Mitochondrial Alterations and Oxidative Stress in an Acute Transient Mouse Model of Muscle Degeneration

**IMPLICATIONS FOR MUSCULAR DYSTROPHY AND RELATED MUSCLE PATHOLOGIES**

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Background: Human muscular dystrophies and inflammatory myopathies share common pathological events. The cardiotoxin (CTX) model displayed acute and transient muscle degeneration and all the cellular events usually implicated in human muscle pathology.

Results: Mitochondrial alterations and oxidative stress significantly contribute to muscle pathogenesis.

Conclusion: The CTX model is valuable in understanding the mechanistic and therapeutic paradigms of muscle pathology.

Muscular dystrophies (MDs) and inflammatory myopathies (IMs) are debilitating skeletal muscle disorders characterized by common pathological events including myodegeneration and inflammation. However, an experimental model representing both muscle pathologies and displaying most of the distinctive markers has not been characterized. We investigated the cardiotoxin (CTX)-mediated transient acute mouse model of muscle degeneration and compared the cardinal features with human MDs and IMs. The CTX model displayed degeneration, apoptosis, inflammation, loss of sarcolemmal complexes, sarcolemmal disruption, and ultrastructural changes characteristic of human MDs and IMs. Cell death caused by CTX involved calcium influx and mitochondrial damage both in murine C2C12 muscle cells and in mice. Mitochondrial proteomic analysis at the initial phase of degeneration in the model detected lowered expression of 80 mitochondrial proteins including subunits of respiratory complexes, ATP machinery, fatty acid metabolism, and Krebs cycle, which further decreased in expression during the peak degenerative phase. The mass spectrometry (MS) data were supported by enzyme assays, Western blot, and histochemistry. The CTX model also displayed markers of oxidative stress and a lowered glutathione reduced/oxidized ratio (GSH/GSSG) similar to MDs, human pathologies, and neurogenic atrophies. MS analysis identified 6 unique oxidized proteins from Duchenne muscular dystrophy samples (n = 6) (versus controls; n = 6), including two mitochondrial proteins. Interestingly, these mitochondrial proteins were down-regulated in the CTX model thereby linking oxidative stress and mitochondrial dysfunction.

We conclude that mitochondrial alterations and oxidative damage significantly contribute to CTX-mediated muscle pathology with implications for human muscle diseases.

Muscular dystrophies (MDs) and inflammatory myopathies (IMs) are neuromuscular disorders arising due to muscle-intrinsic defects. MDs are clinically and genetically heterogeneous, characterized by progressive degeneration of the skeletal muscle (1). Duchenne muscular dystrophy (DMD), dysferlinopathy (Dysf), and sarcoglycanopathy (Sgpy) are common MDs with DMD being the most devastating condition that culminates in premature death (2). Various molecular processes downstream of the genetic mutations in these MDs lead to dystrophic pathology (2, 3). Conversely, IMs are mostly sporadic, characterized by intense and acute inflammation and necrosis (4). Although most MDs have established methods of diagnosis, most of them are not treatable because the molecular basis of muscle degeneration is not completely understood. On the other hand, IMs are treatable disorders, with good clinical outcome. Interestingly, muscle damage in MDs and IMs involve common pathways including cycles of myofiber necrosis/apoptosis, regeneration, and inflammation (4, 5). Muscle degeneration in these conditions in general involves cellular events including sarcolemmal disruption, altered cytoskeletal net-

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3 The abbreviations used are: MD, muscular dystrophy; CTX, cardiotoxin; DMD, Duchenne muscular dystrophy; Sgpy, sarcoglycanopathy; Dysf, dysferlinopathy; IM, inflammatory myopathy; MM, mitochondrial myopathy; ROS, reactive oxygen species; EM, electron microscopy; DNP, dinitrophenyl; EHC, enzyme histochemistry; NADH-TR, nicotinamide adenine dinucleotide tetrazolium reductase; SDH, succinic dehydrogenase; COX, cytochrome oxidase; TA, tibialis anterior; ETC, electron transport chain; CI, mitochondrial complex I; CII, mitochondrial complex II; CIII, mitochondrial complex III; CK, creatine kinase; CS, citrate synthase; D1 etc., day 1 etc.; DAF-FM-DA, 4-amino-5-methylamino-2',7'-difluorofluorescin-diacetate; SMA, spinal muscular atrophy; JC-1, 5,5'-6',6'-tetrachloro-1,1',3,3'-tetraethyl-benzamidazo carboxyanin iodide; CS, citrate synthase; Bicine, N,N-bis(2-hydroxyethyl)glycine; DNPH, dinitrophenyl hydrazine.
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work, aberrant calcium dynamics, oxidative stress, and mitochondrial damage (2, 5, 6). But, the interplay of these processes, their chronology and contribution to degeneration, and the molecular mediators involved are not well elucidated. This is partially due to the absence of reliable models that recapitulate all the molecular, pathological, and symptomatic features common to MDs and IMs.

MDs have been extensively studied using patient muscle biopsies (6, 7) and genetic models (8). However, the models do not replicate the human condition due to species barrier, altered chronology of events, and variable severity of muscle pathology (2). Furthermore, a single model that displays all the cellular events common to MDs and IMs, exhibiting transient degeneration followed by active regeneration and displaying secondary processes such as inflammation and fibrosis, needs to be characterized for mechanistic and therapeutic assessment.

Non-genetic transient animal models that simulate myopathic pathology could be useful to study the pathogenesis. In this regard, two models that display muscle damage/degeneration are the cardiotoxin (CTX) (9) and bupivacaine (10) models. However, the implications of the CTX model for human MD and other myopathies and the role of oxidative stress, calcium dynamics, and mitochondrial dysfunction have not been clearly documented. In our study, we have characterized the muscle damage, secondary mechanisms, and necrotic pathways mediated by CTX in vivo and in vitro compared with human MDs and IMs and investigated the oxidative and mitochondrial changes involved in muscle degeneration.

EXPERIMENTAL PROCEDURES

All chemicals and solvents were of analytical grade. Bulk chemicals and solvents were obtained from Merck and Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Fine chemicals, PCR primers and consumables, tissue culture materials, CTX from Naja mossambica mossambica, protease inhibitor mixture, Nagarse, and α-dinitrophenyl (DNP) antibodies were obtained from Sigma. Proteomic grade trypsin was obtained from Loba Chemie (Mumbai, India). Antibodies cured from Molecular Probes. Tissue culture grade trypsin was obtained from Sigma. Proteomic grade trypsin was obtained from Bio-One GMBH (Frickenhausen, Germany). Amplex Red assay kit, dihydroxycetidium, BODIPY® 581/591 undecanoic acid, and 4-amino-5-methylamino-2′,7′-difluorofluorescein-diace-tate (DAF-FM-DA) were obtained from Invitrogen.

Human Tissue Samples—Patients (n = 103; age = 1.1–65 years) with muscle diseases evaluated at the Neuromuscular Disorders Clinic, NIMHANS, Bangalore, India, during 2006–2012, were selected following diagnostic procedures. The muscle strength of patients (based on the Medical Research Council scale) was recorded by the neurologist and graded 0 to 5 (supplemental “Experimental Procedures”). After obtaining written informed consent, skeletal muscle biopsies from these patients were diagnosed by histopathology. The study included immunohistochemically confirmed cases of DMD (n = 15), Dysf (n = 15), Sgpy (n = 15) and clinically and histologically confirmed cases of spinal muscular atrophy (SMA-1, -2, and -3) (n = 15), DM (n = 15), distal myopathy (Nonaka type) (n = 15), and mitochondrial myopathy (n = 13). As control, paraspinal muscles from patients (n = 12) undergoing spinal surgeries were procured after obtaining written informed consent. The study protocol was approved by the Institutional Ethics Committee. Fresh biopsy samples obtained as explained previously (12) were snap-frozen in isopentane pre-cooled in liquid nitrogen and stored at −80°C and used for histopathological and biochemical studies.

Animal Studies—Experiments were carried out according to the Institutional Guidelines for the Care and Use of Laboratory Animals. The study protocol was approved by the Institutional animal ethics committee. Adult male C57BL/6 mice (10 weeks old; ~30 g each; n ≥ 6 per treatment) maintained under standard conditions were injected either with saline or CTX (single injection; 300 μl of 10 μM in saline) across the tibialis anterior (TA) muscle on one of the hind limbs as described previously (9). CTX was uniformly released along the muscle tissue by injecting the myotoxin while withdrawing the syringe needle (13). Mice were euthanized 1, 2, 3, 5, 7, 11, 14, and 31 days after the CTX injection, and the ipsilateral and contralateral limb muscles were dissected. A fragment of the muscle oriented transversely and snap-frozen in isopentane pre-cooled in liquid nitrogen was used for enzyme and immunohistochemistry, whereas another portion was snap-frozen in liquid nitrogen for RT-PCR and biochemical assays. Tiny pieces were fixed in 3% glutaraldehyde for electron microscopy (EM) and the rest of the tissue was fixed in 10% formalin for histopathology.

Grip Strength Test—Grip strength of the mouse limbs was analyzed by measuring the maximum force exerted (in kg) on a Grip Strength Meter (Columbus Instruments, OH) in tension mode, using the grid assembly of the meter. The readings from 6 trials were averaged.

Histopathology—Cryosections (8-μm thick transverse sections of frozen muscle cut in a cryostat (Leica) at −20°C) were subjected to hematoxylin & eosin (H&E) staining, modified Gomori trichrome staining, enzyme histochemistry (EHC), nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR), succinic dehydrogenase (SDH), succinic dehydrogenase-cytochrome oxidase (SDH-COX), and immunohistochemistry (dystrophin, dysferlin, and α-sarcoglycan). For
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Evans Blue dye fluorescence analysis (excitation = 470 and 540 nm; emission = 680 nm), the cryosections were mounted on coverslips with buffered glycerol and subjected to confocal microscopy (DMIRED-TCS, Leica). Paraffin sections (tissue fixed in 10% formalin, grossed, sequentially dehydrated, embedded in paraffin, and sectioned in a microtome) were subjected to H&E, Masson trichrome, and Alizarin red staining (15). The tissues fixed in 3% glutaraldehyde were processed for EM as described previously (15).

Cell Culture and Cell Viability—C2C12 mouse myoblast cell line was grown as described previously (13). Differentiation was confirmed by the cellular morphology and multinucleation and CK assay (16) using a commercial kit (Olympus Life and Materials, Europa, GmbH). Cells were seeded in 96-well plates (5 x 10⁴/well) for cell viability, oxidative stress, and mitochondrial membrane potential assays and in 90-mm dishes (2 x 10⁶ cells/dish) for biochemical and Ca²⁺ assays (13). Cells were harvested, centrifuged (850 x g, 1 min), and the pellet was resuspended in 1 x phosphate-buffered saline, pH 7.4, containing protease inhibitors. The cell suspension was sonicated (5 s x 4) on ice (Sonics and Materials Inc., CT) and centrifuged (15,000 x g, 10 min, 4°C). The supernatant was utilized for biochemical assays after protein estimation (17). Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (13).

Estimation of Cellular and Mitochondrial Ca²⁺ Levels—Cells (10⁵) seeded on 22-mm coverslips were loaded with 5 μM Fura-2-acetoxyethyl ester (Fura-2AM; a cell permeable Ca²⁺-sensitive fluorescent dye; excitation = 340/380 nm, emission = 510 nm) and 0.8% pluronic acid for 45 min at 37°C in HEPES buffer (20 mM HEPES, 130 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, 1.8 mM CaCl₂, and 15 mM glucose, pH 7.4). The cells were washed with HEPES buffer and incubated in the dark for 15 min. The coverslip was mounted on a fluorescence microscope (Olympus IX 70) and ratiometric fluorescence imaging was performed (TILL Photonics, Germany). Fields containing at least 25–30 cells were selected and alternately excited at 340 and 380 nm and the emitted fluorescence was collected using a band pass filter and fluorescence images were acquired by a 12-bit Peltier-cooled CCD camera. Ratioimetric quantitation of 340/380 nm intensity analyzed using TILL Vision software is a measure of intracellular Ca²⁺ levels (18). CTX (1 or 2 μM) and antagonist (nifedipine at 10 μM) (± 2 mM EGTA) were added to the static bath during experiments. All EGTA experiments were carried out in Ca²⁺-free HEPES buffer.

The mitochondrial Ca²⁺ levels were measured as described previously (19) using Rhod-2AM, a mitochondrial Ca²⁺-sensitive probe. Cells grown on coverslips were loaded with Rhod-2AM (2 μM) for 30 min in HEPES buffer at 35°C and washed with HEPES buffer. The regions of interest were taken from the perinuclear mitochondrial rich areas exhibiting punctate loading of Rhod-2. The cells were excited at 530 nm and emission was collected through a band pass filter. The fluorescence intensity change is represented as arbitrary units. CTX was added to static bath and the fluorescence images were monitored for 30 min at room temperature. The dependence of Rhod-2 fluorescence on Ca²⁺ influx was determined by incubating the cells with CTX ± 2 mM EGTA, followed by quantitation of fluorescence in a fluorimeter (Tecan).

Determination of Membrane Damage—Cells grown in 60-mm dishes to 70% confluence were loaded with 5 μM calcine AM for 45 min at 37°C in HEPES buffer (20). The cells were washed three times with HEPES buffer and incubated with CTX (2 μM) for 0–30 min. Later, the emission spectra of the culture supernatant were recorded at different time points (0.5, 2, 5, 10, and 30 min) at 500–600 nm using an excitation maximum of 480 nm.

Study of Mitochondrial Membrane Potential (Δψm)—C2C12 cells (5 x 10⁵) in 96-well plates were treated with 20 μM 5,5′,6,6′-tetracloro-1,1′,3,3′-tetraethyl-benzamidazolo carbocyanin iodide (JC-1) (a lipophilic fluorescent dye that selectively accumulates in mitochondria and responds to changes in Δψm by forming J aggregates from monomers (21)) and incubated for 30 min at 37°C in dark. JC-1 loaded cells were washed twice with PBS (± 2 mM EGTA) and fluorescence was measured (excitation = 490 nm; emission = 535 and 590 nm, corresponding to monomer and aggregate, respectively) in a plate reader (Tecan) in the kinetic mode (21). Three readings were taken at 1-min intervals after which CTX was added and fluorescence was monitored for 30 min.

Analysis of Reactive Oxygen Species (ROS) in Cells—Total ROS in C2C12 cells was measured using Amplex® red assay kit (Invitrogen) according to the manufacturer’s instructions. Cells in 96-well dishes were loaded with Amplex red and exposed to CTX and the fluorescence corresponding to ROS was recorded in a fluorimeter (Tecan) (excitation = 540 nm; emission = 590 nm). For detection of cellular oxidants, cells loaded with dihydrothidium were exposed to CTX for 1 h, followed by detection of fluorescence in a multiwell fluorimeter (excitation = 518 nm; emission = 606 nm) (22). Quantiﬁcation of the higher oxides of nitric oxide (NO) in C2C12 cells was carried out using DAF-FM-DA as described previously (23). Cells loaded with 5 μM DAF-FM-DA were exposed to CTX followed by quantitation of fluorescence (excitation = 495 nm; emission = 515 nm).

Preparation of Whole Muscle Extracts—Total soluble extract from frozen tissue (50 mg) was prepared (13) followed by protein estimation (17).

Preparation of Mitochondria—Muscle mitochondria were prepared by the method modiﬁed from Bhattacharya et al. (24). Muscle tissue (100 mg) was minced and incubated with 10% nagarse in ionic medium (100 mM sucrose, 10 mM EDTA, 100 mM Tris-HCl, 46 mM KCl, pH 7.4) for 5 min, washed with ionic medium containing 0.5% BSA, homogenized, and centrifuged (500 x g, 10 min, 4°C). The supernatant was centrifuged (12,000 x g, 10 min, 4°C), and the partially puriﬁed mitochondrial pellet was washed twice with ionic medium containing 0.5% BSA and the final pellet was stored at −80°C in suspension medium (230 mM mannitol, 70 mM sucrose, 0.02 mM EDTA, 20 mM Tris-HCl, 5 mM K₂HPO₄, pH 7.4).

Assay of Mitochondrial Complexes I (CI), II (CII), and III (CIII)—CI activity was assayed as described (25) and rotenone-sensitive speciﬁc activity was calculated. CII and CIII were assayed using methods described previously (25).

Citrate Synthase (CS) Assay—The assay was initiated by adding 10 mM oxaloacetate (20 μl) to the reaction mixture contain-
ing 100 mM Tris-HCl, pH 8.1, 0.2 mM DTNB, 0.1% Triton X-100, 0.1 mM acetyl-CoA, and 5 μg of protein and the reaction was monitored at 412 nm (26). CS activity was expressed as nanomole of DTNB/min/mg of protein (MEC = 13.6 mm−1 cm−1).

Aconitase Assay—Aconitase was assayed as previously described (27). The assay mixture containing mitochondrial protein (40 μg), 100 mM Tris-HCl, pH 7.6, and 500 μM cis-aconitate was incubated at room temperature and the enzyme activity was monitored at 240 nm for 15 min. Enzyme activity was calculated using MEC = 3.6 mm−1 cm−1 for cis-aconitate and normalized per mg of protein.

Aspartate Aminotransferase and CK Assays—These assays were carried out using commercial kits (Grenier Bio-One GMBH). Total mitochondrial extract (300 μg) from CTX-injected muscle at d1, d3, and d7 and control were subjected to assays in triplicate, based on the manufacturer’s instructions.

Estimation of Pyruvate, Lactate, NADH, and ADP/ATP Ratio—Total pyruvate and lactate in the muscle extract was estimated by commercial kits (Grenier Bio-One GMBH) according to the manufacturer’s instructions. Muscle tissue (50 mg) from control and CTX-injected animals was extracted in PBS and 10 μl of the total extract was subjected to the assays and normalized per mg of protein. The ADP/ATP ratio in the muscle extract was determined using a commercial kit (Abcam) according to the manufacturer’s instructions.

NADH was estimated according to the method described previously (28). Briefly, NADH was extracted from the muscle homogenate by adding 0.1 N NaOH and neutralized by adding HCl/Bicine to a final concentration of 0.33 M. To 350 μl of neutralized extracts, 310 μl of reaction buffer (0.232 M Bicine, 2 mg/ml of BSA, 1.16 M ethanol, 9.67 mM EDTA, 3.87 mM phenazine ethosulfate, and 1 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added and incubated at 37 °C for 5 min. Then a cycling assay was initiated by the addition of 60 μl of 0.6 mg/ml of alcohol dehydrogenase. After 30 min, the reaction was terminated by adding 300 μl of 12% iodoacetate and absorbance was recorded at 570 nm. Total NADH was normalized to the total protein content.

SDS-PAGE and Western Blot—Total muscle extract (50 μg/lane) was resolved on 12% SDS-PAGE followed by either Coomassie staining of the gel or Western blot (12) using an antibody mixture against five specific mitochondrial proteins (Abcam) according to the manufacturer’s instructions.

Estimation of Total Glutathione (GSH + GSSG), GSH:GSSG Ratio, and Lipid Peroxidation—The total glutathione (GSH + GSSG) assay was carried out as described previously (13). Alternately, the GSH:GSSG ratio was determined by the o-phthalaldehyde method as described earlier (29). The muscle extract (10 μg) was pre-cleared with 5% sulfosalicylic acid and centrifuged (10,000 × g, 10 min at 4 °C). The supernatant was treated with o-phthalaldehyde (1 mg/ml; 10 μl) for 20 min and the fluorescence corresponding to GSH (excitation = 350 nm; emission = 420 nm) was measured and compared with standards. For measuring GSSG, the cleared supernatants were incubated with 5 μl of N-ethylmaleimide for 15 min, followed by addition of 0.15 N NaOH (140 μl). The mixture was treated with o-phthalaldehyde followed by fluorescence measurements.

Lipid peroxidation was quantitated by a thiobarbituric acid reactive substances assay as described earlier (13). Alternately, the extract (10 μl) was incubated with the fluorescent lipid peroxidation sensor 4,4-difuoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (BODIPY® 581/591 undecanoic acid; Invitrogen) and the loss of fluorescence upon interaction with peroxyl radicals was estimated (30) and normalized per mg of protein.

Determination of Protein Carboxylation (Oxyblot)—Tissue supernatant (10 μl at 4 mg/ml of protein) was derivatized with dinitrophenyl hydrazine (DNPH), spotted on a nitrocellulose membrane, and subjected to anti-DNP Western (13). Anti-DNP signal captured in a gel documentation system (Bio-Rad, model 2000) was densitometrically quantified (Quantity One software version 4.2.2, Bio-Rad).

Two-dimensional Gel Electrophoresis (PAGE) and Western Blot—Muscle tissue homogenate (10%) was prepared by a method modified from Sultana and Butterfield (31) in HEPES buffer (10 mM HEPES, pH 7.4, 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH2PO4, 0.6 mM MgSO4) containing protease inhibitors. The extract was sonicated (10 s × 3), centrifuged (10,000 × g, 10 min), and the soluble protein (300 μg) incubated in 4 volumes of 2 N HCl (±10 mM DNPH) for 20 min at room temperature. The sample was precipitated with trichloroacetic acid (final concentration = 30%) and centrifuged (10,000 × g, 5 min). The pellet was washed three times with ice-cold ethanol: ethyl acetate (1:1) and resuspended in two-dimensional lysis buffer (200 μl) (8 M urea, 2 M thiourea, 2% CHAPS, 0.4% amphotol, pH 3–10, 50 mM DTT) and incubated at room temperature for 2 h. The lysate was centrifuged (10,000 × g, 10 min) and the supernatant (180 μl) was loaded onto IPG strips (11 cm, pH 3–10) in the rehydration tray, overlaid with ~800 μl of mineral oil and allowed for overnight rehydration. The strips were subjected to isoelectrofocusing in the IEF cell (Bio-Rad) as follows: step 1, 25 V/20 min (linear ramp); step 2, 8,000 V/2.5 h (linear ramp); and step 3, 8,000 V for 25,000 V-h (rapid ramp). Strips were incubated in equilibration buffer (50 mM Tris-HCl, 6 M urea, 1% (v/v) SDS, 30% (v/v) glycerol and freshly prepared DTT (0.5%)) for 10 min and then in equilibration buffer containing iodoacetamide (4.5%) for 10 min. The equilibrated gels were washed in SDS-PAGE running buffer and electrophoresed on 10% SDS gel (second dimension). SDS gels were placed in fixative solution (50 ml) (10% (v/v) methanol and 7% (v/v) acetic acid) for 1 h at room temperature and stained with SYPRO Ruby stain (50 ml) (2 h at room temperature), destained, and the images captured in the gel documentation system (Bio-Rad) and analyzed by Quantity One software (Bio-Rad) (31). Proteins from SDS-PAGE were transferred onto a nitrocellulose membrane and subjected to anti-DNP Western blot (13).

In-gel Tryptic Digestion and Peptide Extraction—Spots on the SYPRO Ruby-stained two-dimensional gels that corresponded to the anti-DNP Western signal were manually excised and subjected to in-gel tryptic digestion (32, 33). Gel pieces were minced and washed with 500 μl of wash solution (50% acetonitrile and 40 mM ammonium bicarbonate) at room temperature repeatedly with gentle agitation (15 min/wash) until they were completely destained. The gel pieces were dehy-
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dratted in 100% acetonitrile for 5 min. The gel pieces were first rehydrated in 150 μl of reduction solution (5 mM DTT in 40 mM ammonium bicarbonate) for 45 min at 60 °C and incubated with 100 μl of alkylation solution (20 mM iodoacetamide in 40 mM ammonium bicarbonate) for 10 min in the dark at room temperature. The gel pieces were dehydrated in 100 μl of 100% acetonitrile for 5 min. Gel pieces were then incubated in 30 μl of protease digestion solution (20 μg of sequencing grade trypsin reconstituted in 2 ml of 50 mM ammonium bicarbonate) on ice for 30 min. Once the gel pieces were rehydrated with protease digestion solution, excess trypsin was removed and substituted with 40 mM ammonium bicarbonate and incubated overnight at 37 °C. It was then centrifuged (12,000 × g for 30 s) and the supernatant (containing tryptic peptides) was transferred to a sterile tube. The gel pieces were re-extracted with 25–50 μl of extraction solution (50% acetonitrile, 5% formic acid). The extracted peptides were dried in a vacuum evaporator and reconstituted in 20 μl of 0.1% trifluoroacetic acid (TFA) and subjected to tandem mass spectrometric analysis (LC-MS/MS).

**LC MS/MS and Database Analysis**—LC-MS/MS analysis of tryptic peptides was carried out on a LTQ orbitrap Velos Fourier Transform mass spectrometer. Peptides from each gel piece were resolved by reversed-phase liquid chromatography (RP-LC) interfaced with a mass spectrometer using an RP-LC system consisting of a column (75 μm × 2 cm, C18 material 5 μm, 100 Å) and an analytical column (75 μm × 10 cm, C18 material 5 μm, 100 Å) with a nanoflow solvent delivery and electrospray source fitted with an emitter tip (8 μm) and maintained at 2 kV ion spray voltage. Peptides samples (20 μl) were loaded onto the trap column in 0.1% formic acid and 5% acetonitrile. The peptides were resolved on an analytical column using a linear gradient of 7–30% acetonitrile and 0.1% formic acid over 80 min with a flow rate of 350 nl/min. The eluted peptides were subjected to MS analysis on LTQ orbitrap Velos. From each precursor scan, 10 of the most abundant ions in the scan range of m/z 350–1800 were subjected to MS/MS fragmentation (34).

The MS/MS data were searched against the NCBI Ref Seq database (release 52) using human protein database containing 30,083 protein sequences and common contaminants. The parameters selected include (i) trypsin as specific enzyme with maximum of 1 missed cleavage permitted, (ii) fixed post-translational modification was carbamidomethylation of cysteine residues, (iii) oxidation of methionine was set as variable modification, (iv) precursor ion mass range at 400–8,000 Da, and (v) precursor and fragment ions mass tolerance of 20 ppm and 0.1 Da, respectively. The peptide and protein data were extracted using high peptide confidence with target FDR threshold set to 1% at the peptide level.

**mRNA Isolation and Real-time PCR**—All solutions and buffers were prepared in RNase-free diethyl pyrocarbonate-treated water (0.01%). Muscle tissues were lysed in TRIzol reagent (1 ml), mixed with 200 μl of chloroform, and vigorously shaken (15–30 s). The lysis was centrifuged (12,000 × g, 15 min at 4 °C) and the supernatant was mixed with isopropyl alcohol (500 μl) and incubated for 10 min. The mixture was centrifuged (12,000 × g, 10 min at 4 °C) and the RNA pellet was washed with 75% ethanol, dried, and suspended in diethyl pyrocarbonate-treated water (35). cDNA synthesis was carried out using a Superscript III RT kit (Invitrogen) using specific primers (supplemental “Experimental Procedures”). The RNA and primer mixtures were denatured at 65 °C for 10 min and snap chilled. To this mixture, 4 μl of the 5x buffer, 2 μl of 0.1 mM DTT, 1 μl of 10 mM dNTP mixture, 0.5 μl of RNase inhibitor (Roche), and 0.5 μl of SSIII reverse transcriptase was added and mixed. This reaction was incubated first at room temperature (10 min) and then at 50 °C (50 min). The reaction was stopped by heat denaturation at 75 °C (15 min). Real-time PCR was carried out in the Light Cycler 480 II (Roche) (35). The quantitation of the expression of all the genes was normalized to α-actin 1.

**Mitochondrial Proteomics**—Mitochondria from muscle tissue (saline (sal); day 1 CTX-d1; day 3 CTX-d3; day 7 CTX-d7) were resuspended in deionized water, sonicated (30 s), and the protein concentration was estimated (36). Equal protein (250 μg) from each sample was reduced by Tris(2-carboxyethyl) phosphine at 60 °C for 1 h (37). The samples were then treated with the cysteine blocking reagent (methyl methane thiosulfonate) for alkylation, and incubated at room temperature for 10 min. Tryptin digestion was carried out with sequencing grade modified trypsin (Promega) at 37 °C for 16 h and the tryptic digests were evaporated to dryness. Peptides from sal, d1, d3, and d7 were labeled with iTRAQ reagents containing 114, 115, 116, and 117 reporter ions, respectively. Because our study focused on the degenerative phase, data for d1 and d3 compared with sal are presented under “Results.”

The labeled samples were pooled, reconstituted using 10 mM potassium phosphate buffer containing 30% acetonitrile, pH 2.7 (solvent A), and subjected to strong cation exchange chromatography (37). Strong cation exchange fractionation was carried out on a poly-sulfoethyl A column (Poly LC, Columbia, MD) (300 Å, 5 μm, 100 × 2.1 mm) with an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA) with a binary pump, UV detector, and a fraction collector. A linear gradient composed of 0–35% 10 mM potassium phosphate buffer containing 30% ACN, 350 mM KCl, pH 2.7 (solvent B), at a flow rate of 200 μl/min was used for peptide separation. The fractions obtained were dried, reconstituted in 0.1% TFA, and desalted using C18 stage-tips. The desalted samples were subjected to LC-MS/MS analysis.

For LC-MS/MS analysis, the iTRAQ-labeled samples were analyzed on LTQ-Orbitrap Velos mass spectrometer (Thermo Electron, Bremen, Germany) interfaced with Proxene Easy nLC system (Thermo Scientific) (37). Peptides were first loaded onto an enrichment column (2 cm × 75 μm) and further resolved on analytical columns (10 cm × 75 μm) packed in-house with Magic C18 reverse phase material (Michrom Biosciences Inc., Magic C18AQ, 5 μm, 100 Å) prior to introducing the peptides into mass spectrometer. The peptide enrichment was carried

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**TABLE 1**

Details of human samples used in the human grip strength analysis

| Sl no. | Disease | Age (years) | Samples (n) | Gender (male/female) |
|-------|---------|-------------|-------------|----------------------|
| 1     | Control | 28 ± 5      | 10          | 7/3                  |
| 2     | DMD     | 7 ± 3       | 15          | 15/0                 |
| 3     | Dysf    | 25 ± 5      | 15          | 9/6                  |
| 4     | IM      | 38 ± 10     | 15          | 5/10                 |

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A

Muscle strength (grade)

C | DMD | Dysly | IM
---|-----|-------|-----
5 | **2.9** | **2.8** | **3.2**

B

Kb Values

C d1 d2 d3 d5 d7 d11 d14 d31

Days post CTX injection

C | sal | CTX
---|-----|-----

D

P-sal sal d1 d3 d5 d7 d11 d14 d31

E

C DMD Dysly Sgpy IM

F

Centrally nucleated cells (% cellular nuclei)

C d1 d3 d5 d7 d11 d14 d31

G

sal d1 d3 d7 d11 d14 d31 IM
out at a flow rate of 3 μl/min, whereas the peptides were resolved in the analytical column at a flow rate of 350 nl/min employing a linear gradient of 7–30% acetonitrile over 80 min.

Data-dependent MS analysis was carried out by acquiring full scans in the Orbitrap mass analyzer between a mass range of 350 and 1800 at a mass resolution of 60,000 at 400 m/z. The top 20 precursor ions from each survey scan were selected for MS/MS fragmentation and the fragment ions were acquired at a mass resolution of 15,000 at 400 m/z. Fragmentation was carried out using higher-energy collision dissociation mode with normalized collision energy of 41%. Isolation width was set to 2 m/z. The ions selected for fragmentation were dynamically excluded for 30 s. The automatic gain control for full FT MS was set to 1 million ions and for FT, MS/MS was set to 5 × 10^4 ions. The lock mass option was enabled using polydimethylcyclosiloxane (m/z, 445.1200025) ion for accurate mass measurement.

MS/MS data were searched against mouse RefSeq56 data-base (containing 26,707 sequences with common contaminants) using Sequest algorithm in ProteomeDiscoverer 1.3.0.339. The search criteria included oxidation of methionine as dynamic modification and iTRAQ 4-plex modification at peptide N terminus and lysine and methyl thio modification of cysteine as static modifications. A precursor mass tolerance of 20 ppm, fragment mass tolerance of 0.1 Da, and 1 missed cleav-

FIGURE 1. Muscle weakness and degeneration in the CTX mice and human muscle pathologies. A, muscle strength in human Duchenne muscular dystrophy (DMD; n = 15), dysferlinopathy (Dysf; n = 15), and inflammatory myopathies (IM; n = 15) compared with healthy controls (C; n = 10), expressed as grade (0–5) (***, p < 0.001). B, analysis of grip strength in CTX or saline-injected (C) TA muscles of C57BL6 mice assessed from day 1 to day 31 post-injection (d1 to d31), expressed as Kg force (***, p < 0.001). C, analysis of muscle damage by confocal microscopy of Evans Blue dye fluorescence in CTX and saline-injected mouse muscle. P-sal, phase-contrast image of saline-injected muscle; sal, fluorescent images of saline. d1–d7, fluorescent images (magnification ×400).

D, H&E-stained sections of saline-injected (sal) TA muscle and CTX-injected muscle at d1 to d31 (magnification ×400). Infiltration of inflammatory cells into the interstitial spaces are indicated by a star; degenerating muscle fibers are indicated by filled circles; centrally nucleated cells indicated by an arrow. E, H&E staining of human control (C), DMD, Dysf, and IM biopsies (magnification ×400). Central nucleation (arrow), inflammation (star), and necrotic fibers (C) are indicated. F, the number of centrally nucleated cells expressed as % cells/total cells/field in control (C) and CTX-injected muscle at d1–d31. 6 random fields were assessed per time point; **, p < 0.01; ***, p < 0.001 compared with control. G, Masson trichrome staining of saline-injected (sal) and CTX-injected mice muscle (d1–d31). The staining shows the central nucleus (arrow) (magnification ×400). Sections from DMD and Dysf show fibrosis (blue staining around muscle fibers, black arrow) (magnification ×250).

FIGURE 2. Elevated apoptotic markers and inflammatory genes in the CTX mouse model. Gene expression changes (complete name of the genes and primer sequences are given in supplemental “Experimental Procedures”) in the levels of: A, apoptotic markers tnfα, fasL, and litarf; B, cytokines ccl2, ccl3, ccl6, mrc1, Il-1b, and Il-6; and C, cytokine receptors ccr1, ccr4, and ccr5 at d1–d14 in CTX-injected TA muscle of C57BL6 mice analyzed by quantitative RT-PCR. All values were expressed as fold-change over the saline-injected control, carried out in triplicate of 6 experimental sets and averaged; *, p < 0.05; **, p < 0.01; ***, p < 0.001 compared with control.
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Characterization of a Mouse Model of Muscle Degeneration—We characterized an acute transient model of muscle degeneration and compared its cardinal pathological features with human MDs and IMs. We utilized snake CTX (60 amino acids) (38), which causes cardiototoxicity when injected into the blood, but causes tissue injury when administered into the muscle. A single injection of 300 μl of CTX (10 μM) was administered into TA muscle (9) followed by behavioral, histopathological, and inflammatory analyses at 1, 3, 5, 7, 11, 14, and 31 days (d1 to d31) after injection. Although CTX caused immediate, but momentary paralysis in the injected limb, the toxin did not cause any mortality. CTX exposure did not alter the body weight, food and water intake, and other behavioral parameters compared with controls (data not shown).

We investigated whether CTX-induced muscle weakness is as seen in human DMD and IMs (Table 1; Fig. 1A). CTX caused complete loss of grip strength of the injected limb from d1 to d11. The muscle strength was restored by ~40% at d14 and ~90% at d31 indicating recovery of muscle function (Fig. 1B). The reduced muscle strength was associated with muscle damage at d1 as indicated by Evans Blue dye fluorescence, which persisted at d3, but decreased significantly by d7, indicating transient muscle damage (Fig. 1C). Histological analysis (H&E staining) showed widespread damage of the muscle fibers at d1, which persisted until d11 (Fig. 1D). Muscle degeneration represented by myofiber fragmentation and shrinkage of the muscle cells was relatively higher at d3, consistent with previous data (39). However, the muscle structure was significantly recovered at d14 (13), and was comparable with the control at d31. These histopathological features were consistent with human DMD, Dysfy, Sgpy, and IM (Fig. 1E). Masson trichrome staining of the sections indicated that muscle degeneration was not accompanied by apparent fibrosis, possibly because colla-
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FIGURE 4. **Ultrastructural changes in the CTX model and human pathologies.** A, EM analysis of saline (sal)-injected muscle shows LS of sarcolemma (×8,000) (i), T-system (×3,000) (ii), cytoskeleton (×30,000) (iii), and TS of cytoskeleton (×68,000) (iv). The sarcolemmal outer and inner layers are depicted by block arrows (i). Triads of the T-system are indicated by block arrows (ii). The isotropic band (i), anisotropic band (A), Z-line (Z), and M-line (M) of the cytoskeletal sarcomeres are depicted (iii). In the TS of cytoskeleton (iv), the myosin bands are seen as dark dots surrounded by light actin bands. B, EM images from d1 shows (i) inflammatory cells (IC) seen attacking the muscle cell (×2,000), (ii) sarcolemmal breakage (arrow, ×5,000), (iii) disrupted sarcolemma (arrows), homogenized cytoskeleton (star) and IC (×10,000), and (iv) homogenized cytoskeleton (star) (×10,000). C, EM images from d3 show (i) IC outside the degenerating muscle cells with sarcolemmal loss (arrow) (×2,300), (ii) sarcolemmal breach (arrows) and intracellular IC in the muscle cell (×2,700), and (iii) extensive cytoskeletal homogenization (star) and tiny vesicles (filled circle) indicating the degenerating T-system (×30,000). D, EM images from DMD muscle shows homogenized cytoskeleton (arrow) (×7,200), whereas Dysf muscle shows (i) disrupted sarcolemma (arrow) (×30,000) and (ii) vesicles indicating degenerating T-system (×30,000). E, ultrastructural analysis from d7 show (i) variation in fiber size with central nucleus, satellite cells (S), and intact membrane (arrow) (×1,400), (ii) few neutrophils (N) and macrophages (M), fibroblasts (F) with prominent RER, a nerve twig (NT) and blood vessel (BV) (×2,900). F, images from d11 show (i) perinuclear glycogen granules (star) (×6,800), (ii) subperipherally placed nuclei and normal sarcolemma (arrow) (×3,900). G, EM analysis at d14 show (i) centrally nucleated cells (×2,900), (ii) satellite cells (S) and normal sarcolemma (arrow) (×9,300). H, EM analysis at d31 indicating centrally nucleated cells (×6,800) with perinuclear aggregation of mitochondria (arrow) with normal and well developed cristae (×6,800).

gen deposition entails chronic degeneration as seen in human MDs (Fig. 1G). The recovery of muscle architecture was concomitant with the appearance of centrally nucleated regenerating cells (as seen in human DMD and Sgpy; Fig. 1E), which was negligible at d1 and d3, but predominant at d5 to d14 (Figs. 1, E and F). At d7 and beyond, repair and regeneration were evidenced by (i) centrally nucleated cells with perinuclear glycogen granules and (ii) fibroblasts with prominent rough endoplasmic reticulum, engaged in active reconstruction of the extracellular matrix. The number of fibroblasts were decreased by d11 and was not conspicuous at d14 and d31 (data not shown). Activated satellite cells were...
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A

B

C

D

E

F

G

H

I

J

Before CTX

After CTX

Undifferentiated cells

Differentiated cells

Undifferentiated cells

Differentiated cells

Undifferentiated cells

Differentiated cells

Undifferentiated cells

Differentiated cells

Undifferentiated cells

Differentiated cells
prominent at d7, reduced at d11 and d14, and inconspicuous by d31 (Figs. 4, E–H, and 6F).

Muscle degeneration in human MDs is associated with apoptotic (40) and inflammatory mechanisms (41, 42). CTX-injected muscle revealed infiltration of neutrophils, macrophages, and lymphocytes as seen in human DMD and myopathies (Fig. 1, D and E). Inflammatory cells infiltrated the extracellular spaces and phagocytosed the muscle cells at d1–d3 (Fig. 4, B and C), but their number decreased at d7 and was further reduced at subsequent time points (data not shown). The degenerative changes at d1–d14 was associated with elevated expression of apoptotic markers (tnf-α, fasL, and litaF) and inflammatory genes including cytokines (chemokines (ccl2, ccl3, and ccl6) and interleukins (il-6 and il-1β)), mrc1, and cytokine receptors (ccr1, ccr4, and ccr5) (Fig. 2). The apoptotic markers were elevated from d1, with the highest level at d3 followed by reduction to the control level by d14 (Fig. 2A). All cytokines showed increased expression between d1 and d7, which decreased to the control level at d14 (Fig. 2B). The cytokine receptors showed a general trend with elevated expression from d1, peaking at d3 and subsequent reduction at d7, ultimately decreasing to the control level at d14 (Fig. 2C). However, the increase in the relative expression varied among the different genes (43). Up-regulation of the apoptotic and inflammatory genes tested in the CTX model was previously reported in human dystrophies (supplemental Table S1). Based on the behavioral, histopathological, and quantitative RT-PCR data, it could be surmised that CTX-dependent myotoxicity elicited all the cardinal features observed in human pathologies and showed acute transient degeneration at d3.

CTX-injected Muscle Displays Sarcolemmal Disruption and Ultrastructural Perturbations—Muscle tissue from MD patients display loss of sarcolemmal integrity (44), altered cytoskeletal structure, and ultrastructural perturbations (45). The sarcolemmal integrity in the CTX model was ascertained by the status of the sarcolemmal complexes viz., dystrophin, dystrophin-associated, and dysferlin (13). Loss of immunoreactivity of dystrophin, sarcoglycan (dystrophin-associated protein), and dysferlin (dysferlin complex) at d1–d7 indicated sarcolemmal perturbation (Fig. 3A) as observed in human DMD, Sgpy, and Dysfy (Fig. 3B). The restoration of immunoreactivity was visible at d7 and conspicuously re-established at d14–d31.

We investigated whether the transient loss of immunoreactivity of the membrane proteins in the CTX model was due to membrane disruption (supplemental Fig. S2 and Fig. 4). CTX-injected muscle displayed sarcolemmal breach (“leakiness”) at d1 and d3 (Fig. 4, B and C), which was restored at d7 (Fig. 4E). CTX-mediated sarcolemmal disruption also affected the T-system (sarcolemmal invaginations that are continuous with the sarcoplasmic reticulum). CTX caused formation of tiny vesicles, predominantly at d3 (Fig. 4C), probably due to the pinching-off of the damaged sarcolemma, thus destabilizing the T-system. CTX disrupted the cytoskeletal architecture at d1 and d3, as indicated by complete homogenization of the myosin-actin filaments (Fig. 4, B and C). Ultrastructural alterations in the CTX model were similar to features observed in the muscle biopsies of human MDs (Fig. 4D).

Altered Calcium Dynamics following CTX Administration in Vivo and in Vitro—CTX-mediated sarcolemmal disruption consistent with human pathologies could alter the intracellular Ca2+ dynamics. CTX-injected muscles stained positively for alizarin red at d1 and d3 indicating intracellular accumulation of Ca2+, as seen in human DMD (Fig. 5A) (40). We investigated the Ca2+-dependent dye Fura-2AM fluorescence to assess the time-dependent myotoxicity with LD50 of 2 and 1 μM at 40 h in undifferentiated and differentiated cells, respectively (Fig. 5, B and C) (13). CTX treatment of C2C12 cells loaded with the Ca2+-sensitive dye Fura-2AM significantly increased the fluorescence (340/380 nm ratio) within 60 s, indicating elevated intracellular Ca2+ (Fig. 5, D and E). Although this effect was abolished by EGTA treatment, subsequent exposure to Ca2+ (20 μM) increased the fluorescence ratio thus confirming CTX-dependent Ca2+ influx (Fig. 5, F and G). This also suggested that elevated intracellular Ca2+ was probably due to the influx from the extracellular milieu rather than the intracellular stores. Pre-treatment of C2C12 cells with nifidepine, a blocker of L-type voltage-gated Ca2+ channel (most common Ca2+ channel in skeletal muscle) did not prevent CTX-mediated Ca2+ influx suggesting that the immediate Ca2+ rise was voltage-gated Ca2+ channel-independent and is probably due to sarcolemmal disruption to a certain extent (Fig. 5H) along with other unknown mechanisms. When cells loaded with calcine AM were exposed to CTX, the fluorescence signal was detectable in the extracellular medium (Fig. 5I) after 30 min, confirming the loss of membrane integrity. CTX treatment also enhanced Rhod-2 (mitochondrial Ca2+-specific dye) fluorescence indicating Ca2+ influx into the mitochondria (Fig. 5I). These data suggest that CTX causes significant intracellular Ca2+ influx, which subsequently accumulates in the mitochondria.

Evidences for Mitochondrial Dysfunction in the CTX Models and Human Tissues—The dystrophic muscle in human MDs display mitochondrial dysfunction (46). In C2C12 cells, CTX...
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A

Undifferentiated cells

Differentiated cells

B

sal
d1
d3
d7
d11

SDH
d14
d31
DMD
Dysf
IM

C

SDH
-COX

d14
d31
DMD
MD
IM

D

sal
d1
d3
d7
d11

NADH
-TR

d14
d31
DMD
Dysf
IM

E

sal
d1
d3

MGT
MM
Dysf
IM

F

sal
d1
d3
d7

d14
DMD
Dysf

G

H

I

J

ADP/ATP ratio

NAADP

Purinergic

Lactate

mMg protein

mMg protein

mMg protein

mMg protein

mMg protein
treatment decreased the fluorescence signal of JC-1, indicating mitochondrial depolarization. This was abrogated by EGTA, indicating that Ca\(^{2+}\) influx precedes mitochondrial damage (Fig. 6A). Mitochondrial dysfunction was evident in the CTX-injected muscle, as indicated by loss of EHC signal of the mitochondrial activities including SDH, SDH-COX, and NADH-TR activities at d1, and more drastically at d3 (Fig. 6, B–D). At d7, the EHC signal recovered significantly indicating the restoration of mitochondrial function. These changes were comparable with the mitochondrial damage evident in the human dystrophic pathologies (Fig. 6, B–D).

Mitochondrial damage during muscle degeneration could involve either mitochondrial aggregation or altered morphology. Mitochondrial aggregation observed in human myopathies was not evident in the CTX model (Fig. 6E). However, muscle mitochondria showed altered cristae at d3, which was restored at d7 (Fig. 6F). This is consistent with the EM images of muscle biopsies from human DMD and Dysfy (Fig. 6F). Mitochondrial dysfunction was also indicated by the significantly elevated cellular ADP/ATP ratio at d1–d3, which recovered at d7 (Fig. 6G). The mitochondria also displayed lowered NADH content at d1–d7 (Fig. 6H). The overall metabolic activity of the muscle was significantly lowered, as indicated by decreased pyruvate at d1–d7 (Fig. 6I) and decreased lactate content at d1–d3, which recovered at d7 (Fig. 6J).

**Analysis of the Mitochondrial Proteome in the CTX-injected Muscle**—Although dystrophic pathology in the CTX model at d1 and d3 was associated with mitochondrial damage, at d7 and beyond, regenerative processes corroborated with the restored mitochondrial function. To further characterize the global mitochondrial changes associated with CTX-mediated muscle degeneration, we compared mitochondrial proteome changes at d1 (initial degenerative phase) and d3 (peak degenerative phase) compared with saline control (Fig. 7A) by LC MS/MS (supplemental Fig. S1). Data analysis revealed differential expression of 226 mitochondrial proteins at d1 (Fig. 7B and supplemental Table S2), of which, 8 were up-regulated (≥1.5-fold) and 80 proteins were down-regulated (≥0.5-fold), compared with the control (Table 2 and Fig. 7, C–H). There were 7 underexpressed proteins having potential structural/functional interactions with mitochondria, but their mitochondrial localization was not confirmed (Table 3).

Most of the down-regulated proteins at d1 were part of the mitochondrial energy metabolism (Fig. 7B). Among these, the most striking was the down-regulation of 20 subunits of mitochondrial complex I (CI) (Fig. 7D). Interestingly, 17 of these 20 subunits were further down-regulated at d3 (Fig. 7D and Table 2). Apart from this, 29 proteins of other complexes of the electron transport chain and ATP synthesizing machinery (SDH, COX, cytochrome b-cyt, and ATP synthase) were also down-regulated at d1 (Table 2) and among these, 22 proteins were further down-regulated at d3 (Fig. 7E). Five proteins involved in fatty acid oxidation, 4 proteins related to the Krebs cycle, and 22 other mitochondrial proteins were found to be down-regulated at d1 (Fig. 7, F–H, and Table 2). Most of these proteins (3 of 5 fatty acid oxidation proteins, 2 of 4 Krebs cycle proteins, and 14 of 22 other mitochondrial proteins) were relatively up-regulated at d3. Taken together, the lowered expression of the respiratory complexes might predominantly contribute to mitochondrial dysfunction in the CTX model and this effect could be reinforced by other down-regulated proteins.

The proteomics data were corroborated by biochemical and histochromic analysis of the respiratory complexes and other mitochondrial proteins. The enzyme assay of CI in the CTX model confirmed 55% activity at d1, 48% at d3, and ~55% at d7 compared with control (Fig. 8A). Loss of CI activity was also evident in the muscle tissues from Dysfy patients (n = 10) compared with controls (n = 6) (Fig. 8B). The CTX model also displayed loss of CI and CII activities especially at d1–d3, consistent with the poorer activity of these complexes in the muscle tissues from Dysfy patients (n = 10) versus controls (n = 6) (Fig. 8, C–F).

Western blot experiments confirmed the down-regulation of specific subunits of different respiratory complexes at d1–d3 compared with control (Fig. 8, G and H). The down-regulation of other mitochondrial proteins including aspartate aminotransferase (or glutamic oxaloacetic transaminase), mitochondrial CK, and CS was validated by lowered enzyme activity in the CTX model (Fig. 8, I–K). Lowered expression of respiratory complexes in the CTX model was also corroborated by the loss of EHC signal for NADH-TR, SDH, and SDH-COX (Fig. 6, B–D).

**Role of Oxidative Stress in the CTX Model and Human Muscle Diseases**—In accordance with muscle degeneration-associated oxidative stress in human MDs (13), the dystrophic muscle in the CTX model displayed ~2-fold lower glutathione reduced/oxidized ratio (GSH/GSSG) at d1 and d3, which was increased at d7.

**FIGURE 6. Mitochondrial dysfunction in the CTX model and human pathologies.** A, CTX treatment induced mitochondrial dysfunction in C2C12 cells. Cells (undifferentiated and differentiated) loaded with the mitochondria-specific fluorescent dye JC-1 were treated with or without CTX followed by detection of fluorescence at 590 nm. CTX caused elevation in ΔJC-1 fluorescence at 590 nm, which was abrogated by pretreatment with EGTA. **B, p < 0.001** compared with control (C). Assessment of mitochondrial function in the CTX mouse model by EHC staining for SDH (B), SDH-COX (C), and NADH-TR (D) in the cryosections of saline control (sal), CTX-injected muscle at d1–d31, and in human biopsies of DMD, Dysfy, or MD and IM. Loss of EHC signal is indicated by the star in the images from the CTX model and by arrows in human myopathies (magnification ×250). E, modified Gomorri trichrome staining in saline (sal)-injected and CTX-injected (d1 and d3) muscle and in muscle from human pathologies for MM, Dysfy, and IM to analyze mitochondrial aggregation. The characteristic subcellular aggregation (ragged red fibers) usually not observed in MM (arrows) was not observed in the CTX model (magnification ×400) or in Dysfy and IM. F, EM images of mitochondrial alterations in saline-injected (sal) (magnification ×18500) and CTX-injected muscle. Mitochondria with altered cristae and vacuolation were observed at d1 (magnification ×6700) and d3 (×18500) (arrows), whereas images from d7 (×6800) showed fewer abnormalities with normal cristae (arrow) and perinuclear glycogen granules (star). Mitochondrial structure was normal in d14 (×30000) (yellow arrow) and normal sarcocerm (black arrow). Human DMD sample shows muscle degeneration, sarcocermal damage (black arrows), and abnormal mitochondria (yellow arrow) (×7200). The Dysfy sample shows sarcocermal damage (white arrow), normal (black arrow), and abnormal mitochondria (yellow arrow) (×30000). Mitochondrial dysfunction at d1–d7 was also evident from the increased ADP/ATP ratio at d1 and d3 (G) and lowered NADH content at d1–d7 (H); **p < 0.001; ***p < 0.01; and *, p < 0.05 compared with saline (sal) control. The cellular metabolic activity was also significantly affected, as indicated by decreased pyruvate (d1–d7 (l) and decreased lactate content (d1–d3) (J), ***, p < 0.001 and *, p < 0.05 compared with saline (sal) control.
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226 differentially expressed mitochondrial proteins at d1 & d3
- 39 up-regulated: 8 proteins ±1.5 fold
- 187 down-regulated: 80 proteins ±0.5 fold

- 20 subunits of mitochondrial complex I
- 29 proteins of ETC and ATP synthesis
- 6 proteins of β-oxidation of fatty acids
- 4 proteins of Krebs cycle
- 22 other mitochondrial proteins

Down-regulated proteins

Complex I subunits

Electron transport chain and ATP synthesis

Fatty acid oxidation

Krebs cycle related

Other mitochondrial proteins

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TABLE 2—continued

| No. | Ref Seq Acc. | Gene Symbol | Description | Fold change over control | d1/Sal | d3/Sal |
|-----|--------------|-------------|-------------|--------------------------|--------|--------|
| 1   | NP_444349.1  | Ech1        | enoyl-CoA hydratase | 0.49 | 0.70 |
| 2   | NP_492091.1  | Haba        | tri-functional enzyme subunit alpha | 0.41 | 0.27 |
| 3   | NP_014072.2  | Acdh        | long-chain specific acyl-CoA dehydrogenase | 0.35 | 0.49 |
| 4   | NP_663533.1  | Hshb        | tri-functional enzyme subunit beta | 0.33 | 0.37 |
| 5   | NP_024353.2  | Ndufabl     | acyl carrier protein | 0.14 | 0.12 |

(iv) Krebs cycle related

| No. | Ref Seq Acc. | Gene Symbol | Description | Fold change over control | d1/Sal | d3/Sal |
|-----|--------------|-------------|-------------|--------------------------|--------|--------|
| 1   | NP_023836.1  | Pdhb1       | PDH E1 component subunit alpha, somatic form | 0.48 | 0.83 |
| 2   | NP_008720.1  | Cs          | citrate synthase | 0.47 | 0.42 |
| 3   | NP_061289.1  | Mps1        | mitochondrial pyruvate carrier 1 | 0.44 | 0.45 |
| 4   | NP_038849.1  | Idh3a       | isocitrate dehydrogenase [NAD] subunit alpha | 0.30 | 0.30 |

(v) Other mitochondrial proteins

| No. | Ref Seq Acc. | Gene Symbol | Description | Fold change over control | d1/Sal | d3/Sal |
|-----|--------------|-------------|-------------|--------------------------|--------|--------|
| 1   | NP_075612.1  | Chdh3       | coiled-coil domain-containing protein 9OB, mitochondrial isoform 2 | 0.49 | 0.31 |
| 2   | NP_091800.1  | Mrps19      | 39S ribosomal protein L39, mitochondrial protein | 0.46 | 0.41 |
| 3   | NP_766024.1  | Slc25a12    | calcium-binding mitochondrial carrier protein Arai2 isoform 2 | 0.43 | 0.29 |
| 4   | NP_01156390.1| Cckd9b      | coiled-coil domain-containing protein, mitochondrial isoform 2 | 0.43 | 0.48 |
| 5   | NP_00171043.1| Slc25a13    | calcium-binding mitochondrial carrier protein Arai2 isoform 2 | 0.43 | 0.32 |
| 6   | NP_023459.2  | Pgam5       | serine/threonine-protein phosphatase PGAM5, mitochondrial isoform 2 | 0.42 | 0.45 |
| 7   | NP_038927.2  | Timm10      | mitochondrial import inner membrane translocase subunit Tim10 | 0.39 | 0.41 |
| 8   | NP_109642.1  | Lactb       | serine-beta-lactamase-like protein LACTB, mitochondrial precursor | 0.37 | 0.46 |
| 9   | NP_082126.1  | Ociad2      | OCIA domain-containing protein 2 | 0.37 | 0.53 |
| 10  | NP_004429.2  | Nfil1       | NFU1 iron-sulfur cluster scaffold homolog, mitochondrial isoform 2 precursor | 0.34 | 0.44 |
| 11  | NP_013957.1  | Endog       | endonuclease G, mitochondrial precursor | 0.29 | 0.24 |
| 12  | NP_062726.3  | Htra2       | serine protease HTRA2, mitochondrial | 0.25 | 0.41 |
| 13  | NP_00107896.2| Cisd3       | CDGSH iron-sulfur domain-containing protein 3, mitochondrial precursor | 0.25 | 0.20 |
| 14  | NP_002857.1  | Php6        | prohibitin | 0.24 | 0.25 |
| 15  | NP_079668.1  | Synq2p      | synaptotagin-2-binding protein | 0.23 | 0.27 |
| 16  | NP_00123932.1| Slc25a19    | mitochondrial thiamine pyrophosphate carrier isoform 4 | 0.22 | 0.23 |
| 17  | NP_940807.1  | Ckmt2       | creatine kinase M-type, mitochondrial precursor | 0.22 | 0.16 |
| 18  | NP_034455.1  | Got2        | aspartate aminotransferase, mitochondrial | 0.21 | 0.26 |
| 19  | NP_570962.2  | Rmt4p1      | reticulon-4-interacting protein 1, mitochondrial precursor | 0.20 | 0.27 |
| 20  | NP_035824.1  | Vdac1       | voltage-dependent anion-selective channel protein 1 | 0.11 | 0.10 |
| 21  | NP_035825.1  | Vdac2       | voltage-dependent anion-selective channel protein 2 | 0.07 | 0.12 |
| 22  | NP_035992.2  | C1qgap1     | complement component 1 Q subcomponent-binding protein | 0.02 | 0.03 |

FIGURE 7. Proteomic analysis of CTX-injected muscle mitochondria. A, mitochondrial isolation of CTX-injected muscle samples for proteomic analysis. Mitochondria from control (sal) and CTX-injected muscle samples (d1, d3, and d7) were isolated and the purity assessed by electron microscopy (magnification ×23,000). B, schematic diagram depicting the number of mitochondrial proteins displaying altered expression in d1 and d3, compared with saline control. C, relative expression of overexpressed mitochondrial proteins at d1 and d3 compared with control. The description of the abbreviated protein names are provided in Table 2. Relative quantitation of underexpressed mitochondrial proteins is shown in D–H. Relative expression of mitochondrial complex I subunits (D), electron transport and ATP synthesis (E), fatty acid oxidation (F), Krebs cycle related proteins (G), and other underexpressed mitochondrial proteins (H) at d1 and d3 compared with control is shown. The descriptions for the abbreviated protein names are provided in Table 2.
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TABLE 3
Proteins underexpressed in the CTX model that have structural/functional interactions with the mitochondrion (sal: saline control)
Details include ref seq accession, gene symbol, description, and fold-change (d1/sal = 115/114; d3/sal = 116/114).

| No. | Ref Seq accession no. | Gene symbol | Description | Fold change over control |
|-----|-----------------------|-------------|-------------|-------------------------|
| 1   | NP_076186.1           | Rdh14       | Retinol dehydrogenase 14 | 0.85 / 0.66 |
| 2   | NP_849534.2           | Atad3a      | ATPase family AAA domain-containing protein 3 | 0.85 / 0.74 |
| 3   | NP_080841.1           | Apool       | Apolipoprotein O-like precursor | 0.84 / 0.60 |
| 4   | NP_001013031.2        | Dhrs7c      | Dehydrogenase/reductase SDR family member 7C precursor | 0.37 / 0.37 |
| 5   | NP_001165583.1        | Dhrs7b      | Dehydrogenase/reductase SDR family member 7B isomerase 1 | 0.28 / 0.35 |
| 6   | NP_780421.2           | Tmem65      | Transmembrane protein 65 | 0.25 / 0.25 |
| 7   | NP_075720.1           | Stom2l      | Stomatin-like protein 2 | 0.76 / 0.92 |

significantly improved at d7 (Fig. 9A). This is consistent with the lowered GSH/GSSG ratio in muscle tissues from Dysf patients (n = 6) (versus controls (n = 6)) (Fig. 9B). Lowered GSH/GSSG in the CTX model was probably not due to decreased synthesis because expression of the γ-glutamyl cysteine ligase (gcl-c) gene, the rate-limiting enzyme of GSH synthesis was unaltered from d1 to d14 (Fig. 9C). Lowered reduced/oxidized status in the CTX model was associated with elevated lipid peroxidation (by 1.2–2-fold at d1–d7) (Fig. 9D), consistent with elevated lipid peroxidation in the muscle tissues from DMD patients (n = 6) (versus controls (n = 6)) (Fig. 9E) (13). C2C12 cells incubated with CTX displayed elevated cellular oxidants within 30–60 min of toxin exposure. Pretreatment with EGTA decreased the CTX-dependent oxidants, suggesting that oxidative stress is dependent to a limited extent on Ca2+ dynamics (Fig. 9, F–I). CTX treatment also elevated DAF-FM fluorescence (indicator of higher oxides of NO) in C2C12 cells, which was slightly decreased by EGTA treatment (Fig. 9, J–K).

These data indicate a direct correlation between muscle pathology and oxidative stress as previously reported in human MDs (13). We assessed whether the reduced/oxidized markers are altered in non-dystrophic muscle disorders including distal myopathies, mitochondrial myopathies (MMs), IMs, and SMA (Table 4). Except IMs, other pathologies displayed elevated lipid peroxidation (Fig. 9L) and protein oxidation (Fig. 9, N and O). Although GSH depletion was evident in MMs, IMs, and SMA (1 and 2), it was unchanged in distal myopathies and SMA3 (Fig. 9M). Hence, there is credible correlation among muscle diseases, oxidative damage, and GSH depletion. However, the intracellular targets of oxidative damage in human muscle pathology are not completely delineated.

Oxidative damage of proteins, which alters their structure-function relationship and contributes to aging and disease (47) could target specific proteins. Among the pathologies from the current and previous study (13), protein oxidation was strikingly higher in DMD. We set out to identify carbonylated proteins associated with the dystrophic pathology in the total muscle extracts of DMD (n = 6) and control (n = 6) by two-dimensional oxyblot followed by MS analysis. Comparison of two-dimensional blots between DMD and control revealed 23 distinct protein spots among which, spots 17–23 were specific only to DMD, whereas the others were common between DMD and control (Fig. 10A). MS analysis of the spots 17–23 identified 6 potentially oxidized proteins (Fig. 10B and Table 4), two of

which (acotonin hydratase (spot number 17) and dienoyl-CoA isomerase (spot numbers 22 and 23)) were mitochondrial, indicating that oxidative stress in DMD can specifically damage mitochondrial proteins thus impinging on its function. Interestingly, these two proteins were down-regulated in the CTX model at d1 and d3 (supplemental Table S2). Validation of proteomic data by enzyme assay confirmed (i) loss of aconitase activity in DMD muscle (Fig. 10C) and (ii) ROS-dependent inactivation of the aconitase enzyme activity (Fig. 10D).

DISCUSSION

The limited therapeutic option for muscle pathologies has necessitated the investigation of the underlying mechanisms employing various experimental models (8, 48). However, a single model that meticulously mimics all the features of MD and IMs is non-existent (2). Although transgenic models involving higher mammals have breeding and ethical issues (49), transgenic mice are limited by stringent maintenance, restricted ability to recapitulate the human pathology, and species-related variations. For example, the mdx mouse model of DMD displays moderate pathology limited to early stages, predominantly in the diaphragm muscle (8). This is in contrast to the fast progressive pathogenesis in different skeletal muscles, worsening symptoms, and reduced lifespan in DMD patients. Variants of mdx (45) and other MD models have not provided optimal results (7, 46). Furthermore, the MD models should, in addition to genetic mutations, display muscle weakness, persistent inflammation, fibrosis, necrosis and/or apoptosis, and impaired regeneration. This is pertinent to sporadic diseases like IMs, which require non-genetic acute models of muscle inflammation and degeneration. The CTX model described in this study reflects the acute dystrophic pathology and muscle weakness, with downstream events such as sarcocellular disruption, intense inflammation, and necrotic/apoptotic cell death at d1 and d3, as seen in MDs and IMs (Figs. 1 and 11B). The myofiber death is segmental, involving homogenization of cytoskeleton, organelle damage, breakdown of the T-system, and vesicle formation as seen in MDs (Fig. 4). Although CTXs have been studied in cell lines to understand signal transduction, toxin internalization, and Ca2+ dynamics (50–52), direct and comprehensive comparison to human muscle pathologies have not been explored, making it the novel feature of the current study (Fig. 11C).

Regenerative processes in the muscle tissue (53) are inadequate in MD (54) and IM (55). Hence, models that display regenerative processes are important in MD therapeutics.
Because most genetic MD models do not display active regeneration (48), researchers have utilized alternate models including the CTX model to study muscle regeneration (56). The CTX model displayed a regenerative phase that commenced at d7, was fully active from d11 to d14, and nearly complete by d31 (Figs. 4 and 11A).

Muscle degeneration in different pathologies (57, 58) could be both apoptotic and necrotic (59). The CTX model displayed increased expression of apoptotic mediators fasl and tnf-α during the degenerative phase, comparable with the mdx model and human MDs and myopathies (Fig. 2 and supplemental Table S1) (60–62). The inflammatory pathways that contribute...
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Elevated mitochondrial Ca\(^{2+}\) alters mitochondrial homeostasis and turns the ETC leaky, causing increased ROS, mitochondrial depolarization, and ATP depletion, leading to apoptosis (10, 74, 75). Elevated cytosolic Ca\(^{2+}\), abnormal activity of the mitochondrial permeability transition pore, and necrosis is observed in δ-sarcoglycan-deficient hamster cardiomyocytes (76). Analysis of Facio scapulo humeral dystrophy revealed decreased cytochrome c oxidase activity and reduced ATP synthesis (46) and altered mitochondrial permeability transition pore and apoptosis (77). The CTX cell model displayed mitochondrial depolarization and concomitant production of ROS (Figs. 6A and 9, F–I). The mitochondria showed altered cristae (Fig. 6F), loss of NADH-TR, SDH, and SDH-COX activities (Fig. 6, B–D), and lowered activities of respiratory complexes CI, CII, and CIII (Fig. 8, A, C, and E) predominantly at d1 and similar to Dysf (Fig. 8, B, D, and F) and other MDs (6).

Mitochondrial dysfunction in MD and other pathologies could be associated with altered expression of mitochondrial proteins. Comparative proteomics of the mdx TA muscle showed significantly altered mitochondrial proteins in the aged mice (78). Interestingly, muscle from DMD and aged animals share common gene signatures that coordinate mitochondrial metabolism (79). Analysis of the mitochondrial proteome from CTX-injected muscle at d1 and d3 showed down-regulation of mitochondrial proteins involved in energy metabolism and included proteins of the respiratory complexes, ATP synthesis, fatty acid metabolism, and Krebs cycle (Table 2). The subunits of CI formed a major class of the significantly down-regulated proteins thus explaining the drastic reduction in CI specific activity at d1, d3, and d7 (Fig. 8, A and B). Down-regulation of the CI subunits was also confirmed by Western blot (Fig. 8, G and H) and lowered NADH-TR EHC at d1 and d3 (Fig. 6B). Underexpression of different subunits of succinic dehydrogenase and cytochrome oxidase was also supported by lowered CI and CII activities (Fig. 8) and SDH and SDH-COX EHC staining at d1–d3 (Fig. 6).

Intracellular Ca\(^{2+}\) dysregulation and mitochondrial damage might contribute to oxidative stress during MD (5, 80). Altered reduced/oxidized markers has been reported in MD patients and animal models (7, 8, 13, 81). ROS produced in mdx mice and Col6a1\(^+/−\) mice might contribute to muscle pathology (80). Although the role of oxidative damage in MD pathogenesis

**TABLE 4** Details of human samples used in the biochemical analyses of myopathies

| Sl. no. | Disease             | Age (years) | Samples (n) | Gender (male/female) |
|--------|---------------------|-------------|-------------|----------------------|
| 1      | Control             | 28 ± 5      | 10          | 7/3                  |
| 2      | DM\(^{a}\)          | 33 ± 12     | 15          | 13/2                 |
| 3      | MM                  | 29 ± 8      | 13          | 7/6                  |
| 4      | IM                  | 36 ± 10     | 15          | 4/11                 |
| 5      | SMA                 | 11 ± 6      | 15          | 8/7                  |

\(^{a}\) DM, distal myopathy; MM, mitochondrial myopathy; IM, inflammatory myopathy.

FIGURE 9. Oxidative stress associated with CTX-induced muscle pathology and human myopathies. A, determination of the reduced/oxidized status by the GSH/GSSG ratio at d1–d7 following CTX administration compared with saline control (sal); **, \(p < 0.01\); ***, \(p < 0.001\) compared with control. B, lowered GSH/GSSG in human Dysf muscle (n = 6) compared with controls (n = 6); ***, \(p < 0.001\) compared with control (C). C, relative mRNA expression of γ-glutamyl cysteine ligase-catalytic subunit (gcl-c) (primer sequences are given in supplemental “Results”) in the CTX-injected muscle at d1–d14, analyzed by quantitative RT-PCR. The values are expressed as fold-change over the saline-injected control, carried out in triplicate of 6 experimental sets and averaged; n, not statistically significant compared with control. D, elevated lipid peroxidation indicated by the relative fluorescence (AU, arbitrary units) of the peroxidation sensor BODIPY C11 in the CTX-injected muscle at d1–d7 compared with saline control (sal); ***, \(p < 0.001\) compared with control (sal). E, elevated lipid peroxidation (using BODIPY C11 fluorescence) in human DMD muscle compared with control; ***, \(p < 0.001\) compared with control. F and G, changes in total intracellular ROS corresponding to amplex red fluorescence in C2C12 cells (undifferentiated and differentiated) following CTX exposure (±EGTA); **, \(p < 0.05\); ***, \(p < 0.001\) compared with control, H and J, changes in total intracellular oxidants corresponding to dihydroethidium (DHE) fluorescence in C2C12 cells (undifferentiated and differentiated) following CTX exposure (±EGTA); ***, \(p < 0.001\) compared with control. K and L, changes in total intracellular ROS corresponding to amplex red fluorescence in C2C12 cells (undifferentiated and differentiated) following CTX exposure (±EGTA); ***, \(p < 0.001\) compared with control. L–Q, quantitative analysis of redox markers including MDA (L), total GSH (M), and protein oxidation (N and O) in patient muscle biopsies of distal myopathies (DM, n = 15), mitochondrial myopathies (MM, n = 15), IM (n = 15), and spinal muscular atrophies (SMA-1, -2, and -3, n = 15) compared with control (n = 10); **, \(p < 0.05\); ***, \(p < 0.001\); ***, \(p < 0.001\) compared with healthy controls. N, specificity and concentration dependence of the oxyblot method of estimating protein carbonyls by dot blot (carried out in mouse muscle extract, Lane 1, total muscle extract + DNPH; lane 2, extract without DNPH; lane 3, sample without extract + DNPH; lane 4, muscle extract (5 μl) + DNPH; lane 5, muscle extract (10 μl) + DNPH; lane 6, muscle extract (15 μl) + DNPH; lane 7, brain extract (positive control) + DNPH; lane 8, extract + DNPH, without primary antibody (negative control).
FIGURE 10. Proteomic identification of oxidized proteins in DMD. A, total extracts of DMD (n = 6) and control (n = 6) were derivatized with DNPH and subjected to two-dimensional PAGE followed by anti-DNP Western blot. The protein spots on the Western blot were compared with the SYPRO Ruby-stained two-dimensional gel of total protein extract from DMD muscle to identify oxidized proteins common between control and DMD (outlined by a complete circle) and those specific to DMD (outlined by broken circle). There were 23 distinct spots in the DMD blot, of which 8 (numbered 16–23) were not represented in control. Seven spots (numbers 17 to 23) were excised out for analysis by mass spectrometry.

B, list of the spot numbers and the corresponding muscle proteins that are potentially oxidized in DMD patients compared with control. C, the activity of aconitase, one of the proteins carbonylated in the DMD muscle is significantly decreased. Activity indicated in % (100% activity = 0.069 mm/min/mg protein); *, p < 0.05 compared with control (C). D, aconitase activity (in mouse muscle) is inhibited by H_2O_2. **, p < 0.01 compared with untreated control (C).
is not completely understood, it could directly correlate with the severity of the dystrophic pathology (13). Our study showed elevated lipid peroxidation and protein carbonylation in the chronic diseases, distal myopathies, MM and SMA (Fig. 9, L, N, and O).

The CTX model displayed markers of oxidative stress including a lowered GSH/GSSG ratio and elevated lipid peroxidation (Fig. 9, A and D). GSH is essential for cellular differentiation (16) and stress response in the muscle (82). GSH depletion might contribute to oxidative stress in MD (83), cachexia, and muscle atrophy (82) and might increase CTX toxicity in C2C12 cells (13). The GSH level that was low at d1 significantly increased beyond the control level at d3 and d7. This is probably a stress response to lipid peroxidation (78), which in turn might not be due to increased GSH synthesis, because the expression of the GSH synthesizing enzyme γ-glutamyl cysteine ligase (GCL) was unchanged (Fig. 9C).

Analysis of non-dystrophic myopathies in this study showed GSH depletion in MMs, IMs, and more drastically in SMA1 and -2 (Fig. 9M). Following
recovery of muscle structure, the GSH and MDA content in the CTX-injected muscles were restored to control levels indicating a direct correlation of dystrophic pathology with GSH depletion and oxidative stress (data not shown). Hence, GSH depletion could be linked to dystrophic pathology and drugs that boost GSH levels could offer myoprotection. Treatment of C2C12 myotubes with lipoic acid plus coenzyme Q10 increased expression of genes involved in GSH synthesis and recycling (84). Data from our laboratory demonstrated that curcumin, a dietary polyphenol from turmeric, induced GSH synthesis and protected against CTX toxicity both in vitro and in vivo.

MS analysis detected six proteins that were carbonylated only in the DMD muscle (Fig. 10, A and B). One such mitochondrial protein is aconitase hydratase (aconitate), which converts citrate to isocitrate and is indirectly required by GSH reductase for its redox activity (85). Aconitate is susceptible to oxidative damage and is deregulated in DMD (86). Carbonylation of aconitate might inhibit its activity (87). Aconitase activity was significantly down-regulated in DMD muscle compared with control (Fig. 10C). Kinetic assay in mouse muscle extract demonstrated H2O2-dependent inhibition of aconitase activity thus confirming its susceptibility to oxidative stress (Fig. 10D). Interestingly, the CTX model showed down-regulation of aconitate hydratase by 1.4- and 1.7-fold in d1 and d3, respectively, compared with control (supplemental Table S2). The other carbonylated mitochondrial protein in DMD is Δ-(3,5)-(2,4)-Dienoyl-CoA isomerase, mitochondrial precursor (ECH1) (NP_001389.2), which accumulates in the dystrophic muscles (95). Another carbonylated protein α-enolase is a glycolytic enzyme (96) implicated in myoblast fusion and differentiation (97) and is modulated in mdx and muscle injury models (97). Enolase is carbonylated in experimental models of myopathy (87). These data suggest that oxidative damage of specific proteins is pertinent to DMD pathology and most of these proteins were down-regulated in the CTX model.

In summary, the current study has characterized the CTX model of muscle pathology with direct implications for MDs and IMs. The study also emphasized the role of mitochondrial dysfunction and oxidative stress in the model and in muscle pathology.

Acknowledgments—We thank all the patients and their families for the muscle biopsies. The technical help of U. Hemavathy in electron microscopy experiments is gratefully acknowledged. We acknowledge the technical assistance of L. Asmitha, TIFR, Mumbai, India, in RT-PCR experiments. The assistance of Dr. Sonam Kothari in the muscle strength analysis of human patients is gratefully acknowledged. The technical help of Dr. Phalguni Alladi in confocal microscopy is gratefully acknowledged.

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