hdac3 (43); thus, these observations are consistent with the notion that interfering with Hdac3 activity improves skeletal muscle and adipose tissue function.

Our results highlight the pivotal role of class I HDAC activity in the regulation of energy homeostasis. We have shown that pharmacologic inhibition of class I HDACs in the context of obesity and diabetes potentiates mitochondrial function and oxidative capacity in skeletal muscle and adipose tissue. These observations suggest that synthetic class I HDAC inhibitors may have promise in the treatment of obesity and associated disorders.
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A.Ga. and N.M. conceived the study, designed the experimental plan, performed most of the experiments, analyzed data, and wrote and edited the manuscript. A.F. isolated primary brown adipocytes, measured oxygen consumption, performed immunoblots and some molecular experiments, and read and edited the manuscript. E.G., F.G., and C.G. participated in the initial elaboration of the project and read and edited the manuscript. G.C. performed some biological and biochemical experiments and read and edited the manuscript. A.Gu. and E.D. performed electron microscopy analysis, contributed to image interpretation, and read and edited the manuscript. D.R. and C.G. participated in the initial elaboration of the project and read and edited the manuscript. D.C. provided help in histological analysis of muscle sections, Marianna Gaman (Università degli Studi di Milano) for help in electron microscopy analysis, contributed to image interpretation, and read and edited the manuscript. U.G. provided expertise for MRI experiments and read and edited the manuscript. D.R. provided suggestions for some biological experiments and read and edited the manuscript. A.M. synthesized MS275 and read and edited the manuscript. E.S. conceived the study, designed the experimental plan, analyzed data, wrote the manuscript, and edited the manuscript. E.D.F. and M.C. conceived the study, designed the experimental plan, analyzed data, wrote the manuscript, supervised the entire work, and edited the manuscript. E.D.F. and M.C. are the guarantors of this work and, as such, had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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