IN THIS ISSUE, PSYCHOLOGY
Correction for the “In This Issue” summary entitled “Universal displays of pride and shame,” which appeared in issue 33, August 19, 2008, of Proc Natl Acad Sci USA (105:11587–11588).

The authors note that the figure is copyrighted by Bob Willingham and is reprinted with permission. The online version has been corrected. The figure and its corrected legend appear below.

Blind athletes (Right) show pride in victory like sighted athletes (Left). [Reproduced with permission (Copyright 2004, Bob Willingham).]

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PERSPECTIVE

The authors note that a reference was inadvertently omitted from their article. On page 4607, right column, in Conclusion: How Do New Gene Clusters Form?, line 17, the reference callout “(109–111)” should instead read “(109–111, 113).” The added reference appears below.


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DEVELOPMENTAL BIOLOGY

The authors note that a reference was inadvertently omitted from their article. On page 19187, right column, in Conclusion: How Do New Gene Clusters Form?, line 17, the reference callout “(109–111)” should instead read “(109–111, 113).” The added reference appears below.


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APPLIED BIOLOGICAL SCIENCES

The authors note that the author name Christian Steinkulher should have appeared as Christian Steinkulher. The author line has been corrected online. The corrected author line appears below.

Claudia Colussi, Chiara Mozzetta, Aymone Gurtner, Barbara Illi, Jessica Rosati, Stefania Straino, Gianluca Ragone, Mario Pescatori, Germana Zaccagnini, Annalisa Antonini, Giulia Minetti, Fabio Martelli, Giulia Piaggio, Paola Gallinari, Christian Steinkulher, Emilio Clementi, Carmela Dell’Aversana, Lucia Altucci, Antonello Mai, Maurizio C. Capogrossi, Pier Lorenzo Puri, and Carlo Gaetano

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IMMUNOLOGY

The authors note that due to a printer’s error, in the Abstract, beginning on line 6, “‘TDCs include two conventional dendritic cell (DC) subtypes, CD8hiSirplo/hi (CD8hiSirplo+ and CD8hiSirplo– (CD8hiSirplo–), which have different origins. We found that the CD8hiSirplo+ DCs represent a conventional DC subset that originates from the blood and migrates into the thymus’” should instead read: “‘TDCs include two conventional dendritic cell (DC) subtypes, CD8hiSirplo+ (CD8hiSirplo+) and CD8hiSirplo– (CD8hiSirplo–), which have different origins. We found that the CD8hiSirplo+ DCs represent a conventional DC subset that originates from the blood and migrates into the thymus.’”

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HDAC2 blockade by nitric oxide and histone deacetylase inhibitors reveals a common target in Duchenne muscular dystrophy treatment

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The overlapping histological and biochemical features underlying the beneficial effect of deacetylase inhibitors and NO donors in dystrophic muscles suggest an unanticipated molecular link among dystrophin, NO signaling, and the histone deacetylases (HDACs). Higher global deacetylase activity and selective increased expression of the class I histone deacetylase HDAC2 were detected in muscles of dystrophin-deficient MDX mice. In vitro and in vivo siRNA-mediated down-regulation of HDAC2 in dystrophic muscles was sufficient to replicate the morphological and functional benefits observed with deacetylase inhibitors and NO donors. We found that restoration of NO signaling in vivo, by adenosine-mediated expression of a constitutively active endothelial NOS mutant in MDX muscles, and in vitro, by exposing MDX-derived satellite cells to NO donors, resulted in HDAC2 blockade by cysteine S-nitrosylation. These data reveal a special contribution of HDAC2 to NO donors, target the follistatin–myostatin pathway is important for the therapeutic response to NO donors in MDX mice. They also define a common target for independent pharmacological interventions in the treatment of Duchenne muscular dystrophy.

Recent studies have reported on the therapeutic potential of pharmacological interventions that target events downstream to the genetic defect responsible for the disease. For instance, histone deacetylase inhibitors (DIs) and NO donors countered the progression of muscular dystrophy in MDX mice (1, 2). Intriguingly, the beneficial effect of both treatments relies, at least in part, on the formation of myofibers larger than normal and on the transcriptional activation of the myostatin antagonist follistatin (1, 3, 4). This evidence suggests a common mechanism linking the effect of NO donors and that of DIs in dystrophic muscles.

Targeting the follistatin–myostatin pathway by either direct myostatin blockade or follistatin overexpression has revealed valuable therapeutic potential in the treatment of muscular dystrophies (5–8). Thus, the understanding of the mechanism by which compounds of pharmacological interest, such as DIs and NO donors, target the follistatin–myostatin pathway is instrumental to develop effective strategies in the treatment of muscular dystrophies. Follistatin expression in skeletal muscles is regulated by class I histone deacetylases (HDACs) (1, 4), which inhibit the activity of MyoD (9, 10) and additional transcription factors, such as CREB and NFAT, possibly recruited to the follistatin promoter (4).

In the present study we have investigated the individual role of class I HDACs in the progression of muscular dystrophy in MDX mice and evaluated the possibility that DIs and NO signaling could converge on HDAC blockade. We show that class I HDAC2 expression and activity are increased in skeletal muscle from MDX mice. HDAC2 down-regulation by siRNA enhances the ability of MDX-derived-satellite cells to form multinucleated myotubes in vitro and is sufficient to counter the disease progression in vivo, when delivered to MDX muscles.

Finally, we show that HDAC2 S-nitrosylation by NO donors impairs its enzymatic function. These results indicate that HDAC2 is an important common pharmacological target of distinct pharmacological interventions aimed at Duchenne muscular dystrophy (DMD) treatment and suggest a novel mechanism of HDAC2 inhibition by NO-dependent cysteine S-nitrosylation.

Results and Discussion

Previous studies have reported on the impaired NO production in dystrophic muscles due to the disruption of the association between sarcolemmal neuronal NOS and the dystrophin–sarcoglycan complex (11–14). Consistently, the reconstitution of NO levels ameliorates the dystrophic phenotype in MDX mice (14). Additional evidence shows that agents that promote NO release stimulate myoblast fusion into multinucleated myotubes with an increased size via follistatin up-regulation (4)—an effect reminiscent of that observed upon treatment with DIs (2).

We found that the injection of an adenosine vector encoding for a constitutively active endothelial NOS (eNOS) mutant in MDX adductor muscle [supporting information (SI) Fig. S1a] led to the recovery of muscle morphology (Fig. 1) and to the increase in muscle fiber cross-sectional area that are equivalent

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to those observed upon treatment with the class I HDAC inhibitor MS27-275 (Fig. 1B). These results suggest that class I HDACs could be a common target for DIs and NO donors.

The regulation of individual HDACs in dystrophic muscles and their potential role in disease pathogenesis and progression in MDX mice have never been addressed. Thus, we have monitored the global HDAC activity in muscles from normal vs. MDX mice. Whole muscles isolated from MDX mice showed a higher HDAC activity as compared with the normal counterpart (Fig. 2A). This activity was eliminated by the class I DI MS27-275 (Fig. 2A), indicating a deregulated expression and/or activity of class I HDACs in dystrophic muscles. Among the class I HDAC members analyzed by us, only the expression levels of HDAC2 were increased in lysates from whole muscles (Fig. 2B). Likewise, satellite cells derived from single myofibers isolated by MDX mice showed an increased global deacetylase activity (Fig. 2C) and higher expression levels of HDAC2 (Fig. 2D). These data indicate a special contribution from HDAC2 to the pathogenesis and/or disease progression in MDX mice. They also significantly narrow down the spectrum of potential targets of selective pharmacological strategies that could replicate the beneficial effect of DIs in the treatment of DMD. Importantly, an independent screening of siRNA-mediated down-regulation of each individual HDAC (from HDAC1 to HDAC11; data not shown) in primary human skeletal muscle cells showed that HDAC2 siRNA replicates the morphological effect of DIs (fusion into multinucleated myotubes that are larger than controls) more than any other individual HDAC siRNA (G.M., P.G., and P.L.P., unpublished data). Note that this effect was accompanied by the up-regulation of follistatin (Fig. S1b), which is consistent with our previous finding that HDAC2 occupies follistatin promoter in myoblasts (2).

Thus, deregulation of HDAC2 levels and activity appears to be a hallmark of MDX muscles and suggests that selective neutralization of HDAC2 could have a beneficial effect in these mice. siRNA was therefore used to selectively down-regulate HDAC2 in MDX muscles in vitro and in vivo and to assess the effect on regeneration and morphological and functional parameters. HDAC2 knockdown in satellite cells derived from MDX mice was achieved by delivery of siRNA oligonucleotides (Fig. 3A). Satellite cells receiving the HDAC2 siRNA showed enhanced fusion into myotubes when compared with controls (70–80% increase in the fusion index of HDAC2 siRNA-treated MDX satellite cells vs. controls) (Fig. 3B Upper). Down-regulation of HDAC2 was estimated at ~50% by Western blotting analysis (Fig. 3A), which corresponds to a decrease in HDAC2-specific activity paralleled by a global deacetylase activity reduction (Fig. 3B Lower). Thus, in MDX satellite cells, it is sufficient to reduce the levels and activity (Fig. 3B) of the aberrantly expressed HDAC2 to obtain morphological features that are reminiscent of those achieved with NO donors and DIs treatment (3, 4, 13). Consistently, myosin heavy chain (MHC) expression was increased in siRNA-treated MDX satellite cells when compared with control cells (Fig. 3B Lower Left).

Based on this evidence we sought to evaluate whether in vivo down-regulation of HDAC2 in muscles of MDX mice could replicate the beneficial effect of NO donors and DIs. Systemic delivery of HDAC2 siRNAi in MDX mice was achieved by daily i.p. injections for 30 days and led to a down-regulation of HDAC2 in the skeletal muscles that was comparable to that obtained in satellite cells (Fig. 3C). Notably, systemic siRNA-mediated HDAC2 down-regulation resulted in a morphological (Fig. 3D) and functional (Fig. 3D) recovery of dystrophic muscles. Although the strategy adopted in this experiment (systemic delivery of HDAC2 siRNA) does not rule out that HDAC2 down-regulation in other tissues and organs could indirectly contribute to the effect on muscles, this possibility appears remote because HDAC2 is increased only in muscles of MDX mice (data not shown).

The data presented so far suggest that in dystrophic muscles an impaired dystrophin–NO signaling to HDAC2 can provide a selective target for pharmacological interventions. This information assumes a particular relevance within the current effort toward the identification of individual HDACs that should be targeted to achieve the same beneficial effect observed with general DIs.

HDAC2 regulation by NO has recently been reported in neurons (15, 16). Nott et al. (16) identified 2 cysteines at positions 262 and 274 targeted by NO-dependent S-nitrosylation.
The S-nitrosylation of these residues determines an impairment of HDAC2 binding to target DNA sequences (16). A comparison analysis of the amino acidic sequence of all mouse and human class I HDACs revealed that these cysteines are well-conserved among the different class I members, suggesting the possibility that other HDACs, including HDAC1 and HDAC3, could be regulated by S-nitrosylation (see Fig. S2). We therefore investigated whether NO-mediated S-nitrosylation of HDAC1, HDAC2, and HDAC3 could be a relevant intracellular signaling altered in dystrophic muscles. Cysteine-S-nitrosylation of endogenous HDAC2, but not that of HDAC1 and HDAC3, was detected in C2C12 myoblasts exposed to NO donors (Fig. 4A).
This cysteine-S-nitrosylation correlated with a significant reduction in the enzymatic activity of HDAC2 (Fig. 4B). Similarly, HDAC2-S-nitrosylation was increased in MDX adductor muscles infected with the eNOS adenovirus (Fig. 4C), leading to the reduction of HDAC2-specific activity, which is typically elevated in MDX muscles (Fig. 4D). This evidence links the effect of restoring the NO signaling in dystrophin-deficient muscle with a beneficial effect due to HDAC2 inhibition via S-nitrosylation. Surprisingly, we have detected a reduced activity of HDAC1 in lysates from C2C12 myoblasts and from MDX adductor muscle despite the fact that HDAC2 is not S-nitrosylated by DETA-NO (Fig. 4 B–D). In contrast, the enzymatic activity of HDAC3 was unaffected by NO. We further investigate the relationship between NO-mediated S-nitrosylation and the enzymatic activity of HDAC1, HDAC2, and HDAC3 by measuring in vitro the enzymatic activity of the recombinant enzymes purified from _E. coli_ in the presence of NO donors or the D1 SAHA, as control. NO donors markedly reduced the enzymatic activity of HDAC2, minimally influenced that of HDAC1, and were ineffective on HDAC3 (Fig. 4E). In agreement with these results, Western blotting analysis of nitrosylated recombinant HDAC1 and HDAC2 protein showed a strong S-nitrosylation signal in HDAC2 that was not detectable in HDAC1 (Fig. 4F). These results indicate that direct regulation of deacetylase activity by NO-mediated S-nitrosylation is restricted to HDAC2 and that NO-mediated inhibition of HDAC1 can occur by an S-nitrosylation-independent mechanism. Collectively, our findings indicate that HDAC2 inhibition, either via direct blockade or by S-nitrosylation, mediates the beneficial effects of DIs and NO, respectively. This information provides the mechanism that accounts for the overlapping features of 2 distinct pharmacological interventions in DMD.

The key role of class I HDACs in DMD pathogenesis and progression was previously suggested by the beneficial effect exerted by MS27-275 in MDX mice (2). Our current data further extend this notion and indicate that selective blockade of HDAC2 is a primary target for therapeutic strategies.

HDAC2-S-nitrosylation is emerging as a nodal regulatory mechanism for signal-dependent control of gene expression (15, 16). Our finding that HDAC2 is specifically up-regulated in dystrophic muscles offers an interesting model for the exploitation of pharmacological manipulation of HDAC2 via S-nitrosylation as a selective intervention in a genetic disease. (i.e., muscular dystrophies). The precise role of HDAC2 cysteine S-nitrosylation in the regulation of gene expression by cGMP-activated NO signaling (4, 17, 18) during skeletal myogenesis remains to be determined. However, our data indicate that derepression of HDAC2-regulated genes (19–23), such as follistatin, might produce compensatory responses (e.g., increased regeneration) that counterbalance muscle degeneration in muscle wasting disorders. Future work should determine the identity of the genes affected by HDAC2 inhibition via NO-mediated S-nitrosylation and establish whether inhibition of HDAC2 by distinct treatments in dystrophic muscles has synergistic effects. Moreover, the key role of HDAC2 in mediating corticosteroid-regulated gene expression (24, 25) suggests that pharmacological modulation of HDAC2 could also be exploited to modulate the effect of glucocorticoids in the current treatment of DMD (26).

Given the physical and functional relationship between dystrophin (and possibly other components of the dystrophin-associated complex) and NO, and because of the impaired NO signaling in dystrophic muscles, future studies should be devoted to investigating whether reduced HDAC2-S-nitrosylation is responsible for the deregulated gene expression in dystrophic muscles (27). This analysis should determine the contribution of HDAC2 up-regulation in the pathogenesis of muscular dystrophies. Overall, these studies will establish a link between the genetic defect responsible for DMD and epigenetic modifications underlying the disease progression that could be targeted by epigenetic drugs without correcting the primary genetic defect.

Methods

Animals and Treatments. Eight-week-old normal WT C57BL/10 and MDX mice were used in the experiments. MDX mice received i.p. daily injections of MS27-275.
at a dose of 0.5 mg/kg or saline for 21 days. All experimental procedures were approved by the internal Animal Research Ethical Committee (protocol HH39) according to the Italian Ministry of Health and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Cell Cultures and Treatments.** C2C12 myoblasts were maintained at subconfluence in DMEM supplemented with 20% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. DETANO (500 μM) was administered for 2 and 4 h to C2C12 cells in growing conditions. Satellite cells were isolated from single fibers as described in ref. 9 and maintained in growing conditions or induced to differentiate in low-serum medium.

**Infections.** C2C12 myoblasts and satellite cells were infected with either adenoviruses CMV-GFP encoding for the constitutively active isoform of eNOS (eNOS S1177D) or CMV-GFP-null. In in vivo experiments, adductor muscles were injected with 1 × 10^6 pfu per animal of virus according to previously published procedures (28, 29).

**RNA Interference.** Satellite cells were transfected with 3 different oligonucleotides for HDAC2 siRNA (30 nM each; Ambion) by using siPORT NeoFX according to instructions from the manufacturer.

**Protein Analysis.** Western blot was performed on tissue extracts after lysis in 50 mM Tris-HCl (pH 8.0), 125 mM NaCl, 1 mM DTT, 5 mM MgCl2, 1 mM EDTA, 10% glycerol, and 0.1% Nonidet P-40 supplemented with 1 mM PMSF and protease inhibitor mix. The extracts were resolved in SDS/PAGE and transferred on nitrocellulose membrane (Hybond ECL; Amersham) at 50 V for 24 h at 4 °C before immunoblotting assays.