Deletion of Hexose-6-phosphate Dehydrogenase Activates the Unfolded Protein Response Pathway and Induces Skeletal Myopathy*‡§

Received for publication, December 10, 2007, and in revised form, January 23, 2008 Published, JBC Papers in Press, January 25, 2008, DOI 10.1074/jbc.M710067200

Gareth G. Lavery‡, Elizabeth A. Walker§, Nil Turan‡, Daniela Rogoff‡, Jeffery W. Ryder**†, John M. Shelton§, James A. Richardson‡§, Francesco Falciani‡, Perrin C. White‡, Paul M. Stewart‡, Keith L. Parker‡, and Daniel R. McMillan‡§

From the ‡Division of Medical Sciences and the §School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TH, United Kingdom and the Departments of ‡Internal Medicine, ‡Pediatrics, **Physiology, and §Pathology and Molecular Biology, University of Texas Southwestern Medical Center, Dallas, Texas 75390

Hexose-6-phosphate dehydrogenase (H6PD) is the initial component of a pentose phosphate pathway inside the endoplasmic reticulum (ER) that generates NADPH for ER enzymes. In liver H6PD is required for the 11-oxoreductase activity of 11β-hydroxysteroid dehydrogenase type 1, which converts inactive 11-oxo-glucocorticoids to their active 11-hydroxyl counterparts; consequently, H6PD null mice are relatively insensitive to glucocorticoids, exhibiting fasting hypoglycemia, increased insulin sensitivity despite elevated circulating levels of corticosterone, and increased basal and insulin-stimulated glucose uptake in muscles normally enriched in type II (fast) fibers, which have increased glycogen content. Here, we show that H6PD null mice develop a severe skeletal myopathy characterized by switching of type II to type I (slow) fibers. Running wheel activity and electrically stimulated force generation in isolated skeletal muscle are both markedly reduced. Affected muscles have normal sarcermic structure at the electron microscopy level but contain large intrafibrillar membranous vacuoles and abnormal triads indicative of defects in structure and function of the sarcoplasmic reticulum (SR). SR proteins involved in calcium metabolism, including the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA), calreticulin, and calsequestrin, show dysregulated expression. Microarray analysis and real-time PCR demonstrate overexpression of genes encoding proteins in the unfolded protein response pathway. We propose that the absence of H6PD induces a progressive myopathy by altering the SR redox state, thereby impairing protein folding and activating the unfolded protein response pathway. These studies thus define a novel metabolic pathway that links ER stress to skeletal muscle integrity and function.

H6PD§ is a bifunctional enzyme that catalyzes the first two steps of the pentose phosphate pathway (1). It is distinct from its cytosolic homolog, glucose-6-phosphate dehydrogenase, in being localized exclusively to the lumen of the endoplasmic reticulum (ER). H6PD converts glucose 6-phosphate to 6-phosphogluconolactonate with the concomitant production of NADPH, thereby maintaining adequate levels of reductive cofactors in the oxidizing environment of the ER (2, 3).

One critical role for H6PD is providing NADPH to 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), a bi-directional enzyme highly expressed in liver and adipose tissue. 11β-HSD1 catalyzes both dehydrogenation and oxo-reduction of glucocorticoids, but in vivo it acts predominantly as a NADPH-dependent oxoreductase that converts hormonally inactive cortisone to active cortisol (in rodents, 11-dehydrocorticosterone to corticosterone) (4). To investigate the functional interactions of H6PD and 11β-HSD1 in vivo, we produced mice with a targeted inactivation of H6PD and showed that 11β-HSD1 predominantly acts as a dehydrogenase in these mice (6). The resulting cellular resistance to corticosterone leads to activation of the hypothalamic-pituitary-adrenal axis and elevated circulating corticosterone levels (7, 8).

H6PD null mice display fasting hypoglycemia and increased insulin sensitivity. Metabolic abnormalities include decreased glucagon-stimulated hepatocyte glucose output and increased basal and insulin-stimulated glucose uptake in explants from extensor digitorum longus (EDL) muscles, which are normally enriched in relatively insulin-insensitive, glycolytic type IIb fibers. Whereas in vitro experiments implicate impaired glu-

* This study was supported in part by Wellcome Trust Grants 066357 (to P. M. S.) and 074088/2/04/Z (to E. A. W. and P. M. S.), National Institutes of Health Grant DK45440 and the Wilson Center for Biomedical Research (to K. L. P.), and National Institutes of Health Grant DK068101 and the Audre Newman Rapoport Chair in Pediatric Endocrinology (to P. C. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The on-line version of this article (available at http://www.jbc.org) contains a supplemental figure and Tables S1 and S2.

‡ Supported as a postdoctoral fellow by National Institutes of Health Training Grant T32HL007360.

§ To whom correspondence should be addressed: 5323 Harry Hines Blvd., Dallas, TX 75390-9063. Fax: 214-648-9772; E-mail: daniel.mcmillan@UTSouthwestern.edu.

** The abbreviations used are: H6PD, hexose-6-phosphate dehydrogenase; ER, endoplasmic reticulum; EDL, extensor digitorum longus; MOPS, 4-morpholinepropanesulfonic acid; SR, sarcoplasmic reticulum; 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; UPR, unfolded protein response; PGC-1α, proliferator-activated receptor gamma coactivator-1; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase; TA, tibialis anterior; FDR, false discovery rate; NFAT, nuclear factor of activated T-cells; IRE1, inositol-requiring protein 1; sXBP, spliced XBP1; MCIP1, modulatory calcineurin-interacting protein 1; ATF, activating transcription factor; PKR, double-stranded RNA-dependent protein kinase.

3 The abbreviations used are: H6PD, hexose-6-phosphate dehydrogenase; ER, endoplasmic reticulum; EDL, extensor digitorum longus; MOPS, 4-morpholinepropanesulfonic acid; SR, sarcoplasmic reticulum; 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; UPR, unfolded protein response; PGC-1α, proliferator-activated receptor gamma coactivator-1; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase; TA, tibialis anterior; FDR, false discovery rate; NFAT, nuclear factor of activated T-cells; IRE1, inositol-requiring protein 1; sXBP, spliced XBP1; MCIP1, modulatory calcineurin-interacting protein 1; ATF, activating transcription factor; PKR, double-stranded RNA-dependent protein kinase.
corticoid action in the functional abnormalities seen in hepatocytes, it is not known whether the increased glucose uptake in muscle results from glucocorticoid insensitivity or directly from the loss of other actions of H6PD.

Here we show that H6PD null mice suffer a progressive skeletal myopathy. Concurrent changes in metabolism and induction of the unfolded protein response (UPR) pathway are evident by 4 weeks of age, before the most severe histological manifestation of myopathy. These findings highlight a novel role of H6PD in maintaining proper function of the sarcoplasmic reticulum (SR) in skeletal muscle.

**EXPERIMENTAL PROCEDURES**

All animal experiments and procedures were approved by the respective Institutional Animal Care and Use Committees. H6PD null and wild-type mice were housed in standard conditions on a 12-h/12-h light-dark cycle with access to standard rodent chow and water *ad libitum*.

**Microsome Preparations and H6PD Activity**—Liver microsomes were prepared as described (9). SR vesicles were isolated from EDL muscles by Dounce homogenization in 0.3 M sucrose, 1 min intervals. Contaminating 33S tritium were determined by standard Bradford assays. Aliquots were incubated at 23°C for 1 h (TaQ Man assay reagent, Applied Biosystems) as an internal control. Arbitrary units for expression were calculated using 1000 × 2−ΔCt, where ΔCt = (Ct value of the gene of interest) − (Ct value of 18 S rRNA).

Total RNA for reverse transcription-PCR of XBP1 was extracted from wild-type and null quadriceps with RNA-Stat60 (TEL-TEST Inc., Friendswood, TX). cDNA was prepared with an Advantage RT-for-PCR kit (Clontech Laboratories, Mountainview, CA). Oligonucleotide primers for XBP1 amplification were chosen to anneal to separate exons to avoid amplifying contaminating genomic DNA, with sequences of 5′-CTTGTG-GTTGAGAACCAGGAG-3′ (sense) and 5′-ACAGGGTC-CAACTTGTCCAG-3′ (antisense).

**Histological Analysis**—Tissues for routine histology were harvested from anesthetized mice after fixation via transcardiac perfusion with 4% paraformaldehyde. Subsequent paraffin processing, embedding, sectioning, and histological stains were performed by standard procedures (13, 14). Tissues were analyzed by hematoxylin and eosin staining in both null and wild-type mice (n = 3 for each genotype). Soleus, gastrocnemius, and tibialis anterior (TA) muscles were analyzed at 8 and 23 weeks and 14 months of age. Frozen sections were stained with oil red O (with hematoxylin counterstaining) to detect lipid content. Alcoholic formalin fixed tissues were stained with periodic acid-Schiff to detect glycogen (Sigma Aldrich). Frozen sections prepared from unfixed, flash-frozen, hindlimb musculature were stained by metachromatic ATPase (15) to determine fiber type.

**Running Wheel Activity**—Mice (n = 5 for each genotype) were housed individually in a cage equipped with a 25-cm diameter running wheel. Wheel revolutions were recorded for 5 days using a magnetic switch attached to a counter.

**Ex Vivo Muscle Contraction**—Isolated EDL and soleus muscles from wild-type or H6PD null mice were mounted to an isometric force transducer using 4/0 silk sutures. Muscles were immersed in physiological salt solution gassed continuously (95% O2, 5% CO2) and maintained at 33 °C. Optimal length was determined using a series of 200-ms tetany induced by electrical stimulation (25 V, 150 Hz, 0.2-ms pulse duration). Muscles remained quiescent for 30 min after determination of optimal length. Thereafter, muscles were stimulated for 500 ms at 1, 15, 25, 50, 75, 100, 125, 150, and 300 Hz with a 30-min recovery period between measurements. Force development was digitally recorded using a Powerlab 8/S data acquisition unit (AD Instruments). Optimal muscle length and tendon-free mass were used to calculate muscle cross-sectional area. Force generation was expressed as newtons/cm2 of cross-sectional area.

**Electron Microscopy**—Samples of skeletal muscle were fixed by immersion in ice-cold glutaraldehyde phosphate-buffered saline (2.5%/w/v) supplemented with 0.1 mM KCN. Sections were stained in osmium tetroxide, dehydrated, and vacuum-embedded in resin (16). Ultra-thin (80 nm) sections were cut...
Muscles enriched in type IIb fibers, including TA and gastrocnemius, were markedly vacuolated (Fig. 2). Serial sections from TA of 8-week-old mice stained with either hematoxylin-eosin or with metachromatic ATPase staining to determine fiber type analysis clearly show vacuoles in highly glycolytic, fast twitch type IIb fibers, whereas slow twitch type I fibers are spared (Fig. 2, A, A’, B, and B’). Vacuoles were not found in soleus muscle, which contains predominantly types I and Ila fibers. EDL was affected only at later time points (Fig. 2, G and G’).

When soleus muscles from null and wild-type mice were analyzed for fiber type composition using metachromatic ATPase staining (Fig. 3, A, A’, B, and B’), no changes were seen in either the number or fiber content at 8 weeks. At 23 weeks, although there was no change in the total number of fibers present, null mice showed a decrease in type II fibers (null 37.6 ± 1.3% versus wild-type 56.3 ± 1.1%; n = 3, p < 0.0001)

Abnormalities of Activity and Muscle Mass in H6PD Null Mice—Whereas null mice ambulated normally, they were decidedly weaker and more docile than wild-type littermates during routine handling. Null mice had generalized muscle atrophy, with post-mortem muscle tissue weights reduced by 20–40%. Additionally, null mice displayed a prominent kyphosis that intensified with age; however, the kyphotic vertebra showed no obvious skeletal abnormalities (data not shown).

Null and wild-type mice (n = 5) were assessed for spontaneous activity in a running wheel cage. After 5 days null mice had significantly less (p < 0.00002) cumulative activity (166,611 ± 383 revolutions) than wild-type (339,472 ± 750 revolutions, Fig. 1A).

Isolated Mutant Skeletal Muscle Contractions Are Weaker than Normal—Submaximal and maximal isometric force responses were obtained with isolated muscles in response to electrical stimulation. Developed forces were markedly reduced in both fast-twitch EDL and slow-twitch soleus muscles from H6PD null mice (Fig. 1B). In EDL muscles isometric forces were significantly reduced at electrical stimulation frequencies from 75-to 300 Hz, with maximal tetanic force (150 Hz electrical stimulation) reduced by 48% (Fig. 1B). At lower frequencies contractile forces of EDL muscles were similar between wild-type and H6PD null mice. When force values were normalized for maximal responses, a partial leftward shift in the force-frequency curve for mutant EDL muscles was observed at frequencies between 1 and 50 Hz (Fig. 1C). Force generation in slow-twitch soleus muscle from H6PD null mice was also reduced at all electrical stimulation frequencies with twitch force (stimulation at 1 Hz) and maximal tetanic force reduced by 68 and 56%, respectively.

Muscles from Null Mice Are Atrophic and Vacuolated and Display Fiber Type Switching—Skeletal muscle atrophy was clearly evident in the absence of H6PD. At 8 weeks p.o., null mice were markedly weaker and more docile than wild-type littermates. Null mice displayed a prominent kyphosis that intensified with age; however, the kyphotic vertebra showed no obvious skeletal abnormalities (data not shown).

Null and wild-type mice (n = 5) were assessed for spontaneous activity in a running wheel cage. After 5 days null mice had significantly less (p < 0.00002) cumulative activity (166,611 ± 383 revolutions) than wild-type (339,472 ± 750 revolutions, Fig. 1A).

Abnormalities of Activity and Muscle Mass in H6PD Null Mice—Whereas null mice ambulated normally, they were decidedly weaker and more docile than wild-type littermates during routine handling. Null mice had generalized muscle atrophy, with post-mortem muscle tissue weights reduced by 20–40%. Additionally, null mice displayed a prominent kyphosis that intensified with age; however, the kyphotic vertebra showed no obvious skeletal abnormalities (data not shown).

Null and wild-type mice (n = 5) were assessed for spontaneous activity in a running wheel cage. After 5 days null mice had significantly less (p < 0.00002) cumulative activity (166,611 ± 383 revolutions) than wild-type (339,472 ± 750 revolutions, Fig. 1A).

Isolated Mutant Skeletal Muscle Contractions Are Weaker than Normal—Submaximal and maximal isometric force responses were obtained with isolated muscles in response to electrical stimulation. Developed forces were markedly reduced in both fast-twitch EDL and slow-twitch soleus muscles from H6PD null mice (Fig. 1B). In EDL muscles isometric forces were significantly reduced at electrical stimulation frequencies from 75-to 300 Hz, with maximal tetanic force (150 Hz electrical stimulation) reduced by 48% (Fig. 1B). At lower frequencies contractile forces of EDL muscles were similar between wild-type and H6PD null mice. When force values were normalized for maximal responses, a partial leftward shift in the force-frequency curve for mutant EDL muscles was observed at frequencies between 1 and 50 Hz (Fig. 1C). Force generation in slow-twitch soleus muscle from H6PD null mice was also reduced at all electrical stimulation frequencies with twitch force (stimulation at 1 Hz) and maximal tetanic force reduced by 68 and 56%, respectively.

Muscles from Null Mice Are Atrophic and Vacuolated and Display Fiber Type Switching—Skeletal muscle atrophy was clearly evident in the absence of H6PD. At 8 weeks p.o., null mice were markedly weaker and more docile than wild-type littermates. Null mice displayed a prominent kyphosis that intensified with age; however, the kyphotic vertebra showed no obvious skeletal abnormalities (data not shown).

Null and wild-type mice (n = 5) were assessed for spontaneous activity in a running wheel cage. After 5 days null mice had significantly less (p < 0.00002) cumulative activity (166,611 ± 383 revolutions) than wild-type (339,472 ± 750 revolutions, Fig. 1A).

Abnormalities of Activity and Muscle Mass in H6PD Null Mice—Whereas null mice ambulated normally, they were decidedly weaker and more docile than wild-type littermates during routine handling. Null mice had generalized muscle atrophy, with post-mortem muscle tissue weights reduced by 20–40%. Additionally, null mice displayed a prominent kyphosis that intensified with age; however, the kyphotic vertebra showed no obvious skeletal abnormalities (data not shown).

Null and wild-type mice (n = 5) were assessed for spontaneous activity in a running wheel cage. After 5 days null mice had significantly less (p < 0.00002) cumulative activity (166,611 ± 383 revolutions) than wild-type (339,472 ± 750 revolutions, Fig. 1A).

Isolated Mutant Skeletal Muscle Contractions Are Weaker than Normal—Submaximal and maximal isometric force responses were obtained with isolated muscles in response to electrical stimulation. Developed forces were markedly reduced in both fast-twitch EDL and slow-twitch soleus muscles from H6PD null mice (Fig. 1B). In EDL muscles isometric forces were significantly reduced at electrical stimulation frequencies from 75-to 300 Hz, with maximal tetanic force (150 Hz electrical stimulation) reduced by 48% (Fig. 1B). At lower frequencies contractile forces of EDL muscles were similar between wild-type and H6PD null mice. When force values were normalized for maximal responses, a partial leftward shift in the force-frequency curve for mutant EDL muscles was observed at frequencies between 1 and 50 Hz (Fig. 1C). Force generation in slow-twitch soleus muscle from H6PD null mice was also reduced at all electrical stimulation frequencies with twitch force (stimulation at 1 Hz) and maximal tetanic force reduced by 68 and 56%, respectively.
and a concomitant increase in type I fibers (null 62.4% versus wild-type 43.7%, \( p < 0.0001 \)). A large number of type II muscle fibers contained centralized nuclei, which are typically seen in regenerating fibers resulting from injury (Fig. 3B).

Periodic acid-Schiff staining was used as a qualitative indicator of glycogen accumulation in muscle sections. Null muscles showed increased heterogeneity in periodic acid-Schiff staining as compared with wild-type, indicating higher glycogen content in some fibers (Fig. 3C). Increased glycogen concentration did not correlate with vacuolation, as intensely stained fibers did not contain vacuoles (Fig. 3C'). Additionally, as assessed by oil red O staining, the muscle vacuoles did not contain lipid (data not shown).

Vacuoles in Mutant Muscles Consist of Abnormal Sarcoplasmic Reticula—Muscles from null mice had grossly normal sarcomeric structure as assessed by electron microscopy but had prominent vacuoles of variable size containing membranous inclusions (Fig. 4A and C). The location of the vacuoles and the presence of ryanodine receptors (19) with their cytoplasmic domains prominently displayed on the surface of many of the inclusions indicate that they are derived from sarcoplasmic reticula (Fig. 4D).

FIGURE 2. Progressive histological defects in H6PD null skeletal muscle. Transverse muscle sections from wild-type (A–G) and null (A’–G’) mice. Comparing serial TA sections at 8 weeks, stained with hematoxylin and eosin (A and A’) and metachromatic ATPase (B and B’) show vacuoles (arrows) present predominately in type Iib fibers. Asterisks indicate type Ila fibers. Additional TA sections at 23 weeks (C and C’) and 14 months (D and D’) show increased vacuolation, centralized nuclei, and fibrosis. Similar increases are seen in gastrocnemius at 23 weeks (E and E’) and 14 months (F and F’). By 14 months, damage is seen in EDL (G and G’). The 8-week and 14-month samples were cryo-embedded, and the 23-week samples were paraffin-embedded. The reference bar is 80 \( \mu \text{m} \).

FIGURE 3. Fiber type switching and glycogen accumulation in skeletal muscle. Fiber type analysis of (A) wild-type and (A’) null transverse sections of soleus muscle showing a normal distribution of type I fibers (blue) and type Ila fibers (off white) in both null and wild-type sections at 8 weeks of age. By 23 weeks of age, no difference is seen in wild-type muscles (B), but null muscles (B’) show a loss of type Ila fibers with a concomitant increase in type I fibers. Periodic acid-Schiff-stained gastrocnemius sections comparing wild-type (C) with null (C’) muscles show an increase in glycogen content in some null fibers. Note that the vacuoles do not contain glycogen. The reference bar is 80 \( \mu \text{m} \).
terminal SR cisternae flanking a T-tubule, are normally present near the boundary between the A and I bands of skeletal muscle. In H6PD null muscles, triads were frequently missing in areas adjacent to the vacuoles (see the arrows in Fig. 4E). In addition, structurally abnormal triads also contained multiple T-tubules and terminal SR extensions (Fig. 4G), further indicating a disruption of the normal SR structure. Because multimers of some SR proteins are large enough to be visualized by electron microscopy, the loss of electron density in triads of null muscle compared with those of wild-type (Fig. 4F) suggests a change in either expression or localization of SR resident proteins.

The apparent myodegeneration and obvious disturbance of SR structure coupled with the loss of electron density in the triads led us to examine the expression of SR proteins. When Western blots containing crude muscle extracts from wild-type and null muscles were compared, changes in expression of calsequestrin, SERCA, BiP (immunoglobulin heavy chain-binding protein or HSPA5 or GRP78), and calreticulin were detected (Fig. 5A). There was a shift in expression of calsequestrin isoforms, with an increase in the type II (cardiac) and a decrease in the type I (skeletal) isoform. In particular muscles, e.g. planaris and TA, expression of the skeletal isoform was almost absent. SERCA, although expressed at variable levels in wild-type muscles, showed large decreases in all null muscles tested. Conversely, the levels of calreticulin (a calcium binding stress protein) and BiP (an important stress protein responsive to levels of unfolded proteins in the ER) were increased in all null muscles relative to wild type.

Calcineurin Activation Is Involved in Fiber Type Switch in Null Muscles—Calcineurin, a calcium-activated protein phosphatase, is involved in regulating muscle fiber type-specific proteins and myofiber remodeling through its actions on the nuclear
factor of activated T-cells (NFAT) (20). To determine whether calcineurin activation was involved in the fiber type switch in null muscles, we monitored the expression of the products of two calcineurin-regulated genes, an isoform of the MCIP1.4, and peroxisome PGC-1α.

Western blots of crude muscle extracts were probed with an anti-MCIP antibody that recognizes all isoforms (Fig. 5A); constitutive expression of the 1.1 isoform at 37 kDa served as an internal control. The 1.4 isoform, at 24 kDa, was expressed at low levels in wild-type muscle but was up-regulated in all null muscles with the exception of TA. Basal levels of PGC-1α expression varied between wild-type muscles, but relative expression was up-regulated in all corresponding null muscles (Fig. 5A).

The UPR Is Activated in Mutant Muscles—Increased BiP expression in null muscles is indicative of ER stress; in particular, the accumulation of unfolded proteins in the ER subsequently activates the UPR pathway. A subset of the UPR pathway is initiated by signal transduction cascades originating through the inositol-requiring protein 1 (IRE1, ERN1). Activation of the IRE1 pathway triggers an inducible post-transcriptional splicing event that produces the functional transcription factor, spliced XBP1 (sXBP1). We used reverse transcription-PCR to confirm splicing and the subsequent presence of the activated transcription factor sXBP1 (Fig. 5B). The expression of unspliced and, therefore, inactive XBP1 is revealed by the presence of a 274-bp ampiclon. A very low level of spliced and activated XBP1 (sXBP1), indicated by a 248-bp ampiclon, is seen in wild-type muscle, but a clear induction is detected in null muscle.

Microarray Analysis Confirms Abnormal Expression of Genes Responding to Stress in the Endoplasmic Reticulum—We carried out microarray analysis on TA and soleus muscles from 4-week-old mice (these data sets have been deposited in the NCBI Gene Expression Omnibus with the accession number GSE10347). Using a threshold FDR of 0.05, 323 probe sets representing 266 independent genes differed in expression between wild-type and mutant TA muscles. Only 64 probe sets representing 56 genes differed in soleus muscles, and only 23 probe sets differed in both types of muscle (supplemental Table S1).

To identify functional relationships among differentially expressed genes, we queried a knowledge base (Ingenuity Systems, Redwood City, CA) (21) that had listings for 4142 genes with known functions in the endoplasmic reticulum. Enrichment analysis was used to find functional relationships such as physical interactions or influences on gene expression. A more extensive network is displayed in supplemental Fig. 1, B, expression of selected UPR pathway genes in muscles from mice at 4 weeks of age shows induction of 2.5–5-fold in TA but not soleus (sol) muscle. Further increases in expression detected at 14 weeks in TA muscle indicate progressive ER stress.

HSP90B1 (glucose-regulated protein 94), calreticulin, and DDIT3 (CHOP10-C/EBP-homologous protein). All 4 genes were overexpressed by 2–5-fold in TA muscles from 4-week-old null mice, whereas expression in the soleus was comparable with that in wild-type samples (Fig. 6B). These changes persisted in 14-week-old mice; the same genes were overexpressed 5–18-fold in TA muscles from null compared with wild-type mice.

H6PD Is Expressed and Generates NADPH in Skeletal Muscle—H6PD mRNA was not detected in mutant muscle; however, in wild-type mice H6PD mRNA levels were ~25% that in liver. As measured by NADPH production, H6PD activity was present in isolated microsomes from liver and muscle of wild-type but not mutant mice. Normalized H6PD activity in muscle was ~45% that in liver (not shown).

**DISCUSSION**

Deletion of H6PD Affects Skeletal Muscle—Deletion of H6PD resulted in both decreased spontaneous locomotor activity and a dramatic increase in spontaneous locomotor activity. This is consistent with the severe skeletal myopathy observed in null mice. The UPR is activated in mutant muscles, indicating ER stress. It is possible that the increased BiP expression is responsible for the increased locomotor activity, as BiP is a known regulator of ER stress.

**H6PD Deletion Causes a Severe Skeletal Myopathy**
activity in the whole animal and reduced force generation in isolated muscles. In addition, null mice displayed a prominent kyphosis that intensified with age, a relatively common phenotype in mouse models of neuromuscular diseases such as the mdx mouse (22) and the kyphoscoliosis (ky) mouse (23). Because normal extension by the animal or gentle manipulation by a handler corrected the curvature, it is likely that the inherent skeletal muscle weakness also underlies this phenotype.

**Metabolic Implications of Fiber Type Switching**—A significant fiber type switch from type II (fast) to type I (slow) was apparent in soleus muscles of null mice. Although not yet fully characterized, the switch apparently occurs in a relatively narrow time window from 8 to 23 weeks. Skeletal muscle fiber type switching due to specific regulation of slow fiber-specific genes is controlled by the calcineurin pathway (24). Calcineurin, a calmodulin-dependent protein phosphatase that senses intracellular calcium levels, dephosphorylates the NFAT, which translocates to the nucleus and regulates a cascade of muscle remodeling genes (25). The expression of downstream calcineurin/NFAT-regulated genes provides a reliable measure of calcineurin activation. We chose to monitor MCIP1.4, an isoform of the MCIp1, also known as DSCR1 (26) and RCAN1 (27), transcribed from an alternative calcineurin-regulated promoter (28), and peroxisome PGC-1α (29, 30), both known to be involved in fiber type formation as markers of calcineurin/NFAT pathway activation.

With the notable exception of TA, MCIP1.4 expression is up-regulated in all muscles examined from H6PD null mice. Although some transcripts of MCIP1 reportedly are directly UPR-inducible (31), this is unlikely to pertain here, as both BiP and calreticulin are induced in null TA muscles (reflecting UPR pathway activation), and PGC-1α expression is similarly up-regulated in all muscles examined. In addition to calcineurin, PGC-1α expression is induced by a variety of stimuli including glucagon and certain external stimuli, e.g. exercise and fasting (for review, see Ref. 32). Although additional stimuli may play a role in induction of PGC-1α in muscle cells, the improved insulin sensitivity in H6PD null mice argues against a role for glucagon. Taken together, these results indicate that calcineurin activation, presumably a consequence of increased intracellular calcium concentration, is likely responsible for the fiber type switch.

Skeletal muscle glycogen content is abnormal in null mice. Activation of calcineurin is one plausible mechanism to explain this, because transgenic mice overexpressing constitutively active mutant calcineurin also have increased glycogen deposition in muscle and increased basal and insulin-stimulated glucose uptake (33). The link between the calcineurin pathway and glycogen metabolism is supported by the fact that both abnormal calcineurin activation and higher glycogen content are particularly found in muscles composed mainly of type II fibers (e.g. gastrocnemius). However, it is not known whether other effects of H6PD deficiency influence glycogen deposition or whether the effects of calcineurin activation on glycogen deposition are solely a consequence of increased basal and insulin-stimulated glucose uptake or also reflect other dysregulated cellular processes.

**H6PD Deletion Causes a Severe Skeletal Myopathy**

_With the notable exception of TA, MCIP1.4 expression is up-regulated in all muscles examined from H6PD null mice._

_Although some transcripts of MCIP1 reportedly are directly UPR-inducible (31), this is unlikely to pertain here, as both BiP and calreticulin are induced in null TA muscles (reflecting UPR pathway activation), and PGC-1α expression is similarly up-regulated in all muscles examined. In addition to calcineurin, PGC-1α expression is induced by a variety of stimuli including glucagon and certain external stimuli, e.g. exercise and fasting (for review, see Ref. 32). Although additional stimuli may play a role in induction of PGC-1α in muscle cells, the improved insulin sensitivity in H6PD null mice argues against a role for glucagon._

_Taken together, these results indicate that calcineurin activation, presumably a consequence of increased intracellular calcium concentration, is likely responsible for the fiber type switch._

_Skeletal muscle glycogen content is abnormal in null mice. Activation of calcineurin is one plausible mechanism to explain this, because transgenic mice overexpressing constitutively active mutant calcineurin also have increased glycogen deposition in muscle and increased basal and insulin-stimulated glucose uptake (33). The link between the calcineurin pathway and glycogen metabolism is supported by the fact that both abnormal calcineurin activation and higher glycogen content are particularly found in muscles composed mainly of type II fibers (e.g. gastrocnemius). However, it is not known whether other effects of H6PD deficiency influence glycogen deposition or whether the effects of calcineurin activation on glycogen deposition are solely a consequence of increased basal and insulin-stimulated glucose uptake or also reflect other dysregulated cellular processes._

**H6PD Deficiency Causes a Novel Myopathy Associated with the UPR Pathway**—The UPR pathway is a well defined, multiarmed homeostatic response that is initiated by a variety of cellular insults affecting the transit of secretory proteins through the ER. Ultimately, such insults result in the accumulation of unfolded proteins in the ER, i.e. a state of ER stress (for review, see Refs. 34–37). Recently, ER stress and the UPR pathway have been implicated in a variety of pathophysiological conditions, including Alzheimer disease, Parkinson disease, and some forms of diabetes and hepatic diseases (for review, see Ref. 37). In addition, up-regulation of UPR proteins has been detected in some skeletal myopathies including some forms of inclusion body myositis (IBM) (39, 40) and inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia (IBM-PFD) (41).

Deletion of H6PD induces a robust ER stress response as evidenced by increased expression of components of the UPR pathway to compensate for ER protein overload. The decrease in SERCA protein expression is consistent with translational attenuation of non-innerRibosome entry site (IRES)-containing transcripts resulting from activation of the PKR-like ER kinase pathway, whereas the downstream target of activating transcription factor 4 (which contains an IRES), the C/EBP-homologous protein (CHOP10 or DDIT3), is induced.

The activating transcription factor 6 (ATF6) pathway is chiefly responsible for the induced expression of protein chaperones. Consistent with ATF6 pathway activation, we saw induction of ATF6 and protein chaperones including BiP (immunoglobulin heavy chain-binding protein or HSPA5 or GRP78), HSP90B1 (or glucose-regulated protein 94), and HSP40 (or DnaJb9). Calreticulin, induced at both the protein and gene expression levels in null muscles, is likely a component of the ATF6 pathway.

XBP1, a component of the IRE1 pathway, regulates expression of many genes (42), including several involved in ER-associated protein degradation (43, 44). Indeed, we detected increased expression of genes involved in protein degradation, including the members of the Der1p-like protein (Derlin1 and Derlin 3) family.

The structural aspects of the myopathy (the occurrence of large membranous vacuoles visible on hematoxylin-eosin-stained sections and the triad junction abnormalities seen by electron microscopy) can be readily explained by activation of the ER stress response. sXBP1 is involved in the synthesis of phosphatidylcholine, the predominant phospholipid in the ER membrane. Overexpression of sXBP1 in NIH-3T3 cells results in a significant increase in ER surface area (45). Our results showing ryanodine receptor-containing membranes in the vacuoles, and expression of sXBP1 in null muscles are consistent with the role of XBP1 in membrane biogenesis. Perturbation of the normal SR structure by the markedly increased membrane volume likely is responsible for both the loss of triads in areas adjacent to the vacuoles and the observed abnormal triad structures.
H6PD Deletion Causes a Severe Skeletal Myopathy

The vacuoles appear to be restricted to predominantly fast-twitch muscles as they are found early in TA and gastrocnemius/plantaris but not in soleus. Likewise, gene expression analysis indicates that the ER stress response is much more severe in TA than in soleus and progressively worsens with age. Intrinsic differences in metabolism between fiber types may underlie these observations. In particular, the SR is the site of calcium uptake, storage, and release for muscle contraction, all of which are processes sensitive to redox state. Thus, it is plausible that changes in metabolism and redox status act in concert to influence the phenotype. The electron density of the triads of null mice is decreased compared with that of wild-type triads. Given that calsequesmin, a low affinity, high capacity calcium-binding protein localized in the terminal SR assembles into calcium-dependent polymers large enough to be seen by electron microscopy (46), an obvious prediction is that there is an accompanying decrease in calsequesmin expression. Rather than a decrease, however, there is a shift in isoform expression. Null muscles showed decreased expression of the type 1 (skeletal) isoform with accompanying overexpression of the Type 2 (cardiac) isoform, which is encoded by a separate gene. The significance of the change is unclear. The triad junctions constitute the calcium release units in skeletal muscle (47). The combination of decreased SERCA expression, altered calsequesmin expression, and perturbations in triad structure may play a role in the putative calcium signaling and subsequent calcineurin activation involved in fiber type switch.

Why Does H6PD Deficiency Cause Myopathy?—The increased deposition of glycogen in skeletal muscle may contribute to the observed pathology as humans with glycogen storage disease II (Pompe disease) develop weakness of skeletal muscles in addition to involvement of the heart and diaphragm. However, neither affected humans nor mouse models of glycogen storage disease II develop vacuoles in muscle fibers. Similarly, mice transgenic for the glycogen synthase gene display neither muscle weakness nor obvious muscle pathology other than glycogen deposition (48). Thus, although a role for glycogen deposition in the myopathy of H6PD null mice cannot be excluded, it is unlikely to be the primary cause.

At present, we cannot rule out the possibility that the observed phenotype is affected by local glucocorticoid inactivation in muscle due to the gain in 11β-HSD1 dehydrogenase function. However, 11β-HSD1 is expressed in muscle at only ~5% of that of the levels in liver, suggesting that it does not significantly modulate glucocorticoid availability in muscle. Moreover, deletion of H6PD does not phenocopy other genetic models lacking glucocorticoids in development and during adulthood, arguing against a pathogenetic role for glucocorticoids (10, 38).

Alternatively, we propose that the loss of H6PD and the consequent change in NADPH/NADP+ ratio is a specific stressor that affects the redox balance and ultimately disrupts the normal protein-folding environment of the SR (Fig. 7). The subsequent accumulation of unfolded proteins activates the UPR pathway in an effort to relieve the stress and restore homeostasis. Activation of the UPR pathway slows general protein trans-
molecular basis for the phenotype remain to be elucidated. We anticipate that the H6PD knock-out mouse will provide a novel genetic model in which to explore further the interplay between ER redox state and the cellular stress responses.

Acknowledgments—We thank Dr. Beverly Rothermel for kindly sharing reagents and for invaluable discussions and Kelli Black, Emily Harris, Amanda Keith, and Heather Powell for excellent technical support.

REFERENCES

1. Mason, P. J., Stevens, D., Diez, A., Knight, S. W., Scopes, D. A., and Vul- 

2. Banhegyi, G., Marcelongo, P., Fulcieri, R., Hinds, C., Burchell, A., and 

3. Picciarella, S., Czegle, I., Lizak, B., Margittai, E., Senesi, S., Papp, E., Csa- 

4. Tomlinson, J. W., Walker, E. A., Bujalska, I. J., Draper, N., Laverty, G. G., 

5. Rogoff, D., Rydén, J. W., Black, K., Yan, Z., Burgess, S. C., McMullan, D. R., 

6. Laviery, G. G., Hauton, D., Hewitt, K. N., Brice, S. M., Sherlock, M., Walker, 

7. Rogoff, D., Rydén, J. W., Black, K., Yan, Z., Burgess, S. C., McMullan, D. R., 

8. Kotelevtsev, Y., Holmes, M. C., Burchell, A., Houston, P. M., Schmoll, D., 

9. Hori, H., Nembai, T., Miyata, Y., Hayashi, T., Ueno, K., and Koide, T. (1999) J. Bioch. (Tokyo) 126, 723–730

10. Kotelevtsev, Y., Holmes, M. C., Burchell, A., Houston, P. M., Schmoll, D., 

11. Bush, E., Fieltz, J., Melvin, L., Martinez-Arnold, M., McKinsey, T. A., 

12. Bujalska, I. J., Draper, N., Michailidou, Z., Tomlinson, J. W., White, P. C., 

13. Shehan, D. C., and Hrapchak, B. B. (1980) Theory and Practice of Histo-

14. Woods, A. E., and Ellis, R. C. (1996) Laboratory Histopathology, A Com- 

15. Ogilvie, R. W., and Feeback, D. L. (1990) Stain Technol. 65, 231–241

16. O’Brien, K. M., Skillbeck, C., Sidell, B. D., and Egginton, S. (2003) J. Exp.

17. Jain, N., Thatte, J., Bricale, T., Ley, K., O’Connell, M., and Lee, J. K. (2003) 

18. Reiner, A., Yekutieli, D., and Benjamini, Y. (2003) Bioinformatics 19, 

19. Saito, A., Seiler, S., Chu, A., and Fleischer, S. (1984) J. Cell Biol. 99, 

20. Bassel-Duby, R., and Olson, E. N. (2006) Annu. Rev. Biochem. 75, 19–37

21. Calvano, S. E., Xiao, W., Richards, D. R., Feliciano, R. M., Baker, H. V., Cho, 

22. Chin, E. R., Olson, E. N., Richardson, J. A., Yang, Q., Humphries, C., Shel- 

23. Mason, R. M., and Palfrey, A. J. (1984) J. Orthop. Res. 2, 333–338

24. Chan, E. R., Olson, E. N., Richardson, J. A., Yang, Q., Humphries, C., Shel- 

25. Olson, E. N., and Williams, R. S. (2000) BioEssays 22, 510–519

26. Fuentes, J. J., Pritchard, M. A., Planas, A. M., Bosch, A., Ferrer, I., and 

27. Rothermel, B. A., Vega, R. B., and Williams, R. S. (2003) Trends Cardio-

28. Lin, J., Wu, H., Tarr, P. T., Zhang, C. Y., Wu, Z., Boss, O., Michael, L. F., 

29. Lin, J., Wu, H., Tarr, P. T., Zhang, C. Y., Wu, Z., Boss, O., Michael, L. F., 

30. Puigserver, P., and Spiegelman, B. M. (2002) Nature 418, 797–801

31. Leahy, K. P., Davies, K. J., Dull, M., Kort, J. J., Lawrence, K. W., and Crawford, D. R. (1999) Arch. Biochem. Biophys. 368, 67–74

32. Corton, J. C., and Brown-Borg, H. M. (2005) J. Gerontol. A Biol. Sci. Med.

33. Ryder, J. W., Bassel-Duby, R., Olson, E. N., and Zierath, J. R. (2003) J. Biol.

34. Wu, J., and Kaufman, R. J. (2006) Cell Death Differ. 13, 374–384

35. Bernales, S., Papa, F. R., and Walter, P. (2000) Annu. Rev. Cell Dev. Biol. 16, 487–508

36. Schroder, M. (2006) Mol. Biotechnol. 34, 279–290

37. Yoshida, H. (2007) FEBS J. 274, 630–658

38. Coll, A. P., Challis, B. G., Lopez, M., Piper, S., Yeo, G. S., and O’Rahilly, S. (2005) Diabetes 54, 2269–2276

39. Vattemi, G., Engel, W. K., McFerrin, J., and Askanas, V. (2004) Am. J.

40. Liewluck, T., Hayashi, Y. K., Ohsawa, M., Kurokawa, R., Fujita, M., Nogu- 

41. Watts, G. D., Wymers, J., Kovach, M. J., Mehta, S. G., Mumm, S., Darvish, D., Peirstonk, A., Whyte, M. P., and Kominos, V. E. (2004) Nat. Genet. 36, 377–381

42. Acosta-Alvear, D., Zhou, Y., Blais, A., Tsikitis, M., Lents, N. H., Arias, C., 

43. Travers, K. J., Patil, C. K., Wodicka, L., Lockhart, D. J., Weissman, J. S., and 

44. Oda, Y., Okada, T., Yoshida, H., Kaufman, R. J., Chen, R. O., Brownstein, B. H., Cobb, J. P., Tschoeke, S. K., Miller-

45. Sriburi, R., Jackowski, S., Mori, K., and Brewer, J. W. (2004) J. Cell Biol. 

46. Franzini-Armstrong, C., Kenney, L. J., and Varriano-Marston, E. (1987) 

47. Duhlunty, A. F. (2006) Clin. Exp. Pharmacol. Physiol. 33, 763–772

48. Manchester, J., Skurat, A. V., Roach, P., Haushka, S. D., and Lawrence, J. C. Jr. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10707–10711

H6PD Deletion Causes a Severe Skeletal Myopathy

875–885

85.

84.

83.

82.

81.

80.

79.

78.

77.

76.

75.

74.

73.

72.

71.

70.

69.

68.

67.

66.

65.

64.

63.

62.

61.

60.

59.

58.

57.

56.

55.

54.

53.

52.

51.

50.

49.

48.

47.

46.

45.

44.

43.

42.

41.

40.

39.

38.

37.

36.

35.

34.

33.

32.

31.

30.

29.

28.

27.

26.

25.

24.

23.

22.

21.

20.

19.

18.

17.

16.

15.

14.

13.

12.

11.

10.

9.

8.

7.

6.

5.

4.

3.

2.

1.

MARCH 28, 2008•VOLUME 283•NUMBER 13 JOURNAL OF BIOLOGICAL CHEMISTRY 8461