Nrf2-Mediated Regulation of Skeletal Muscle Glycogen Metabolism

Akira Uruno,^a Yoko Yagishita,^a Fumiki Katsuoka,^b Yasuo Kitajima,^c Aki Nunomiya,^d Ryoichi Nagatomi,^c Jingbo Pi,^e Shyam S. Biswal,^f Masayuki Yamamoto^{a,b}

Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan^a; Department of Integrative Genomics, Tohoku Medical Megabank Organization, Tohoku University, Sendai, Miyagi, Japan^b; Division of Biomedical Engineering for Health and Welfare, Tohoku University Graduate School of Biomedical Engineering, Sendai, Miyagi, Japan^c; Department of Medicine and Science in Sports and Exercise, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan^d; School of Public Health, China Medical University, Shenyang, Liaoning, China^e; Department of Environmental Health Sciences, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland, USA^f

Nrf2 (NF-E2-related factor 2) contributes to the maintenance of glucose homeostasis *in vivo*. Nrf2 suppresses blood glucose levels by protecting pancreatic β cells from oxidative stress and improving peripheral tissue glucose utilization. To elucidate the molecular mechanisms by which Nrf2 contributes to the maintenance of glucose homeostasis, we generated skeletal muscle (SkM)-specific *Keap1* knockout (*Keap1*MuKO) mice that express abundant Nrf2 in their SkM and then examined Nrf2 target gene expression in that tissue. In *Keap1*MuKO mice, blood glucose levels were significantly downregulated and the levels of the glycogen branching enzyme (*Gbe1*) and muscle-type PhK α subunit (*Phka1*) mRNAs, along with those of the glycogen branching enzyme (GBE) and the phosphorylase *b* kinase α subunit (PhK α) protein, were significantly upregulated in mouse SkM. Consistent with this result, chemical Nrf2 inducers promoted *Gbe1* and *Phka1* mRNA expression in both mouse SkM and C2C12 myotubes. Chromatin immunoprecipitation analysis demonstrated that Nrf2 binds the *Gbe1* and *Phka1* upstream promoter regions. In *Keap1*MuKO mice, muscle glycogen content was strongly reduced and forced GBE expression in C2C12 myotubes promoted glucose uptake. Therefore, our results demonstrate that Nrf2 induction in SkM increases GBE and PhK α expression and reduces muscle glycogen content, resulting in improved glucose tolerance. Our results also indicate that Nrf2 differentially regulates glycogen metabolism in SkM and the liver.

The tight regulation of glucose homeostasis is essential for the maintenance of biological functions. As glucose metabolites are major energy sources for skeletal muscle (SkM) contraction (1–3), SkM requires an efficient supply of glucose metabolites during exercise. Perturbations in SkM glucose metabolism often provoke metabolic disorders; e.g., impaired glucose tolerance and diabetes mellitus. SkM and liver store glucose as glycogen (4), which is used to generate glucose metabolites when energy is required; consequently, efficient SkM glycogen utilization is an important factor in exercise and the maintenance of glucose homeostasis (4).

Our bodies are continuously exposed to toxic chemicals (often electrophiles) and oxidative stress from the environment; these stresses are termed environmental stresses (5). While these environmental stresses are known to provoke tissue damage, they also affect cellular metabolism and energy production (5). The Keap1-Nrf2 system protects our bodies against these environmental stresses (6, 7). Nrf2 (NF-E2-related factor 2) belongs to the cap 'n' collar subfamily of basic region-leucine zipper-type transcription factors (8). Under unstressed conditions, Keap1 (Kelch-like ECH-associated protein 1) constitutively represses Nrf2 activity (9) by acting as an adaptor subunit for cullin-3-based ubiquitin E3 ligase (7). This E3 ligase complex efficiently ubiquitinates Nrf2, leading to its rapid proteasomal degradation (10). When cells are exposed to either electrophilic toxic chemicals or reactive oxygen species, the cysteine residues of Keap1 are modified, leading to the loss of Keap1 ubiquitin ligase activity. Consequently, Nrf2 is stabilized, accumulates in the nucleus (11–16), and heterodimerizes with small Maf proteins to activate the transcription of cytoprotective enzymes and bind antioxidant response elements (AREs) and electrophile response elements (EpREs) (17).

Nrf2 contributes to the maintenance of metabolic homeostasis

(5); for example, Nrf2 induction in pancreatic β cells markedly suppresses oxidative-stress-mediated dysfunction (18, 19). Furthermore, recent chromatin immunoprecipitation (ChIP)-sequencing (seq) analysis demonstrated that Nrf2 also binds the regulatory elements of various metabolism-related genes (20). Consistent with this binding, Nrf2 induces the expression of pentose phosphate pathway enzymes in proliferating cells (21) and upregulates metabolism-related *Fgf21* gene expression (22). These observations suggest that Nrf2 regulates the expression of many metabolism-related genes independently of its role in regulating the oxidative and electrophilic stress response (5, 23).

Previously, we determined that Nrf2 induction in SkM lowers blood glucose levels both under feeding conditions and during an insulin tolerance test (ITT), improves immobility during an open-field test, and suppresses body weight gain in a diet-induced obesity model (18); however, the precise mechanisms by which Nrf2 improves both SkM glucose metabolism and locomotor activity remained unclear. To address this issue, we identified Nrf2 target genes in SkM that are involved in the improvement of glucose

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Address correspondence to Akira Uruno, uruno@med.tohoku.ac.jp, or Masayuki Yamamoto, masiyamamoto@med.tohoku.ac.jp.

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utilization. We found that Nrf2 regulates the expression of two important glycogen metabolism-related genes, *Gbe1* and *Phka1*. *Gbe1* encodes glycogen branching enzyme (GBE) and contributes to the transfer of one glycogen chain to another via an α -1-6 bond (4). The loss of GBE provokes type IV glycogen storage disease (GSD) (24–26), and *Gbe1* hypomorphic mutant mice display a type IV GSD-like phenotype (27). Phosphorylase *b* kinase (PhK) is an important regulator of glycogen metabolism that activates glycogen phosphorylase activity to enhance glycogen breakdown (28). The *Phka1* gene encodes one of four PhK subunits, the muscle-type PhK α subunit (PhK α), and loss of PhK has been linked to type IX GSD (29, 30). Our present analysis demonstrates that Nrf2 significantly activates SkM and liver glycogen metabolism. Intriguingly, induction of GBE in SkM and liver provokes a distinct glycogen storage phenotype, indicating that the Nrf2-mediated enhancement of SkM glucose utilization is an important mechanism for resolving diabetes mellitus.

MATERIALS AND METHODS

Animals. Two independent lines of *Keap1*-floxed mice were utilized in this study. One line, *Keap1^{FA}*, was established in our laboratory (31) and the other, *Keap1^{FB}*, was established at Johns Hopkins University (32). Conditional (33) and global *Nrf2* knockout (6) (*Nrf2^F* and *Nrf2^{-/-}*, respectively) mouse lines were generated as previously described. *MuCre* mice were supplied by the RIKEN Bioresource Center (34), and *Alb-Cre* mice were supplied by the Jackson Laboratory. In addition, *db* mice were purchased from CLEA Japan. C57BL/6J and ICR mice were purchased from Japan SLC. *Keap1^{FA}*, *Keap1^{FB}*, *Nrf2^F*, *MuCre*, *Alb-Cre*, and *db* mice were backcrossed into the ICR background for at least four generations. *Nrf2^F*, *MuCre*, and *Nrf2^{-/-}* mice were also backcrossed into the C57BL/6J background for at least 10 generations. Mice were fed standard chow (2.31 kcal/g; Labo MR Stock; Nosan). For the *db/db* mouse study, mice were fed another standard chow (3.59 kcal/g; MF; Oriental Yeast). All of the mice were handled according to the regulations for animal experiments and related activities at Tohoku University.

Cell culture. C2C12 myoblasts were purchased from Sumitomo-Dainippon-Pharma. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mmol/liter glucose, 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. When the C2C12 myoblasts reached confluence, the culture medium was replaced with differentiation medium (DMEM supplemented with 2% horse serum) and the cells were cultured for an additional 72 h to facilitate myotube differentiation.

Reagents. Diethyl maleate (DEM) was purchased from Wako Pure Chemicals. *tert*-Butylhydroquinone-diethyl (tBHQ) was purchased from Sigma. Sulforaphane (SFN) was purchased from MP Biochemicals. CDDO-Im was a generous gift from Mochida Pharmaceuticals (18).

Glucose and insulin measurements. Blood glucose levels were measured with a OneTouch Ultra View meter (Life Scan), and plasma insulin levels were measured with an insulin enzyme-linked immunosorbent assay kit (Morinaga Institute) according to the manufacturer's instructions. The intraperitoneal-administration glucose tolerance test (ipGTT) and the ITT were previously described (18).

Microarray expression analysis. Microarray expression analysis was performed as previously described (22). Briefly, RNA samples were extracted from soleus and gastrocnemius muscles from 9-week-old male *Keap1^{MuKO-B}* and control *Keap1^{FB/FB}* mice fed *ad libitum* with Sepasol-RNA I Super G reagent (Nacalai Tesque) according to the manufacturer's instructions. Agilent 4 $\times$ 44K whole-mouse genome oligonucleotide microarray slides were hybridized, washed, and scanned with an Agilent microarray scanner according to the manufacturer's protocol. The expression data were analyzed with GeneSpring software (Silicon Genetics). Genes that were differentially expressed in *Keap1^{MuKO-B}* mouse SkM were identified by using a cutoff of a 1.5-fold change and a

statistically significant difference ($P < 0.05$) from SkM from *Keap1^{FB/FB}* mice according to the Benjamini-Hochberg method ($n = 3$).

qPCR. Total RNA was isolated from mouse SkM, livers, brains, and differentiated C2C12 myotubes cultured in six-well plates with Sepasol-RNA I Super G reagent. Total RNA was used for random 6-mer- and oligo(dT)-primed reverse transcription (RT) with PrimeScript RT master mix (TaKaRa Bio) according to the manufacturer's instructions. The resulting templates were then used for real-time quantitative PCR (qPCR) with Thunderbird qPCR Mix (Toyobo) and a QuantStudio 6 Flex real-time PCR system (Life Technologies). Information about the primer and TaqMan probe sequences is available upon request. The primers and probes are described in Table S1 in the supplemental material.

Immunoblot analysis. SkM (150 to 200 mg) was minced well on ice with scissors and added to 1 ml of cell lysis buffer (20 mmol/liter Tris-HCl [pH 7.5], 5 mmol/liter EDTA, 10 mmol/liter Na₄P₂O₇, 1% NP-40, and cComplete protease inhibitor cocktail [Roche]) (18). The samples were incubated on ice overnight and sonicated for 30 s, and cell debris was removed by centrifugation at $18,800 \times g$ for 10 min. For nuclear protein preparations, differentiated C2C12 myotubes cultured in 100-mm dishes were treated with either 100 nmol/liter CDDO-Im or 0.1% dimethyl sulfoxide (DMSO) for 3 h, washed three times with phosphate-buffered saline (PBS), and lysed in 100 μ l of cell lysis buffer (20 mmol/liter HEPES [pH 7.6], 20% glycerol, 10 mmol/liter NaCl, 1.5 mmol/liter MgCl₂, 0.2 mmol/liter EDTA, 1 mmol/liter dithiothreitol [DTT], 1 mmol/liter phenylmethylsulfonyl fluoride, and cComplete protease inhibitor cocktail [Roche]). After 5 min on ice, the nuclear fractions were collected by centrifugation at $1,000 \times g$ for 5 min and the supernatants were removed. The pellets were mixed with 100 μ l of 1 $\times$ sodium dodecyl sulfate (SDS) loading buffer (62.5 mmol/liter Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 50 mmol/liter DTT, 0.01% bromophenol blue) (35, 36) and then sonicated for 30 s. The cell debris was removed by centrifugation at $18,800 \times g$ for 5 min.

The denatured samples were separated on 10 to 20% gradient SDS-polyacrylamide gels (PAGE; Atto) and transferred onto Immobilon P polyvinylidene difluoride membranes (Millipore). The membranes were then blocked with 5% nonfat dry milk and probed with rabbit monoclonal anti-GBE (clone EP11113, Abcam ab180596; 1:2,000 dilution), rabbit polyclonal anti-PhK α 1 (Abcam ab96752; 1:2,000 dilution), goat polyclonal anti-NQO1 (Abcam ab2346; 1:2,000 dilution), mouse monoclonal anti- α -tubulin (clone DM1A; Sigma-Aldrich T6199; 1:5,000 dilution), rat monoclonal anti-Nrf2 (clone 103; 1:100 dilution) (22), and mouse monoclonal anti-lamin B1 (clone L-5; Invitrogen 332000; 1:2,000 dilution) antibodies. The bands were visualized with either ECL-prime (GE Healthcare) or Western Lightning Plus-ECL (PerkinElmer) reagents and a ChemiDoc MP ImageLab system (Bio-Rad).

CT. Mice were anesthetized with isoflurane and scanned with a Latheta (LCT-200) experimental-animal computed tomography (CT) system (Aloka). Continuous-slice images of the whole body were collected, and the lean and fat areas in CT images were estimated by Hounsfield units (HU) with Latheta software.

Mitochondrial DNA content. SkM (gastrocnemius and soleus muscles) was freshly removed and digested with proteinase K. Total DNA was then purified by standard methods with phenol, chloroform, and isoamyl alcohol. The mitochondrial DNA and genomic DNA contents of SkM were determined by qPCR with the following primers (for mitochondrial DNA encoding cytochrome *c* oxidase I, forward primer 5'-ACTATACTA CTACTAACAGACCG-3' and reverse primer 5'-GGTCTTTTTCCTGGAGT-3'; for genomic DNA encoding 18S rRNA, forward primer 5'-CTCAACACGGGAAACCTCAC-3', reverse primer 5'-CGCTCCACC AACTAAGAACG-3', and probe 5'-6-carboxyfluorescein-AGGATTG ACAGATTGATAGC-6-carboxytetramethylrhodamine-3') (18, 37). The mitochondrial DNA content was normalized to the genomic DNA content.

Measurement of oxygen consumption. Oxygen consumption in SkM was measured as previously reported (18). SkM (gastrocnemius and so-

leus muscles) was freshly removed. Krebs-Ringer bicarbonate buffer (KRBB) was aerated with a 5% CO₂–95% O₂ gas mixture. SkM was added to the KRBB (1.2 ml) containing 5.6 mmol/liter glucose in a 24-well SDR sensor OxoDish (PreSens), and changes in oxygen concentration in KRBB were measured every 10 s for 4 min at 37°C with an SDR2 sensor dish reader (PreSens). Oxygen consumption was determined by measuring the change in the oxygen content of the buffer normalized to tissue weight for 4 min.

Treadmill tests. Before treadmill tests, mice were exposed to the treadmill (MK-680; Muromachi Kikai Co., Ltd.). Training started at 10 m/min for 10 min and 22 m/min for 15 min; the following day, the treadmill test was performed (see Fig. 9A). Exercise capacity was estimated by determining the all-out running speed on the treadmill when mice could not or would not run forward despite any stimuli for 20 s. The protocol consisted of a starting speed of 10 m/min for 10 min, followed by an increased of 2 m/min every 30 s (see Fig. 9B).

ChIP and ChIP-seq analysis. ChIP and ChIP-seq experiments were performed as described previously (20). Briefly, differentiated C2C12 myotubes were cultured in 100-mm dishes and treated with 100 nmol/liter CDDO-Im or 0.1% DMSO for 3 h. Cross-linking with 1% formaldehyde was performed for 10 min at room temperature and subsequently quenched with 0.125 mol/liter glycine for 5 min. The samples were then lysed, and the nuclear fractions were extracted and sonicated with a Sonifier 250 sonicator for 1,000 s (Branson) to generate 220-bp DNA fragments. For immunoprecipitation, the samples were incubated with an anti-Nrf2 monoclonal antibody (clone D1Z9C; Cell Signaling Technology) overnight at 4°C and then heated overnight at 65°C with shaking to reverse the cross-links.

For ChIP-seq analysis, the purified DNA was used to prepare next-generation sequencing libraries with an Ovation SP Ultralow Library system and a Modrian SP+ workstation (TaKaRa). The qMiSeq (quantitative MiSeq) method was used to quantify the libraries and to verify their quality (38). The resulting libraries were analyzed with a HiSeq 2500 sequencing system (Illumina). ChIP-seq enrichment mapping and peak calling were performed with Bowtie and MACS (version 1.4).

For manual ChIP analysis, the purified DNA was analyzed by real-time PCR with Thunderbird qPCR mix (Toyobo) and a QuantStudio 6 Flex real-time PCR system (Life Technologies). The values obtained for the immunoprecipitated samples were normalized to those of the input DNA. For descriptions of the primers used, see Table S2 in the supplemental material.

Plasmids. A chimeric construct (*Gbe1* –993/+180-luc) harboring mouse *Gbe1* genomic DNA and firefly luciferase cDNA (pGL4.15; Promega) was constructed. Mouse *Gbe1* genomic DNA was PCR amplified from mouse tail genomic DNA with PrimeSTAR (TaKaRa). PCR-based mutagenesis with PrimeSTAR (TaKaRa) was used to engineer ARE mutations into the *Gbe1* –993/+180-luc construct as follows (substitutions are underlined): wild type, TGCTTCATCACTGTGATTAA GCA; ARE1-m, TATTTCAATTCTGTGATTAAAGCA; ARE2-m, TGCT TCATCACTGGTTTAAACTA; ARE1/2-m, TATTTCAATTCTGGTTT AAACATA. Mouse *Gbe1* cDNA was amplified from mouse SkM cDNA by RT-PCR with PrimeSTAR (TaKaRa) and then cloned into the pF9A expression vector (*Gbe1*-pF9A; Promega).

Transfection assays. For reporter transfection experiments, 1×10^5 C2C12 myoblasts were seeded into 24-well plates and transfected with 500 µg of *Gbe1* –993/+180-luc and 100 µg of Tk-pRL (thymidine kinase promoter-driven *Renilla* luciferase) with X-tremeGENE 9 (Roche) according to the manufacturer's instructions. For stable transfections, 1×10^5 C2C12 myoblasts were seeded into six-well plates and transfected with 200 µg of either *Gbe1*-pF9A or empty pF9A and selected with 1,000 µg/ml G418.

Glycogen content. We measured mouse SkM, mouse liver, and C2C12 myotube glycogen contents by the phenol-sulfuric acid method (39). One hundred to 150 mg of mouse SkM (soleus and gastrocnemius muscles) and liver was used for measurements. Differentiated C2C12

myotubes cultured in 100-mm dishes were treated with either 100 nmol/liter CDDO-Im or 0.1% DMSO for 48 h, washed three times with PBS, and collected by scraping in PBS.

Samples were transferred into 500 µl of Na₂SO₄-saturated 30% KOH solution and heated at 100°C for 8 min to achieve complete lysis. Ethanol (95%, 600 µl) was added to the samples, and the samples were then incubated on ice for 5 min before centrifugation at $840 \times g$ for 15 min. The supernatants were removed, and the glycogen precipitates were resuspended in distilled water (200 µl for SkM, 1,000 µl for liver, 50 µl for C2C12 myotubes). Aliquots of glycogen solution (50 µl for SkM and C2C12 myotubes, 5 µl of sample plus 45 µl of H₂O for liver) were mixed with 50 µl of a 5% phenol solution. Concentrated sulfuric acid (250 µl) was then rapidly added, and absorbance at 490 nm was measured with a plate reader.

Glycogen release of G1P. Two aliquots of a purified glycogen solution (40 µg of glycogen in 80 µl of H₂O) were added to 100 µl of a reaction cocktail (100 mmol/liter KH₂PO₄, 3 mmol/liter MgCl₂, 0.2 mmol/liter EDTA, 10 µg/ml α-D-glucose 1,6-bisphosphate potassium salt hydrate [Sigma], 1 mmol/liter β-NADP [Wako Pure Chemicals], 2 U/ml glucose-6-phosphate [G6P] dehydrogenase [Sigma], 2 U/ml phosphoglucomutase [Sigma]) with oyster glycogen (Wako Pure Chemicals) as a positive control. The absorbance at 340 nm (*A*₃₄₀) was measured every minute for 5 min at 30°C. The samples were then mixed with 20 µl of an enzyme solution (40 mmol/liter β-glycerophosphate and 80 mmol/liter cysteine solution [pH 6.8] with or without 1 U/ml phosphorylase *a* [Sigma]), and the *A*₃₄₀ was measured for an additional 30 min. Glucose-1-phosphate (G1P) release was determined from the concentration of NADP (NADPH) by measuring the difference in *A*₃₄₀ in the presence and absence of phosphorylase *a*.

2-DG uptake. For the *in vivo* study, after mice were fasted for 16 h, 2-deoxyglucose (2-DG) was intraperitoneally administered (1 g/kg of body weight). Mice were then refed, and SkM was freshly removed from mice after 4 h of 2-DG administration. Intracellular 2-DG–6-phosphate levels in SkM were measured by utilizing capillary electrophoresis time of flight mass spectrometry (CE-TOF/MS) as previously reported (40). For the *in vitro* study, differentiated C2C12 myotubes were cultured in six-well plates, supplied with serum-free DMEM containing 25 mmol/liter glucose, incubated for 6 h, and then supplied with Krebs-Ringer-phosphate-HEPES buffer (pH 7.5) supplemented with 2% bovine serum albumin. After 1 h, 2-DG (1 mmol/liter) was added and the cells were further incubated for 30 min before intracellular 2-DG–6-phosphate levels were measured with a 2-DG uptake measurement kit (Cosmo Bio Co., Ltd.).

Statistical analysis. All data are presented as the mean ± the standard error of the mean (SEM). Statistical analysis was performed by either Student's *t* test or analysis of variance, followed by Fisher's least significant difference *post hoc* test for multiple comparisons.

Nucleotide sequence accession number. ChIP-Seq data were deposited in the Gene Expression Omnibus database under accession no. GSE78780.

RESULTS

SkM-specific Nrf2 expression improves glucose tolerance. We previously generated one line of *Keap1*^{MuKO} mice (18, 34) to conditionally delete exons 4 to 6 of *Keap1* (19, 31). However, the *Keap1* flox allele unexpectedly expressed *Keap1* mRNA less than the wild-type allele did, and the decrease in *Keap1* expression activated Nrf2 signaling (41), hampering our attempts to specifically evaluate SkM glucose metabolism. Consequently, we also utilized another line of *Keap1* knockout mice with a conditional deletion of exons 2 and 3 (32, 42). An important difference between these two floxed mouse lines is that the former exhibits a mild knock-down phenotype before Cre-mediated deletion while the latter does not. Both lines of *Keap1* floxed mice were used in this study and are referred to as *Keap1*^{1FA} and *Keap1*^{1FB}, respectively.

FIG 1 Glucose metabolism in mice with SkM-specific Nrf2 induction. (A) Blood glucose levels and AUCs measured by ipGTT in *Keap1FB/FB* and *Keap1MuKO-B* mice ($n = 8$ or 9). Glucose (2 g/kg of body weight) was administered intraperitoneally to 9-week-old male mice after 16 h of fasting. (B) Blood glucose levels of *Keap1FB/FB* and *Keap1MuKO-B* mice either fed *ad libitum* or under fasting conditions ($n = 9$). (C) Plasma insulin levels of 9-week-old male *Keap1FB/FB* and *Keap1MuKO-B* mice ($n = 8$ or 9) fed *ad libitum*. (D) Body weights of 9-week-old male *Keap1FB/FB* and *Keap1MuKO-B* mice ($n = 9$). (E) *Keap1* mRNA expression in SkM and liver tissues from *Keap1FB/FB* and *Keap1MuKO-B* mice ($n = 9$). The data were normalized to *Hprt*, and the expression levels in *Keap1FB/FB* mice were set as 1. (F) Blood glucose levels and AUCs measured by ipGTT in *Nrf2F/F::Keap1FB/FB* and *Nrf2F^{del}/F^{del}::Keap1MuKO-B* mice ($n = 7$). Glucose (2 g/kg of body weight) was intraperitoneally administered to 9-week-old male mice after 16 h of fasting. (G) Blood glucose levels of *Nrf2F/F::Keap1FB/FB* and *Nrf2F^{del}/F^{del}::Keap1MuKO-B* mice either fed *ad libitum* or under fasting conditions ($n = 7$). (H) Body weights of 9-week-old male *Nrf2F/F::Keap1FB/FB* and *Nrf2F^{del}/F^{del}::Keap1MuKO-B* mice ($n = 7$). The data were normalized to *Hprt*, and the expression levels in *Nrf2F/F::Keap1FB/FB* mice were set as 1. Error bars show the mean $\pm$ SEM. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ (versus the control). All of the data were collected from ICR background mice.

We first performed an ipGTT with *Keap1MuKO-B* mice. These mice exhibited significant repression of blood glucose and the AUC (area under the curve) compared with control *Keap1FB/FB* mice lacking Cre (Fig. 1A). Under fed conditions, *Keap1MuKO-B*

mice had significantly lower blood glucose and plasma insulin levels than control *Keap1FB/FB* mice (Fig. 1B and C). Additionally, *Keap1MuKO-B* mice had slightly lower body weights than *Keap1FB/FB* mice (Fig. 1D). As expected, *Keap1* mRNA levels

FIG 2 Energy consumption in mice with SkM-specific Nrf2 induction. (A) CT images of 28-week-old male *Keap1FB/FB* and *Keap1MuKO-B* mice. Note that the epididymal fat masses of these mice were almost comparable. (B) Estimated whole-body lean and fat mass volumes by HU in the continuous-slice CT images ($n = 5$). (C to E) Tissue weights (C), oxygen consumption (D), and mitochondrial DNA content (E) in dissected SkM ($n = 5$). Oxygen consumption levels were normalized to tissue weight (D). The mitochondrial DNA content data were normalized to genomic DNA content, and the average content of *Keap1FB/FB* mice was set as 1 (E). Error bars show the mean $\pm$ SEM. *, $P < 0.05$ (versus the control); n.s., not significant. All of the data were collected from ICR background mice.

were markedly reduced in SkM from *Keap1MuKO-B* mice but were unchanged in the livers (Fig. 1E). These results indicate that the SkM-specific *Keap1* knockout in *Keap1MuKO-B* mice significantly improved glucose tolerance.

We also performed an ITT with *Keap1MuKO-B* mice. Blood glucose levels after 60 min of insulin administration and the AUC were decreased in *Keap1MuKO-B* mice compared with control *Keap1FB/FB* mice (see Fig. S1 in the supplemental material). These data are consistent with our previous data obtained with *Keap1MuKO-A* mice fed a high-fat diet (18) and indicate that Nrf2 induction enhances insulin sensitivity.

To determine whether the *Keap1* knockout-mediated improvement in glucose tolerance could be attributed to Nrf2, we crossed conditional *Nrf2* knockout (*Nrf2F/F*) mice (33) with *Keap1MuKO-B* mice to generate *Nrf2F^{del}/F^{del}::Keap1MuKO-B* and control *Nrf2F/F::Keap1FB/FB* mice. As measured by ipGTT, *Nrf2F^{del}/F^{del}::Keap1MuKO-B* mice displayed mildly elevated blood glucose levels (Fig. 1F). *Nrf2F^{del}/F^{del}::Keap1MuKO-B* mice also exhibited increased blood glucose levels under fasting conditions (Fig. 1G). In contrast, the mice had comparable body weights (Fig. 1H). *Keap1* and *Nrf2* expression levels were reduced in SkM from *Nrf2F^{del}/F^{del}::Keap1MuKO-B* mice (Fig. 1I). These results support our hypothesis that the reduction in blood glucose levels observed in *Keap1MuKO-B* mice required Nrf2.

As *Keap1FA/FA* mice exhibited both reduced *Keap1* expression and Nrf2 induction independently of the presence of Cre recombinase, we measured the levels of expression of *Keap1* and *Nqo1*, a faithful Nrf2 target gene (6), in *Keap1FB/FB* mice. *Keap1FB/FB*, *Keap1FB/+*, and wild-type mice exhibited comparable *Keap1* and *Nqo1* expression levels in their SkM, livers, and brains and had similar body weights (see Fig. S2A and B in the supplemental material). In contrast, *Keap1FA/-* mouse body weight gains were substantially suppressed (41). While the body weights of *Keap1FA/-* mice were lower than those of *Keap1FA/+* mice, this reduction in body weight was not observed in *Keap1FB/-* mice (see Fig. S2C). These results indicate that the *Keap1FB* allele does not produce a *Keap1* hypomorphic phenotype.

SkM-specific Nrf2 induction increases energy consumption. As body weights were decreased in *Keap1MuKO-B* mice, we evaluated their systemic lean and fat mass volumes by CT. While obvious morphological changes in CT images were not observed (Fig. 2A), the estimated systemic lean mass volumes, but not fat mass volumes, were decreased in *Keap1MuKO-B* mice (Fig. 2B). These data indicate that the decrease in body weight is due to the decline in lean mass volume.

To evaluate whether energy consumption is changed in *Keap1MuKO-B* mouse SkM, we dissected SkM from the mice. The

FIG 3 Glucose metabolism in mice with SkM-specific Nrf2 deletion. (A) Blood glucose levels and AUCs measured by ipGTT in *Nrf2F/F* and *Nrf2MuKO* mice ($n = 6$). Glucose (2 g/kg of body weight) was administered intraperitoneally to 8-week-old male mice after 16 h of fasting. (B) Blood glucose levels of *Nrf2F/F* and *Nrf2MuKO* mice either fed *ad libitum* or under fasting conditions ($n = 6$). (C) Body weights of *Nrf2F/F* and *Nrf2MuKO* mice ($n = 6$). Error bars show the mean $\pm$ SEM. **, $P < 0.01$; *, $P < 0.05$ (versus *Nrf2F/F* mice). All of the data were collected from C57BL/6J background mice.

SkM tissue weights of *Keap1MuKO-B* mice were lower than those of *Keap1FB/FB* mice (Fig. 2C), consistent with the estimated lean mass volumes in CT images. We next measured oxygen consumption in the dissected SkM and found that oxygen consumption in *Keap1MuKO-B* mouse SkM was higher than that in *Keap1FB/FB* mouse SkM (Fig. 2D). We also evaluated the mitochondrial DNA content of dissected SkM. The mitochondrial DNA content was comparable in *Keap1MuKO-B* and *Keap1FB/FB* mouse SkM (Fig. 2E), indicating that Nrf2 induction in SkM increases energy consumption without an increase in mitochondrial DNA content.

SkM-specific Nrf2 deletion aggravates glucose tolerance. To evaluate the contributions of Nrf2 to glucose metabolism, we generated *Nrf2F^{del}/F^{del}::MuCre* (*Nrf2MuKO*) mice. We performed ipGTT of the *Nrf2MuKO* mice and found that the blood glucose levels were higher in *Nrf2MuKO* mice than in *Nrf2F/F* mice after 60 and 120 min of glucose administration (Fig. 3A). The AUC of blood glucose during the ipGTT was also elevated in *Nrf2MuKO* mice (Fig. 3A). Under fed conditions, *Nrf2MuKO* mice displayed higher blood glucose levels than *Nrf2F/F* mice did (Fig. 3B). In contrast, fasting blood glucose levels in *Nrf2MuKO* mice were comparable to those of control mice (Fig. 3B), while *Nrf2F^{del}/F^{del}::Keap1MuKO-B* mice showed an elevation of blood glucose under fasting conditions (Fig. 1G). *Nrf2MuKO* mice had mildly lower body weights than control *Nrf2F/F* mice (Fig. 3C). These results indicate that the SkM-specific Nrf2 knockout mildly aggravates glucose tolerance and increases body weight.

Nrf2 regulates the expression of genes related to glycogen metabolism. As we were particularly interested in determining how glucose tolerance is improved in *Keap1MuKO-B* mice, we examined the expression profiles of Nrf2 target genes that regulate glucose metabolism in *Keap1MuKO-B* and *Keap1FB/FB* control mice. Through microarray analysis, we identified 111 genes that were upregulated and 102 genes that were downregulated in SkM from *Keap1MuKO-B* mice compared to SkM from *Keap1FB/FB* mice (see Table S3 in the supplemental material). We also performed gene ontology analyses with GeneSpring software and found that Nrf2 induction upregulates oxidation-reduction-related gene groups and downregulates seven groups of genes (see Table S4). Notably, we identified two glycogen metabolism-related genes, *Gbe1* and *Phka1*, that were upregulated in *Keap1MuKO-B* mice (see Table S3).

Using qPCR to validate our microarray results, we observed that *Gbe1*, *Phka1*, and *Nqo1* expression levels were all significantly increased in SkM from *Keap1MuKO-B* mice (Fig. 4A). Importantly, concomitant Nrf2 knockout in *Nrf2F^{del}/F^{del}::Keap1MuKO-B* mice severely abrogated the increased mRNA expression levels.

We previously reported that *Keap1FA/FA* and *Keap1FA/FA::Cre* mice displayed graded declines in *Keap1* expression (41). In this study, we exploited this trait to determine how graded Nrf2 induction impacts SkM glycogen metabolism. Indeed, *Keap1* expression was repressed in SkM from *Keap1FA/FA* mice and was further reduced in *Keap1MuKO-A* mice (Fig. 4B). Similarly, *Nqo1* expression was incrementally increased in *Keap1FA/FA* and *Keap1MuKO-A* mice (Fig. 4B). These data indicated that we could exploit the *Keap1MuKO-A* mouse line to generate graded reductions in SkM *Keap1* expression. Using these mice, we measured *Gbe1* and *Phka1* expression levels and observed that *Gbe1* mRNA expression was incrementally enhanced in *Keap1FA/FA* and *Keap1MuKO-A* mice and that *Nqo1* levels exhibited a very similar pattern (Fig. 4B). *Phka1* mRNA expression was also induced in SkM from *Keap1MuKO-A* mice; however, the induction was less dramatic, suggesting that *Phka1* induction requires a strong Nrf2 signal.

We next evaluated the contribution of Nrf2 to *Gbe1* and *Phka1* mRNA expression by using *Nrf2MuKO* mice. In SkM from *Nrf2MuKO* mice, *Nrf2*, *Gbe1*, *Phka1*, and *Nqo1* expression levels were significantly reduced (Fig. 4C). GBE, PhK α 1, and NQO1 protein levels were markedly increased in SkM from *Keap1MuKO-B* mice (Fig. 4D), while GBE, PhK α 1, and NQO1 baseline expression levels were reduced in SkM from *Nrf2MuKO* mice (Fig. 4E), indicating that Nrf2 induces the expression of the genes encoding GBE and PhK α 1.

Nrf2 induction reduces SkM glycogen content. As Nrf2 induction ameliorates SkM glucose utilization (18), we asked whether *Gbe1* and *Phka1* induction resulted in increased SkM glycogen content or steady-state G1P utilization. To answer this question, we measured SkM glycogen contents under fasting and fasting-and-refeeding (F/R) conditions (summarized in Fig. 5A). While the glycogen content was elevated in SkM from control *Keap1FB/FB* mice after 12 h of fasting and 6 h of refeeding, the increase was substantially abrogated in SkM from *Keap1MuKO-B* mice (Fig. 5B). Furthermore, *Keap1MuKO-B* mice did not have significantly increased glycogen content, indicating that Nrf2-mediated induction of GBE and PhK α 1 instead reduces SkM glycogen content.

We also evaluated the expression of additional glycogen metabolism-related genes in SkM from *Keap1MuKO-B* mice. Levels

FIG 4 Nrf2 regulates the expression of glycogen metabolism-related enzymes. (A) *Gbe1*, *Phka1*, and *Nqo1* mRNA expression levels in SkM from 9-week-old male *Keap1FB/FB*, *Keap1MuKO-B*, *Nrf2F/F::Keap1FB/FB*, and *Nrf2F^{del}/F^{del}::Keap1MuKO-B* mice ($n = 6$ to 9). (B) *Keap1*, *Gbe1*, *Phka1*, and *Nqo1* mRNA expression levels in SkM from 9-week-old male *Keap1FA/+*, *Keap1FA/FA*, and *Keap1MuKO-A* mice ($n = 6$). (C) *Nrf2*, *Gbe1*, *Phka1*, and *Nqo1* mRNA expression levels in SkM from 6-week-old male *Nrf2F/F* and *Nrf2MuKO* mice ($n = 4$). The data were normalized to *Hprt*, and the expression level in each control was set as 1. (D, E) Immunoblot analysis of GBE, PhK α 1, NQO1, and α -tubulin (TUB) in SkM from 9-week-old male *Keap1FB/FB* and *Keap1MuKO-B* mice ($n = 6$) (D) and 6-week-old *Nrf2F/F* and *Nrf2MuKO* mice ($n = 6$ or 7) (E). Protein expression was quantified and normalized to TUB. The expression level in each control was set as 1. Error bars show the mean $\pm$ SEM. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ (versus the control). All of the data were collected from ICR background mice.

of the mRNAs encoding the PhK β subunit (*Phkb*), the PhK γ subunit (*Phkg1*), amylo-1,6-glucosidase and 4- α -glucanotransferase (*Agl*), glycogen synthase (*Gys1*), phosphoglucomutase (*Pgm2*), and UDP-glucose pyrophosphorylase (*Ugp2*) were comparable in SkM from *Keap1MuKO-B* and *Keap1FB/FB* mice (data not shown). With these results in mind, we hypothesized that *Gbe1* and *Phka1* induction facilitated glycogen utilization by activating both the branching enzyme and phosphorylase.

In this scenario, we expect to observe a reduction in the number of glucose molecules in the outer layers of glycogen that can be rapidly utilized by phosphorylase (4). To test this hypothesis, we examined rabbit phosphorylase α -mediated release of G1P from *Keap1MuKO* mouse muscle glycogen. Indeed, G1P release was much weaker in *Keap1MuKO-B* mice than in *Keap1FB/FB* mice (Fig. 5C, left side). Consistent with this finding, the rate of G1P release in *Keap1MuKO-B* mice was strongly reduced (Fig. 5C, right side). These results support our contention that Nrf2 induction reduces steady-state SkM glycogen levels and promotes SkM glucose uptake (see below) and utilization.

Nrf2 differentially regulates SkM and liver glycogen metabolism. The liver and SkM, two major organs responsible for glycogen metabolism (4), are known to store and utilize glycogen differently. We examined liver glycogen metabolism by crossing *Keap1FB* mice with *Alb-Cre* mice to generate liver-specific *Keap1* knockout mice, referred to as *Keap1LKO-B* (*Keap1FB/FB::Alb-Cre*) mice. In *Keap1LKO-B* mouse livers, *Nqo1* and *Gbe1* expression levels were inversely related to *Keap1* expression (Fig. 6A).

This trend was recapitulated in another mouse line, *Keap1LKO-A* (*Keap1FA/FA::Alb-Cre*), in which incremental reductions in *Keap1* expression in *Keap1FA/FA* and *Keap1LKO-A* mouse livers were associated with incremental increases in *Nqo1* and *Gbe1* mRNA expression levels in *Keap1LKO* (Fig. 6B).

To measure liver glycogen content, we used the protocol described in Fig. 6C. Under F/R conditions, *Keap1FB/FB*, *Keap1LKO-B*, *Keap1FA/+*, and *Keap1LKO-A* mice had greater liver glycogen contents, and both *Keap1LKO-B* and *Keap1LKO-A* mice exhibited greater liver glycogen content than the respective *Keap1FB/FB* and *Keap1FA/+* control mice (Fig. 6D). In contrast, the blood glucose levels in these mice were not different under F/R conditions (Fig. 6E). As measured by ipGTT, *Keap1LKO-B* and *Keap1FB/FB* mice had comparable blood glucose levels (Fig. 6F). These results indicate that although Nrf2 induction led to increased liver glycogen content, this increase did not substantially contribute to an improvement in glucose tolerance, further supporting our contention that the liver and SkM metabolize glycogen differently.

Nrf2 in the liver contributes to the maintenance of blood glucose levels during fasting. Because glycogen in the liver plays a critical role in the maintenance of blood glucose levels during fasting, we evaluated the roles of Nrf2 on blood glucose regulation under fasting conditions. We first determined the effects of F/R cycles on the expression of the *Gbe1* and *Phka1* mRNAs by using the protocol demonstrated in Fig. S3A in the supplemental material. To confirm a successful F/R cycle, we examined glycogen content and *Fasn* mRNA expression in the liver. The glycogen content and *Fasn* expression were repressed during fasting and induced under F/R conditions (see Fig. S3B and C). We also evaluated *Gbe1* expression and found that *Gbe1* mRNA expression was strongly repressed during fasting and was dramatically increased by refeeding (see Fig. S3C). *Nqo1* expression was mildly repressed during fasting and induced under F/R conditions in the liver (see Fig. S3C). While the *Nqo1* mRNA level in SkM was higher under both fasting and F/R conditions than under the fed condition, *Gbe1* and *Phka1* gene expression in SkM was not altered during F/R cycles (see Fig. S3D). These data indicate that *Gbe1* expression is regulated during F/R cycles in the liver.

We next determined whether Nrf2 contributes to blood glucose regulation during fasting. We performed time course studies of fasting with 32-week-old *Keap1LKO-B* and *Keap1MuKO-B* mice and measured blood glucose levels at various time points. Interestingly, blood glucose levels were higher in *Keap1LKO-B* mice than in *Keap1FB/FB* mice at 8 and 16 h of fasting (see Fig. S4A in the supplemental material). In contrast, *Keap1MuKO-B* mice displayed lower blood glucose levels than *Keap1FB/FB* mice at 0, 8, and 16 h of fasting (see Fig. S4B). These data indicate that Nrf2 in the liver contributes to the maintenance of blood glucose levels.

In vitro chemical Nrf2 induction. We next asked whether chemical-based Nrf2 induction also promoted *Gbe1* and *Phka1* gene expression. To answer this question, we treated C2C12 myotubes with the chemical Nrf2 inducers DEM, tBHQ, SFN, and CDDO-Im. All four Nrf2 inducers promoted *Gbe1* and *Nqo1* mRNA expression in C2C12 myotubes, and tBHQ, SFN, and CDDO-Im promoted *Phka1* mRNA expression (Fig. 7A). Furthermore, CDDO-Im induced *Gbe1*, *Phka1*, and *Nqo1* expression in a dose-dependent manner (Fig. 7B).

CDDO-Im-mediated induction of *Gbe1* and *Phka1* expres-

FIG 6 Nrf2 and glycogen metabolism in the liver. (A and B) *Keap1*, *Nqo1*, and *Gbe1* mRNA expression profiles in the livers of 15-week-old male *Keap1*^{FB/FB} and *Keap1*^{LKO-B} mice (A; *n* = 6) and *Keap1*^{FA/+}, *Keap1*^{FA/FA}, and *Keap1*^{LKO-A} mice (B; *n* = 6). The data were normalized to *Gapdh*, and the expression levels in the controls were set as 1. (C) Protocol for evaluation of liver glycogen metabolism. (D and E) Liver glycogen content (D) and blood glucose levels (E) of *Keap1*^{FB/FB} mice under fasting (*n* = 3) and F/R conditions (*n* = 8), in *Keap1*^{MuKO-B} mice under F/R conditions (*n* = 8), in *Keap1*^{FA/+} mice under fasting and F/R conditions, and in *Keap1*^{MuKO-A} mice under F/R conditions (*n* = 6). Glycogen content was normalized to tissue weight. (F) Blood glucose levels of *Keap1*^{LKO-B} and *Keap1*^{FB/FB} mice (*n* = 10 or 11) as measured by ipGTT. Glucose (2 g/kg of body weight) was intraperitoneally administered to 9-week-old male mice after 16 h of fasting. Error bars show the mean ± SEM. ***, *P* < 0.001; **, *P* < 0.01; *, *P* < 0.05. *n. s.*, not significant. All of the data were collected from ICR background mice.

sion began as early as after approximately 3 h of incubation and peaked at 12 h (Fig. 7C), while treatment with DMSO (vehicle) did not affect *Gbe1* and *Phka1* mRNA expression (Fig. 7C). *Nqo1* expression levels began to increase 3 h after CDDO-Im addition and gradually increased over the 24-h incubation period (Fig. 7C).

We also evaluated the effects of Nrf2-inducing chemicals on C2C12 myotube glycogen content. In contrast to our results with livers (Fig. 6D), CDDO-Im reduced C2C12 myotube glycogen

content (see Fig. S5 in the supplemental material). These results indicate that chemical Nrf2 inducers also promote *Gbe1* and *Phk1* expression and negatively regulate glycogen content in C2C12 myotubes.

In vivo CDDO-Im induction of Nrf2. We next examined whether Nrf2 chemical inducers could promote *Gbe1* and *Phk1* mRNA expression *in vivo* by orally administering CDDO-Im to wild-type and *Nrf2* knockout (6) mice. After CDDO-Im treatment, *Gbe1*, *Phk1*, and *Nqo1* mRNA levels were significantly in-

FIG 7 Effects of chemical Nrf2 induction on *Gbe1* and *Phka1* expression. (A) Effects of Nrf2-inducing chemicals on *Gbe1*, *Phka1*, and *Nqo1* gene expression. C2C12 myotubes were treated with 0.1% DMSO (vehicle [Veh]), 100 μ Mol/liter DEM, 50 μ Mol/liter tBHQ, 10 μ Mol/liter SFN, or 100 nmol/liter CDDO-Im for 24 h. The data were normalized to *Hprt*, and the expression levels in vehicle-treated cells were set as 1 ($n = 4$ each). (B) Dose-response analysis. C2C12 myotubes were treated with either 0.1% DMSO (vehicle in dose 0) or CDDO-Im for 24 h. The data were normalized to *Hprt*, and the expression levels in the vehicle-treated groups were set as 1 ($n = 4$ each). (C) Time course analysis. C2C12 myotubes were treated with either 0.1% DMSO or 100 nmol/liter CDDO-Im for the times indicated. The data were normalized to *Hprt*, and the expression levels in the time zero groups were set as 1 ($n = 4$ each). Error bars show the mean $\pm$ SEM. ***, $P < 0.001$; *, $P < 0.05$ (versus the control).

creased in the SkM of wild-type mice (Fig. 8A). Similarly, CDDO-Im induced *Gbe1* and *Nqo1* mRNA expression in wild-type mouse livers (Fig. 8B). These effects were nearly fully abrogated by simultaneous *Nrf2* knockout (Fig. 8). These results unequivocally demonstrate that Nrf2 regulates *Gbe1* and *Phka1* mRNA expression in mouse SkM and liver.

Nrf2 enhances exercise capacity. To evaluate the role of Nrf2 in exercise capacity, we conducted a treadmill test with mice treated by oral administration of CDDO-Im by using the protocols described in Fig. 9A and B. CDDO-Im increased their maximum running speed and distance on the treadmill compared to the vehicle control (Fig. 9C). We also measured blood glucose levels and found that CDDO-Im decreased glucose levels compared to the vehicle control (Fig. 9D).

We next determined the effect of CDDO-Im on glycogen content in SkM and liver. The SkM tissue weights of the CDDO-Im and vehicle groups were comparable (Fig. 9E). We also measured

the glycogen contents of SkM and liver. While CDDO-Im decreased the glycogen content of SkM before treadmill exercise, the glycogen content was strongly decreased after treadmill exercise in both the CDDO-Im and vehicle groups (Fig. 9F). In contrast, CDDO-Im significantly increased the glycogen content of livers before treadmill exercise, and CDDO-Im slightly increased the content after treadmill exercise (Fig. 9F). These data indicate that the glycogen content is decreased in SkM and increased in the liver by chemical Nrf2 induction.

We also determined the expression of Nrf2 target genes in SkM and found that CDDO-Im increased the expression of *Gbe1* and *Nqo1* mRNAs both before and after treadmill exercise (Fig. 9G). While CDDO-Im enhanced *Phka1* mRNA expression in SkM before treadmill exercise, it failed to increase its expression after exercise (Fig. 9G). The reason for the loss of *Phka1* induction after exercise is unclear. These data indicate that chemical Nrf2 induction activates Nrf2 signaling both before and after forced exercise

FIG 8 CDDO-Im effects on *Gbe1* and *Phka1* in vivo. (A, B) Time course analysis of the effects of CDDO-Im administration in mouse SkM (A) and liver (B). Wild-type (WT) and Nrf2 knockout mice received either CDDO-Im (30 μ mol/kg of body weight) or the vehicle orally, and SkM and livers were collected at the times indicated ($n = 3$ each for 3 and 6 h, $n = 4$ for 12 h). CDDO-Im (30 μ mol/kg of body weight) and the vehicle were readministered to the 12-h groups 6 h after the first administration. The data were normalized to *Hprt*, and the expression levels in the vehicle-treated wild-type mice were set as 1. Error bars show the mean $\pm$ SEM. ***, $P < 0.001$; **, $P < 0.01$ (versus the vehicle-treated wild type). All of the data were collected from C57BL/6J background mice.

and regulates *Gbe1* and *Phka1* expression, except for the *Phka1* expression after exercise.

Regulation of *Gbe1* and *Phka1* transcription. To determine how Nrf2 regulates *Gbe1* transcription in SkM, we performed ChIP-seq analysis of chromatin samples from CDDO-Im-treated C2C12 myotubes with an anti-Nrf2 antibody. This analysis revealed that Nrf2 binds two regulatory regions, one 0.4 kb upstream and one 15 kb downstream of the *Gbe1* transcription start site (TSS) (Fig. 10A). These *Gbe1* regulatory regions contain three highly conserved AREs; the 0.4-kb upstream region contains ARE1 and ARE2, while the 15-kb downstream region contains ARE3 (43). Importantly, these motifs are conserved among primates and rodents and ARE1 is more similar to the ARE consensus sequence than to either ARE2 or ARE3 (see Fig. S6A and B in the supplemental material).

We validated Nrf2 binding by manual ChIP analysis and confirmed that Nrf2 binding to the AREs in the *Gbe1* 0.4-kb upstream region and *Nqo1*-ARE (a positive locus) markedly increased in C2C12 myotubes following a 3-h CDDO-Im treatment (Fig. 10B). In contrast, although Nrf2 bound the *Gbe1* 15-kb downstream region, CDDO-Im treatment did not significantly enhance binding (Fig. 10B). Nrf2 accumulated in C2C12 myotube nuclei following the 3-h CDDO-Im treatment (Fig. 10C).

To further examine the contributions of ARE1 and ARE2 to *Gbe1* transcriptional activity, we linked genomic regions proximal to the *Gbe1* TSS (−993 to +180) to cDNA encoding luciferase (Fig. 10D). Following reporter transfection, addition of CDDO-Im to C2C12 myoblast culture medium incremen-

tally increased luciferase reporter activity (Fig. 10E). These results indicate that the regulatory region proximal to *Gbe1* contains CDDO-Im-responsive ARE elements.

We also prepared a series of mutant reporters. Mutation of either ARE1 (ARE1-m) or ARE2 (ARE2-m) markedly attenuated CDDO-Im-mediated induction of luciferase activity, and reporter activity was completely abrogated by the simultaneous mutation of ARE1 and ARE2 (ARE1/2-m) (Fig. 10F). These results demonstrate that both ARE1 and ARE2 play a critical role in *Gbe1* gene regulation.

Phylogenetic inspection of *Phka1* regulatory elements revealed a highly conserved ARE 1.6 kb upstream of the TSS. This ARE is conserved among primates, rodents, dogs, and horses (see Fig. S6C). In our manual ChIP analysis, Nrf2 binding to the *Phka1* ARE increased in C2C12 myotubes following 3 h of CDDO-Im treatment (Fig. 10G), suggesting that Nrf2 may bind to the ARE and regulate *Phka1* expression.

Nrf2 increases glucose uptake in SkM. Because we have reported that glucose utilization is increased in the global Nrf2 induction model *Keap1FA*− mice (18), we evaluated whether Nrf2 enhances glucose uptake in SkM. We measured 2-DG–6-phosphate levels in SkM after 4 h of 2-DG administration by CE-TOF/MS (40). Intracellular 2-DG–6-phosphate levels in SkM were significantly higher in *Keap1FA*− mice than in *Keap1FA*+/+ mice (Fig. 11A), indicating that Nrf2 increases glucose uptake in SkM.

To determine the importance of SkM in blood glucose regulation in *Keap1FA*− mice (18), we crossed *Keap1FA*− and

FIG 9 Effect of Nrf2 induction on exercise capacity. (A and B) Protocols for CDDO-Im administration (A) and the treadmill test (B) to estimate exercise capacity. (C) Treadmill test results of 8-week-old male mice after treatment with CDDO-Im or the vehicle ($n = 6$ or 7). Maximum running speed and distance were determined. (D) Blood glucose levels after the treadmill test ($n = 6$ or 7). (E to G) SkM tissue weights (E); glycogen contents in SkM and liver (F); and expression levels of *Gbe1*, *Phka1*, and *Nqo1* in SkM (G) before and 1 day after treadmill exercise ($n = 6$ or 7). The glycogen contents were normalized to tissue weights (F). The mRNA data were normalized to *Hprt*, and the expression levels in the vehicle-treated pretreadmill mice were set as 1. Error bars show the mean $\pm$ SEM. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ (versus vehicle-treated mice). All of the data were collected from C57BL/6J background mice.

Nrf2^{MuKO} mice to generate *Keap1*^{FA/+::Nrf2}^{F/F}, *Keap1*^{FA/-::Nrf2}^{F/F}, and *Keap1*^{FA/-::Nrf2}^{MuKO} mice as described in Fig. 11B. *Keap1* mRNA expression was moderately decreased and *Nqo1* mRNA expression was induced in *Keap1*^{FA/-::Nrf2}^{F/F} mice compared with *Keap1*^{FA/+::Nrf2}^{F/F} mice (see Fig. S7 in the supplemental material). In *Keap1*^{FA/-::Nrf2}^{MuKO} mice, the expression of both *Keap1* and *Nrf2* was severely repressed, resulting in a decrease in *Nqo1* expression (see Fig. S7). We next performed ipGTT. *Keap1*^{FA/-::Nrf2}^{F/F} mice had lower blood glucose levels

than control *Keap1*^{FA/+::Nrf2}^{F/F} mice (Fig. 11C). Importantly, blood glucose levels during the ipGTT were strongly elevated in *Keap1*^{FA/-::Nrf2}^{MuKO} mice (Fig. 11C). These data support our contention that Nrf2 in SkM plays critical roles in lowering the effects of blood glucose. We also determined expression levels in SkM and found that *Gbe1* expression was much greater in *Keap1*^{FA/-::Nrf2}^{F/F} mice than in control mice (Fig. 11D). In contrast, *Phka1* expression was only slightly greater in *Keap1*^{FA/-::Nrf2}^{F/F} mouse SkM (Fig. 11D), the expression of both *Gbe1* and

FIG 10 Nrf2 transcriptional regulation of *Gbe1* and *Phka1* expression. (A) Screen shots of ChIP-seq profiles around the *Gbe1* and *Nqo1* TSSs from two independent experiments (Exp.) with CDDO-Im (100 nmol/liter, 3 h)-treated C2C12 myotubes and anti-Nrf2 antibody. (B) Manual ChIP analysis of *Gbe1* (kb -0.4 and kb +15 from the TSS) and *Nqo1* (a positive locus). Nrf2-DNA complexes were immunoprecipitated with either anti-Nrf2 antibody ($n = 4$) or control IgG ($n = 3$) from nuclear extracts of C2C12 myotubes cultured with either 0.1% DMSO or 100 nmol/liter CDDO-Im for 3 h. The data are percentages of the input DNA. (C) Immunoblot analysis of Nrf2 and lamin B from nuclear extracts of C2C12 myotubes cultured in 0.1% DMSO or 100 nmol/liter CDDO-Im for 3 h. (D) Schematic of the chimeric reporter containing the 5'-flanking region of the mouse *Gbe1* gene and luciferase cDNA. SV40, simian virus 40. (E and F) Luciferase reporter analysis in C2C12 myoblasts. C2C12 myoblasts were transiently transfected with reporter vectors and cultured for 24 h with the concentrations of CDDO-Im indicated. Analysis of luciferase reporter expression in response to various doses of CDDO-Im (E) and mutational analysis of *Gbe1* AREs (F). The transcriptional activities at dose 0 (E, 0.1% DMSO vehicle) and of the vehicle-treated wild-type reporter (F) were set as 1 ($n = 4$). Data are relative firefly luciferase activity (test) normalized to *Renilla* luciferase activity (control). (G) Manual ChIP analysis of *Phka1* (kb -1.6 from the TSS) in nuclear extracts from C2C12 myotubes cultured with 0.1% DMSO or 100 nmol/liter CDDO-Im for 3 h with anti-Nrf2 antibody ($n = 4$) or control IgG ($n = 3$). The data are the percentages of the input DNA. Error bars show the mean $\pm$ SEM. ***, $P < 0.001$ (versus the vehicle).

FIG 11 Nrf2 enhances glucose uptake in SkM. (A) 2-DG uptake in SkM of 8-week-old male *Keap1FA/+* and *Keap1FA/-* mice for 4 h. After 16 h of fasting, 2-DG (1 g/kg of body weight) was intraperitoneally administered. The data are intracellular 2-DG-6-phosphate levels normalized to tissue weight ($n = 7$), and the level in the control was set as 1. (B) Profiles of *Keap1FA/-::Nrf2MuKO* mice. (C) Blood glucose levels and AUCs in ipGTT of *Keap1FA/+::Nrf2F/F*, *Keap1FA/-::Nrf2F/F*, and *Keap1FA/-::Nrf2MuKO* mice ($n = 5$ or 6). Glucose (2 g/kg of body weight) was administered intraperitoneally to 16-week-old male mice after 16 h of fasting. (D) *Gbe1* and *Phka1* mRNA expression levels in SkM ($n = 5$ or 6). The data were normalized to *Hprt*, and the expression levels in the controls were set as 1. Error bars show the mean $\pm$ SEM. ***, $P < 0.001$; *, $P < 0.05$ (versus the control). †, $P < 0.001$ (versus *Keap1FA/-::Nrf2F/F* mice). All of the data were collected from ICR background mice.

Phka1 was repressed in *Keap1FA/-::Nrf2MuKO* mice compared to that in *Keap1FA/-::Nrf2F/F* mice (Fig. 11D). These data indicate that Nrf2 preferentially induces *Gbe1* expression in SkM.

GBE and glucose metabolism. We next assessed the contribution of GBE induction to C2C12 myotube glucose uptake by transfecting C2C12 myoblasts with either the GBE expression vector *Gbe1*-pF9A or empty pF9A and selecting stably transfected cells with G418. In this assay, we utilized bulk-selected cells in a pool. As shown in Fig. S8A in the supplemental material, three pools of *Gbe1*-pF9A-transfected C2C12 myotubes expressed GBE protein at levels that were approximately 6-fold higher than those in myotubes transfected with control pF9A. To assess the contribution of GBE to improved glucose tolerance, we examined 2-DG uptake in cells that were stably transfected with GBE. After 30 min of incubation with 2-DG, 2-DG-6-phosphate levels were significantly higher in *Gbe1*-pF9A-transfected C2C12 myotubes than in control pF9A-transfected myotubes (see Fig. S8B). These results support our contention that Nrf2-mediated induction of GBE improves glucose uptake and explain, at least in part, the mechanisms underlying the Nrf2-mediated suppression of blood glucose levels.

SkM-specific Nrf2 induction lowers blood glucose levels in diabetic model mice. Finally, to evaluate whether Nrf2 in SkM improves glucose metabolism under diabetic conditions, we crossed *Keap1MuKO-A* mice with diabetic model *db/db* mice

and generated *db/db::Keap1MuKO-A* mice. The *db/db* mice lack the leptin receptor and develop a severe increase in blood glucose (44). The high blood glucose level was significantly lower in *db/db::Keap1MuKO-A* mice than in control *db/db::Keap1FA/FA* mice (Fig. 12A). In contrast, the body weights of *db/db::Keap1MuKO-A* and *db/db::Keap1FA/FA* mice were comparable (Fig. 12B). These data indicate that Nrf2 induction in SkM lowers blood glucose by improving insulin resistance under diabetic conditions.

DISCUSSION

In a mouse model of diet-induced obesity, *Keap1MuKO* mice harboring high levels of Nrf2 in their SkM are known to exhibit significantly improved glucose utilization and enhanced locomotor activity (18). We performed a series of microarray expression assays to clarify the molecular mechanisms underlying the contribution of Nrf2 to glucose metabolism and locomotor activity. Through this analysis, we found that Nrf2 induced the expression of *Gbe1*, *Phka1*, and a set of cytoprotective genes in SkM. Following Nrf2 induction, the SkM glycogen content was significantly decreased and forced GBE expression in C2C12 myotubes enhanced 2-DG uptake. From these results, we conclude that in SkM, Nrf2 induces GBE and *PhkA* subunit expression, reducing the SkM glycogen content and resulting in increased glucose uptake and utilization, which promotes exercise capacity and loco-

FIG 12 SkM-specific induction of Nrf2 improves diabetes mellitus. Blood glucose levels (A) and body weights (B) of 8- to 9-week-old control *db/db::Keap1FA/FA* ($n = 9$; all females) and *db/db::Keap1MuKO-A* ($n = 5$; 4 females and 1 male) mice fed *ad libitum* are shown. Error bars show the mean $\pm$ SEM. *, $P < 0.05$ (versus the control). All of the data were collected from ICR background mice.

motor activity. These metabolic changes are summarized in Fig. 13. On the basis of our data, we propose that Nrf2 regulation of the *Gbe1* and *Phk1* genes is the critical molecular mechanism for improving SkM glucose utilization.

Glycogen branching is one of the most influential factors in determining SkM glycogen levels (45, 46). Here, we reproducibly observed upregulation of SkM GBE and PhK α 1 expression and repression of glycogen content *in vivo* in both genetic and pharmacological Nrf2 induction models. Furthermore, cultured C2C12 myotubes treated with CDDO-Im also induced Nrf2 protein levels, along with *Gbe1* and *Phk1* mRNA expression. As phosphorylase kinase functions to positively regulate glycogen phosphorylase (28, 47), it is quite plausible that PhK α 1 contributes to the Nrf2-induced reduction of SkM glycogen content. Moreover, glycogen phosphorylase overexpression in SkM has been reported to reduce glycogen content and to increase 2-DG uptake (48), suggesting that the enhanced phosphorylase-mediated glycogen breakdown in SkM stimulates glucose uptake, leading to reduced blood glucose levels. Consistent with this idea, we observed reduced blood glucose levels in response to Nrf2-mediated increased GBE and PhK α 1 expression. Together, these results support the notion that *in vivo*, Nrf2-mediated reductions in blood glucose levels and SkM glycogen content result from GBE and PhK α 1 induction.

We found that Nrf2 induction also enhanced *Gbe1* gene expression in mouse liver *in vivo* and in Hepa1 cells *in vitro* (data not shown). The *Phk1* gene encodes the muscle-type isoform of the PhK α subunit, while *Phk2* encodes the hepatic isoform (49); however, we did not observe Nrf2 binding to

FIG 13 Schematic representation of Nrf2 regulation of liver and SkM glycogen and glucose metabolism. Nrf2-mediated GBE and PhK α 1 induction accelerates SkM glycogen branching and breakdown. In SkM, Nrf2 enhances glycogen release of G1P and promotes glucose uptake, exercise capacity, and locomotive activity. In contrast, Nrf2 enhances expression of GBE but not PhK α 1 in the liver, promoting glycogen storage and contributing to the maintenance of blood glucose levels during fasting.

Phka2 regulatory regions in our ChIP-seq analysis of Hepa1 cells (20). In contrast, we observed CDDO-Im-mediated suppression of *Phka2* mRNA expression in mouse liver *in vivo* (A. Urano, unpublished data). This observation suggests that Nrf2 induction downregulates liver glycogen phosphorylase activity. Consistent with this hypothesis, *Keap1* knockout induced liver Nrf2, resulting in increased glycogen content. Similarly, CDDO-Im increased Hepa1 cell glycogen content *in vitro* (data not shown). Together, these results demonstrate distinct functions for Nrf2 in SkM and the liver. Induction of Nrf2 reduces SkM glycogen content by inducing GBE and PhK α 1. In contrast, the induction of Nrf2 increases liver glycogen content by activating the expression of GBE but not PhK α 2.

Glycogen degradation and phosphorylase activity are also differentially regulated in SkM and the liver. While phosphorylase is activated by PhK under the control of hormones (e.g., catecholamine in SkM [50] and glucagon in the liver [51]), phosphorylase activity is also regulated positively by AMP and negatively by ATP and G6P (4, 52). As exercise increases the intracellular AMP concentration, phosphorylase activity in SkM is much more easily increased during exercise than in the liver. In fact, phosphorylase activity is reported to be higher in SkM than in the liver (53). We surmise that the difference in phosphorylase activity in SkM and the liver may contribute to the distinct phenotypes of glycogen metabolism in SkM and the liver upon induction of Nrf2.

It should be noted that liver glycogen metabolism and muscle glycogen metabolism cooperatively contribute to reduce blood glucose. Glucose-6-phosphatase (G6Pase) catalyzes the conversion of G6P to glucose (54). An important observation is that the liver and SkM differentially express G6Pase (4); this difference seemingly underlies the cooperative contributions of the different regulatory mechanisms. In other words, because SkM does not express G6Pase, glycogen breakdown in muscle does not increase blood glucose levels but instead reduces blood glucose levels via accelerated uptake. In direct contrast, hepatocytes abundantly express G6Pase and hepatic glycogen breakdown increases blood glucose levels (4).

In this study, we have demonstrated that Nrf2 in SkM plays a critical role in blood glucose regulation. While Nrf2 regulates *Gbe1* and *Phka1* gene expression, microarray analyses have revealed that Nrf2 also regulates the expression of many genes in SkM, but contributions of the genes to glucose metabolism have not been elucidated. In addition, we have demonstrated in this study that Nrf2 in the liver plays an important role in the maintenance of blood glucose levels during fasting. Although Nrf2 in the liver contributes to the elevation of blood glucose levels in nondiabetic mice, it has also been reported that Nrf2 in the liver lowers blood glucose through suppression of gluconeogenesis under diabetic conditions (18, 55).

In conclusion, this study demonstrates for the first time that Nrf2 induces GBE and PhK α 1 expression and is tightly associated with glycogen metabolism. Nrf2 promotes SkM glycogen utilization and glucose uptake and concomitantly facilitates liver glycogen storage. Consequently, glycogen metabolism is one of the critical mechanisms by which Nrf2 reduces blood glucose levels and protects against diabetes mellitus onset and/or progression.

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