Distinct and additive effects of sodium bicarbonate and continuous mild heat stress on fiber type shift via calcineurin/NFAT pathway in human skeletal myoblasts

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Yamaguchi T, Omori M, Tanaka N, Fukui N. Distinct and additive effects of sodium bicarbonate and continuous mild heat stress on fiber type shift via calcineurin/NFAT pathway in human skeletal myoblasts. Am J Physiol Cell Physiol 305: C323–C333, 2013. First published May 22, 2013; doi:10.1152/ajpcell.00393.2012.—Ingestion of sodium bicarbonate (NaHCO3) is known to enhance athletic performance, probably via increased extracellular buffering capacity. At present, little is known about the direct effects of NaHCO3 on myogenesis, especially in vitro. Here, we examined the effects of NaHCO3 and the combined effects of NaHCO3 and continuous mild heat stress (CMHS) at 39°C on the differentiation of human skeletal muscle myoblasts (HSMMs). Levels of myosin heavy chain (MyHC) type I mRNA increased with increasing NaHCO3 concentrations; in contrast, those of MyHC IIX decreased. The NaHCO3-induced fast-to-slow shift was additively enhanced by CMHS. Likewise, intracellular calcium levels and expression of three factors, nuclear factor of activated T cells c2 (NFATc2), NFATc4, and peroxisome proliferator-activated receptor-γ coactivator-1α, were upregulated with increasing NaHCO3 concentrations; moreover, these effects of NaHCO3 were additively enhanced by CMHS. Overexpression experiments and small interfering RNA-mediated knockdown experiments confirmed that NFATc2 and NFATc4 were involved in MyHC I regulation. The present study provided evidence that NaHCO3 and CMHS distinctly and additively induced a combined effects of NaHCO3 and continuous mild heat stress on fiber type shift via calcineurin/NFAT pathway in human skeletal myoblasts.

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soleus muscle (2). Because athletic performance is closely associated with the balance of muscle fiber types and mitochondrial function, we hypothesized that fiber type switching is affected by NaHCO₃ supplementation via specific NFAT isoforms. We previously reported that continuous mild heat stress (CMHS) imposed by incubation at 39°C induces a fast-to-slow fiber type shift in mammalian myoblasts (38). Therefore, the present study aimed to determine whether NaHCO₃ supplementation and CMHS have additive or synergistic effects on fast-to-slow fiber type shifts. We also determined the effects of NaHCO₃ supplementation and CMHS on the expression of molecules associated with mitochondria.

MATERIALS AND METHODS

Cell culture. We used commercially available human skeletal muscle myoblasts (HSMMs) of normal human quadriceps muscle obtained from five Caucasian males, 16, 22, 24, 25, and 26 yr of age (CC-2358; Lonza, Walkersville, MD). HSMMs were cultured in culture dishes using the SkGM-2 BulletKit according to the manufacturer’s protocol (CC-2580; Lonza, Walkersville, MD). HSMMs were cultured in air mixed with 5% CO₂. During the experiments, the culture temperature was monitored continuously using a thermometer with a precision of ±0.1°C.

NaHCO₃ supplementation and heat stress exposure. HSMMs were seeded in growth medium and incubated at 37°C until they reached 60–70% confluency. The medium was then changed to DM to induce myotube formation. To determine the effect of varying concentrations of NaHCO₃, the DM was supplemented with 11, 22, 33, or 44 mM NaHCO₃ (GIBCO). To determine the effects of CMHS, the cells were cultured at 37 or 39°C in water-jacketed incubators with humidified air mixed with 5% CO₂. During the experiments, the culture temperature was monitored continuously using a thermometer with a precision of ±0.1°C.

RNA interference. FlexiTube small interfering RNAs (siRNAs) for NFATc1 (HS_NFATC1_7 and HS_NFATC1_8), NFATc2 (HS_NFATC2_2 and HS_NFATC2_5), NFATc3 (HS_NFATC3_3 and HS_NFATC3_5), and NFATc4 (HS_NFATC4_4 and HS_NFATC4_7) were purchased from Qiagen (Hilden, Germany). Allstars Negative Control siRNA (Qiagen) was used as a negative control. HSMMs at 50% confluence on 48-well plates were transfected with 5 pmol of NFATc siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. For each targeted gene we selected two sequences with high silencing efficiency. All experiments were performed with both sequences. After transfection, the medium was changed to DM containing 14 mM NaHCO₃ at 37°C, which is the standard concentration in DM according to the manufacturer’s protocol for HSMMs. Cells were harvested after 72 h.

Overexpression experiments. Transient transfections of expression vectors were performed using Lipofectamine 2000 according to the manufacturer’s instructions. A total of 2.5 × 10⁵ cells/well was transfected with 0.5 µg of HaloTag control vector, or HaloTag expression vector carrying NFATc2 (pFN21AB0454, FHC11780) or NFATc4 (pFN21AB0442, FHC03802) (Promega, Madison, WI) on a 24-well plate. Lipofectamine 2000 was used at an amount of 1.0 µ/ml. After transfection, the medium was changed to DM containing 14 mM NaHCO₃. Cells were harvested after 72 h.

pH measurement. All pH measurements were performed using an Accumet AR10 pH meter (Fisher Scientific, Pittsburgh, PA) in an incubator (5% CO₂) at 37°C.

Western blot analysis. Western blot was carried out with the following primary antibodies: PGC-1α (1:1,000, ST1202; Calbiochem, San Diego, CA), MyHC I (1:4,000, clone NOQ7.5.4D; Sigma-Aldrich, St. Louis, MO), MyHC IIi (1:500, clone MY-32; Sigma-Aldrich), and β-actin (1:1,000, clone AC-15; Sigma-Aldrich). Cells were washed twice with PBS and then lysed with 2× Laemmli sample buffer. Protein concentrations were quantified using the BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL) with BSA as the standard. Aliquots containing 10 µg of total protein were separated using 8–12% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was then blocked using Tris-buffered saline with 0.05% Tween 20 (TBST) containing 3% skim milk for 1 h and incubated with an appropriately diluted primary antibody in TBST at 4°C overnight. After incubation, membranes were washed three times in TBST and incubated for 1 h with a horseradish peroxidase-labeled secondary antibody (1:5,000, sc-2030 or sc-2031; Santa Cruz Biotechnology, Santa Cruz, CA). The immunoreactive bands were visualized on X-ray films (Fuji Photo Film, Tokyo, Japan) using an Immobilon Western Detection Reagent (Millipore, Billerica, MA).

Quantitative real-time RT-PCR analysis. Total RNA from HSMMs was isolated using the PureLink RNA Mini kit (Invitrogen). After DNase treatment, cDNAs were obtained by reverse transcription of 2 µg of total RNA (Sensiscript RT kit; Qiagen). Quantitative real-time RT-PCR (qPCR) was performed using the SYBR Premix Ex Taq (Perfect Real Time) Premix (TaKaRa Bio, Otsu, Japan) or the Light-Cycler FastStart DNA Master SYBR Green I Master (LightCycler; Roche, Germany) in a fluorescent temperature cycler (LightCycler; Roche). Primer sequences were obtained from previous publications (6, 15, 35, 38) (Table 1). The qPCR protocol included initial denaturation at 95°C for 10 min (LightCycler FastStart) or 10 s (SYBR Premix), followed by 40–50 PCR cycles of 95°C for 5 s and 60°C for 20 s (SYBR Premix), or 95°C for 10 s, 60°C for 10 s, and 72°C for 12 s (LightCycler FastStart). SYBR green fluorescence emissions were determined after each cycle, and the amount of cDNA was quantified by the 2⁻¹Ct method.
using the LightCycler software version 3.5 (Roche). All PCR analyses were performed in triplicate. The housekeeping gene β-actin was used as a control template for normalizing relative change of each mRNA in qPCR. The normalized expression level was compared with the control sample.

Fluorescent staining. For immunofluorescent staining, cells were incubated in culture dishes containing collagen type I-coated cover glasses (25 mm type; Iwaki, Tokyo, Japan). At appropriate times, the cells were washed with warm PBS at 37°C and fixed by incubation at room temperature in FME (4% formaldehyde, 2 mM MgCl2, and 5 mM EGTA in PBS) for 10 min. Cells were washed three times with PBS and permeabilized with FME containing 0.3% Triton X-100 for 10 min at room temperature. The cells were then washed three times with PBS and incubated in PBS containing 1% BSA, 0.02% sodium azide, and an appropriately diluted primary antibody for MyHC (type I, clone NOQ7.5.4D; type II, clone MY-32), NFATc1-c4 (1:100; c1, 7A6, sc-7294; c2, G1-D10, sc-7295; c3, F-1, sc-8405; c4, H-74, sc-13036; Santa Cruz Biotechnology) or COX I (1:200, OxPhos Complex IV Subunit I, clone ID6E1A8; Invitrogen) at 4°C overnight. The cells were washed three times with PBS and incubated in PBS containing 1% BSA, 0.02% sodium azide, and an appropriately diluted primary antibody for MyHC (type I, clone NOQ7.5.4D; type II, clone MY-32), NFATc1-c4 (1:100; c1, 7A6, sc-7294; c2, G1-D10, sc-7295; c3, F-1, sc-8405; c4, H-74, sc-13036; Santa Cruz Biotechnology) or COX I (1:200, OxPhos Complex IV Subunit I, clone ID6E1A8; Invitrogen) at 4°C overnight. The cells were washed three times with PBS, and then reacted for 1 h with fluorescence-conjugated secondary antibody Alexa fluor 488 or Alexa fluor 594 (1:500; A11029 or A21424; Invitrogen). To stain functioning mitochondria in living cells, we used MitoTracker Red CMXRos (PA-3017; Lonza) according to the manufacturer’s protocol; this stain yields a fluorescent signal the intensity of which is dependent on mitochondria in living cells, we used MitoTracker Red CMXRos

Measurement of fusion index. The fusion index was defined as the ratio of the number of DAPI stained nuclei in myotubes with three or more nuclei to the total number of DAPI stained nuclei in each field. This percentage was determined by counting 1,000 nuclei per dish on 3 independent cultures for each condition.

Intracellular calcium level measurements. HSMMS were loaded with fura-2 AM (5 μM, F-1225; Invitrogen) in modified Hanks’ solution for 45 min at 37°C. They were then washed three times with modified Hanks’ solution, incubated in that solution for 20 min at 37°C, and kept in modified Hanks’ solution during analysis. The cells were viewed on an inverted fluorescence microscope (IX71; Olympus) equipped with an on-stage incubation chamber (INUG2-ONICS; Tokai Hit, Shizuoka, Japan) that kept the cells at either 37°C or 39°C under a 5% CO2 atmosphere. The excitation wavelengths of fura-2 AM are 340 and 380 nm, while the emission wavelength is 510 nm. An intensified CCD camera (ORCA-R2; Hamamatsu Photonics, Shizuoka, Japan) was used for imaging. Image intensities were recorded every second to give a ratio of emitted intensities at excitation wavelengths of 340 and 380 nm, F340/F380. Intracellular calcium level ([Ca2+]i) was determined from the F340/F380 ratio. For each experiment, the fluorescence signals of 10 cells were averaged for analysis.

Statistical analysis. The effect of temperature on peak/baseline [Ca2+]i was determined using an unpaired Student’s t-test. The fusion index and the effects of a single incubation with NaHCO3, siRNA, or NFATc isoform overexpression on MyHC expression were deter-

Fig. 1. Effects of sodium bicarbonate (NaHCO3) concentration on myosin heavy chain (MyHC) isoform expression in human skeletal muscle myoblasts (HSMMS). A: relative expression of MyHC I, MyHC IIa, and MyHC IIx mRNA in HSMMS cultured in differentiation medium (DM) containing 11, 22, 33, or 44 mM NaHCO3 at 37°C for 5 days. Expression levels of MyHC isoforms mRNA were determined by quantitative (q)PCR. Results are normalized to β-actin expression. Each column represents means ± SE of 3 independent experiments. B: pH of DM containing indicated concentrations of NaHCO3 (left) and pH of DM containing 11 mM NaHCO3 and indicated concentrations of NaOH (right). Results are means ± SE of 3 independent experiments. C: Western blot analysis of indicated protein in HSMMS cultured for 7 days in DM containing 11 mM NaHCO3 and indicated concentrations of NaOH using a specific antibody for MyHC I (clone NOQ7.5.4D) or MyHC II (clone MY32). β-Actin was used as a loading control.
Fig. 2. Effects of continuous mild heat stress (CMHS) on NaHCO₃-induced fiber type shift and cell fusion in HSMM cultures. A: relative expression of MyHC I, MyHC IIA, or MyHC IIX mRNA in HSMMs cultured in DM for 5 days under indicated conditions. Expression of respective MyHC isoforms mRNA was determined by qPCR. Results were normalized to β-actin expression. Each column shows the means ± SE of 5 independent experiments per treatment. Statistical analyses involved two-way ANOVA and Bonferroni post hoc test. **P < 0.01 refers to comparisons to cells cultured with 11 mM NaHCO₃ at respective temperatures; ##P < 0.01, main effect of temperature (37 vs. 39°C).

B: HSMMs were cultured for 7 days in DM under indicated conditions, and the presence of MyHC I or MyHC II was visualized by immunofluorescent staining (scale bar = 100 µm).

C: relative expression of sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase 1 (SERCA1) and SERCA2a mRNA in HSMMs cultured in DM for 5 days under indicated conditions. Expression levels were determined by qPCR and normalized to β-actin expression. Each column shows the means ± SE of 5 independent experiments per treatment. Statistical analyses involved two-way ANOVA and Bonferroni post hoc test. **P < 0.01, main effect of temperature (37 vs. 39°C).

D: HSMMs were cultured for 24 or 48 h in DM under indicated conditions. The fusion index of each culture was examined. Each column shows the means ± SE of 3 independent experiments per treatment. Statistical analyses involved one-way ANOVA and Dunnett’s post hoc test at the respective times. *P < 0.05 and **P < 0.01 refer to comparisons to cells cultured with 11 mM NaHCO₃ at 37°C at the respective times.
mined using a one-way ANOVA followed by Dunnett’s test. The effects of both NaHCO3 and CMHS on gene expression were analyzed using a two-way ANOVA followed by Bonferroni’s post hoc test. Statistical significance was set at $P < 0.05$ (GraphPad Prism Statistical Software version 5; GraphPad Software, San Diego, CA).

RESULTS

NaHCO3 alters expression levels of MyHC isoforms in HSMMs. To investigate the direct effects of NaHCO3 on the expression of MyHC isoforms in HSMMs, we incubated HSMMs at 37°C in DM supplemented with 11, 22, 33, or 44 mM NaHCO3. Expression of MyHC I or MyHC II isoforms was analyzed by qPCR after 5 days of differentiation. Levels of MyHC I mRNA were significantly higher in the cells cultured with 33 or 44 mM NaHCO3 than in the control cells cultured in DM containing 11 mM NaHCO3; in contrast, levels of MyHC IIX mRNA were significantly lower in the cells cultured with 33 or 44 mM NaHCO3 than in the control cells. Expression levels of MyHC IIa were not significantly affected by differences in NaHCO3 concentrations (Fig. 1A).

![Graph showing effects of NaHCO3 on MyHC isoform expression](image)

Fig. 3. Effects of CMHS and/or changes in NaHCO3 concentration on the expression of peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α and PGC-1α targets. A: HSMMs were cultured in DM for 5 days under indicated conditions. Expression of PGC-1α mRNA was determined by qPCR and normalized to β-actin expression (top). Cell lysates were prepared from cells cultured in DM for 7 days, and Western blot analysis was performed using a specific antibody for PGC-1α. β-Actin was used as a loading control (bottom). B: relative expression of nuclear respiratory factor-1 (NRF-1), mitochondrial transcription factor A (mtTFA), and cytochrome c oxidase I (COX I) mRNAs in HSMMs cultured in DM under indicated conditions for 5 days. Expression levels were determined by qPCR and normalized to β-actin cDNA levels. Each column represents means ± SE of 5 independent experiments per treatment. Statistical analyses involve two-way ANOVA and Bonferroni post hoc test. *$P < 0.05$ and **$P < 0.01$ refer to comparisons to cells cultured with 11 mM NaHCO3 at respective temperatures; **$P < 0.01$, main effect of temperature (37 vs. 39°C). C: HSMMs were cultured for 7 days in DM under indicated conditions, and the presence of COX I protein was visualized by immunofluorescence staining using a monoclonal antibody against OxPhos Complex IV Subunit I (clone ID6E1A8). Mitochondrial membrane potential of live cells was assessed via MitoTracker Red CMXRos staining (400 nM). Representative fluorescent images are shown (scale bar = 100 μm).
We next studied the effects of differences in extracellular pH on shifts in MyHC isoforms. The pH of the DM containing 11 mM NaHCO₃ was 7.4 and that of DM with 44 mM NaHCO₃ was 8.1. To regulate the pH within that range without using NaHCO₃, we added NaOH or sodium citrate at varying concentrations to DM that contained 11 mM NaHCO₃ (Fig. 1A). Differentiation of HSMMs was induced for 7 days, and the protein levels of MyHC I and MyHC II were determined by Western blot analysis. In this experiment, the MyHC I protein levels were modestly affected by changes in NaOH concentration, while those of MyHC II decreased only when the cells were cultured in media containing 8 mM NaOH (Fig. 1B). Similarly, sodium citrate had no significant effects on MyHC levels were modestly affected by changes in NaOH concentration, while those of MyHC II decreased only when the cells were cultured in media containing 8 mM NaOH (Fig. 1C). Similarly, sodium citrate had no significant effects on MyHC switching (data not shown).

**CMHS enhances NaHCO₃-induced changes in MyHC isoform expression and in the fusion process in HSMMs.** To test whether the NaHCO₃-induced fast-to-slow shift of MyHC isoforms was enhanced by CMHS, we incubated the cells in DM supplemented with 11, 22, or 33 mM NaHCO₃ at 37°C or 39°C. We examined the expression of each MyHC isoform at the mRNA level. The levels of MyHC I mRNA were significantly higher at 33 mM NaHCO₃ at 39°C (33 mM/39°C) than at 11 mM/39°C, while those of MyHC IIX were unaffected by CMHS or an increase in the NaHCO₃ concentration (Fig. 2A).

Changes in levels of MyHC I and IIX were the main effect of CMHS. For fluorescent immunostaining, HSMMs were incubated in DM for 7 days at 11 mM/37°C, 33 mM/37°C, or 33 mM/39°C. Representative images are shown in Fig. 2B. For myotubes expressing MyHC I, the strongest staining was evident when cells were cultured at 33 mM/39°C; the next strongest staining was observed in myotubes cultured at 33 mM/37°C, and the weakest staining was in myotubes at 11 mM/37°C. In contrast, different culture conditions had little effect on the staining intensity of the myotubes expressing MyHC II.

To further examine the changes in gene expression in myotubes, we assessed the mRNA levels of SERCA1 and SERCA2a in HSMM-derived myotubes after 5 days of differentiation. Levels of SERCA2a mRNA were significantly higher in myotubes that had differentiated at 33 mM/37°C than in those that had differentiated at 11 mM/37°C; in contrast, SERCA1 expression was unaffected by the increase in NaHCO₃ concentration or by CMHS (Fig. 2C). Differences in SERCA2a expression were the main effect of CMHS.

To investigate the effects of NaHCO₃ and heat stress on the fusion of HSMMs in cultures, we incubated the cells for 24 or 48 h in DM at 11 mM/37°C, 33 mM/37°C, or 33 mM/39°C and measured the fusion index of HSMM cultures. The fusion index...
was highest at 33 mM/39°C, next highest at 33 mM/37°C, and lowest at 11 mM/37°C at both 24 h and 48 h (Fig. 2D).

**NaHCO₃ and CMHS alter the expression of mitochondria-related genes in HSMMs.** To address whether NaHCO₃ and CMHS affect the expression of mitochondria-related genes, we examined the expression of PGC-1α and of three downstream targets of PGC-1α-NRF-1, mtTFA, and COX I. Levels of PGC-1α transcripts were significantly higher in the cells cultured at 33 mM/37°C or at 33 mM/39°C than in cells cultured at 11 mM/37°C or at 11 mM/39°C, respectively. Differences in PGC-1α mRNA expression were the main effect of CMHS. Levels of PGC-1α protein were higher at higher NaHCO₃ concentrations, and this trend was more obvious in the cells cultured at 39°C than in those cultured at 37°C (Fig. 3A). Levels of COX I mRNA were significantly higher in the cells cultured at 33 mM/39°C than in the cells cultured at 11 mM/39°C. Levels of mtTFA mRNA or COX I mRNA were higher in cells cultured at 39°C than in those cultured at 37°C,

**Fig. 5.** Effects of CMHS and/or changes in NaHCO₃ concentration on the expression of nuclear factor of activated T cells (NFAT) isoforms. A: HSMMs were cultured in DM for 5 days under indicated conditions, and the cells were harvested. Levels mRNAs encoding NFAT isoforms in HSMMs were determined by qPCR and normalized to β-actin expression. Each column represents means ± SE of 5 independent experiments per treatment. Statistical analyses involved two-way ANOVA and Bonferroni post hoc test. *P < 0.05 refers to comparisons to cells cultured with 11 mM NaHCO₃ at respective temperatures; **P < 0.05 and ***P < 0.01, main effect of temperature (37 vs. 39°C). B: HSMMs were cultured for 7 days in DM under indicated conditions. NFAT isoforms were visualized by immunofluorescence with the use of specific antibodies (scale bar = 50 μm).
but levels of NRF-1 mRNA tended to increase only with increasing NaHCO₃ concentrations (Fig. 3B). To further examine the levels of COX I protein and the mitochondrial membrane potential, HSMMS were maintained in DM under conditions of 11 mM/37°C, 33 mM/37°C, or 33 mM/39°C and then stained with the anti-COX I antibody or MitoTracker Red CMXRos. Figure 3C shows representative images of HSMMS after 7 days of differentiation. Both COX I staining and MitoTracker Red CMXRos staining were most intense in the cells cultured at 33 mM/39°C; staining was next most intense in cells cultured at 33 mM/37°C and least intense in cells cultured at 11 mM/37°C.

NaHCO₃ and CMHS alter calcium signaling in HSMMS. We next examined whether NaHCO₃ and CMHS affected [Ca²⁺].
in HSMMs. In this experiment, we added NaHCO₃ to the media and studied the changes in [Ca²⁺]. Supplementation with NaHCO₃ (11 mM) resulted in an immediate increase in [Ca²⁺], which was more obvious in cells cultured at 39°C than in those cultured at 37°C (Fig. 4A, left). Next, we determined the difference between peak [Ca²⁺] and baseline [Ca²⁺]. Peak [Ca²⁺] was divided by baseline [Ca²⁺], and the change in [Ca²⁺] was expressed as a percentage increase over baseline. The peak/baseline [Ca²⁺] at 39°C (1.25 ± 0.01) was significantly greater than that at 37°C (1.07 ± 0.02; Fig. 4A, right).

To examine the role of Cn in the fiber type shift and PGC-1α expression, we compared Cn activity in HSMMs cultured under different culture conditions. We examined the expression of myocyte-enriched calcineurin-interacting protein 1 (MCIP1) mRNA to estimate Cn activity (6, 39). The expression of MCIP1 mRNA was significantly higher in the cells cultured at 33 mM/37°C or 33 mM/39°C than in the cells cultured at 11 mM/37°C or 11 mM/33°C, respectively (Fig. 4B). Differences in MCIP1 mRNA expression were the main effect of CMHS. To further examine the role of Cn, we treated the cells with each of two specific Cn inhibitors, cyclosporine A (Calbiochem) or FK506 (Cayman Chemical, Ann Arbor, MI). HSMMs were incubated in DM containing either 0.25–0.5 μM cyclosporine A or 0.5–1.0 μM FK506 for 7 days. These Cn inhibitors attenuated the levels of MyHC I and PGC-1α proteins but not of MyHC II protein (Fig. 4C).

Roles of NFAT isoforms in fiber type determination. To investigate the combined effect of NaHCO₃ and CMHS on the expression of NFAT isoforms, we assessed the expression of NFAT mRNAs in HSMMs cultured under several different conditions. Figure 5A shows that all four NFAT isoforms were expressed at substantial levels in HSMMs. At either 37 or 39°C, the levels of NFATc2 and of NFATc4 mRNA were significantly higher in the cells cultured with 33 mM NaHCO₃ than in control cells cultured with 11 mM NaHCO₃. Changes in levels of NFATc2 and NFATc4 were the main effect of CMHS. In contrast, levels of NFATc1 and NFATc3 mRNA did not differ significantly among the cells cultured under different conditions. To confirm these findings at the protein level, we conducted immunofluorescent staining of the cells. For this, HSMMs were incubated for 7 days in DM at 11 mM/37°C, 11 mM/33°C, 33 mM/37°C, or 33 mM/39°C. The cells were stained with antibodies against individual NFAT isoforms for analysis of immunofluorescence. Of the three culture conditions, the fluorescence intensities of NFATc2 and NFATc4 were the most intense in the cells cultured at 33 mM/39°C and next most intense in those cultured at 33 mM/37°C. Fluorescence was least intense in the cells cultured at 11 mM/37°C (Fig. 5B).

In contrast, the fluorescence intensities of NFATc1 or of NFATc3 differed only modestly among cell cultures under these three conditions.

To elucidate the roles of individual NFAT isoforms as potential regulators of MyHC isoforms in HSMMs, we treated the cells with siRNA to knockdown the levels of individual NFAT isoforms and examined the expression of MyHC isoforms. The specificity and efficiency of knockdown of each NFAT isoform was confirmed by qPCR (Fig. 6A). NFATc2 siRNAs or NFATc4 siRNAs reduced the mRNA levels of MyHC I but not those of MyHC IIX or MyHC IIa. siRNA-mediated knockdown of NFATc1 or NFATc3 did not alter the mRNA levels of any of the MyHC isoforms (Fig. 6B). Figure 6C shows representative immunostaining for individual NFATc isoforms in HSMMs after 72 h of transfection with siRNA. MyHC I staining intensity was obviously reduced by knockdown of NFATc2 or NFATc4 but only marginally by knockdown of NFATc1 or NFATc3. The knockdown of any NFAT isoform did not significantly affect the MyHC II signal.

To further investigate the role of NFATc2 and NFATc4 in MyHC isoforms regulation in HSMMs, we examined the effects of NFATc2 or NFATc4 overexpression on MyHC isoform expression in HSMMs (Fig. 6D and E). Western blot analysis showed that the levels of MyHC I protein were significantly higher in cells that overexpressed NFATc2 or NFATc4 than in cells that did not. Moreover, the levels of MyHC II protein did not differ between cells regardless of whether they overexpressed NFATc2 or NFATc4.

DISCUSSION

Athletic performance often improves after ingesting NaHCO₃ (10, 22, 28, 32). Changes in the expression of MyHC and SERCA isoforms indicated that NaHCO₃ supplementation dose dependently induced fast-to-slow fiber type shifts in HSMMs, which supports our hypothesis. The NaHCO₃-induced shift in myofiber type was enhanced by CMHS. This effect was additive, because the effects of NaHCO₃ and CMHS did not significantly interact in any analysis (data not shown). We previously reported that CMHS can induce a fast-to-slow shift in fiber type (38). The present study shows that increased NaHCO₃ concentrations also induce a fast-to-slow shift in MyHC isoform expression in HSMMs.
Calcium signaling is involved in the determination of muscle fiber type. Kubis et al. (18) reported that a modest but sustained rise in [Ca\(^{2+}\)] caused by low concentrations of Ca\(^{2+}\) ionophore A-23187 induced a fast-to-slow fiber type shift in rabbit primary skeletal muscle cells, and Cn has been implicated in a fast-to-slow fiber type shift (25, 33). The expression of MCIP1 is rapidly and robustly regulated by Cn and thus is recognized as a highly accurate marker of Cn activity (6, 39). Here, we showed that [Ca\(^{2+}\)] and MCIP1 expression were increased by NaHCO\(_3\) supplementation and that these increases were augmented by CMHS. Furthermore, Cn inhibitors caused a slow-to-fast shift in MyHC expression. These results are consistent with the notion that changes in calcium signaling are a pivotal mechanism behind the fast-to-slow fiber type shift caused by NaHCO\(_3\) supplementation or CMHS.

Each of the four NFAT isoforms plays a distinctive role in the development of skeletal muscle in rodents. NFATc3 activity is critical for the generation of primary myofibers (16), whereas NFATc2 regulates skeletal muscle growth (12). NFATc2-null and NFATc3-null mice have an essentially normal composition of muscle fiber types (12, 16). Somatic gene transfer into adult skeletal muscle of a constitutively active NFATc1 enhances the promoter activity of MyHC I and inhibits that of MyHC IIb. Conversely, the expression of MyHC IIa and MyHC IIx is inhibited by siRNAs that target NFATc4 (33). Together, these findings indicate that NFATc2, c3, or c4, whereas the fast-glycolytic MyHC-IIb is only inhibited by siRNAs that target NFATc4 (33). Together, these findings indicate that NFATc2, c3, and c4 are all transcriptionally active in rat fast-type skeletal muscles. Consistent with these findings in vivo, increased [Ca\(^{2+}\)] in mouse skeletal myoblasts induces the recruitment of p300 to NFATc1, which increases NFATc1 transcriptional activity and enhances MyHC I expression (23). Here, we confirmed that all four NFATc isoforms were expressed at substantial levels in these primary cultures of human skeletal muscle. We then found that changes in NFATc2 and NFATc4 levels were involved in the regulation of MyHC I in HSMMS. Although NFATc1 is a regulator of MyHC I gene expression during the fast-to-slow fiber type shift in rodent skeletal muscle cells (33), the results of our RNA interference experiment indicated that NFATc1 is not involved in the fast-to-slow shift in HSMMS. The activities of NFAT proteins are regulated not only by expression levels but also by their subcellular localization and phosphorylation. Thus further studies are required to clarify the details of the involvement of NFAT proteins in the regulation of MyHC isoforms in HSMMS.

Mitochondrial function depends on the coordinated expression of the mitochondrial and nuclear genomes. PGC-1\(\alpha\) and the transcription factors NRF-1 and miTFA that are downstream of PGC-1\(\alpha\) play important roles in the coordinated expression of these two genomes. These three proteins are therefore considered to be key regulators of mitochondrial biogenesis (5, 6). Our results showed that NaHCO\(_3\) supplementation increased PGC-1\(\alpha\) mRNA and protein levels and increased the mitochondrial membrane potential. Moreover, NaHCO\(_3\)-induced changes were augmented by CMHS. Several pathways have been implicated in the signaling that acts upstream of PGC-1\(\alpha\) transcription, such as the Cn and CaMK, p38 MAPK, Sirtuin 1, and AMP-activated protein kinase pathways (5, 26). In addition, endurance exercise readily induces PGC-1\(\alpha\) expression in skeletal muscle in humans (31). Here, we showed that Cn inhibitors attenuated PGC-1\(\alpha\) protein levels. However, the protein levels of PGC-1\(\alpha\) and staining for mitochondrial membrane potential were essentially unaltered by the siRNA-mediated knockdown of individual NFAT isoforms (data not shown), implying that Cn, but not NFAT, is involved in mitochondrial biogenesis and function in human skeletal muscle.

The present study found that NaHCO\(_3\) enhanced and CMHS additively enhanced MCIP1 expression and HSMMS fusion. The activation of Cn is linked to the induction of myogenic differentiation that leads to myoblast fusion via myogenin and myocyte enhancer factor-2 (17). We previously reported that CMHS enhances myogenin expression and HSMMS fusion, leading to a fast-to-slow shift in fiber type (38). Myogenin is implicated in the fast-to-slow fiber-type shift (4, 14) and in a shift of enzyme activity from glycolytic to oxidative metabolism in the skeletal muscles of transgenic mice (14). NaHCO\(_3\) might affect a fast-to-slow fiber-type shift and the expression of molecules associated with mitochondria, leading the differentiation rate of HSMMS through a pathway involving Cn and myogenin.

Accumulating evidence suggests that NaHCO\(_3\) ingestion improves performance during single bouts of anaerobic exercise, repeated short-duration sprints, single-effort prolonged sprints, and prolonged, intermittent exercise at high intensity (10, 22, 28, 32). McNaughton and Thompson (21) described differences in performance during short-duration, high-intensity cycling between acute and chronic NaHCO\(_3\) loading in humans. Chronic NaHCO\(_3\) ingestion for 6 days allowed participants to perform more work (21) and 0.3 g of NaHCO\(_3\) per kg of body weight are the apparent accepted dose (32). Thus the dose and duration of NaHCO\(_3\) ingestion might be important factors when attempting to improve athletic performance. We showed that NaHCO\(_3\) supplementation dose dependently enhanced the expression of mitochondrial-related genes and the mitochondrial membrane potential in HSMMS. Bishop et al. (2) reported that oral NaHCO\(_3\) administration before each bout of exercise for 5 wk resulted in improved mitochondrial mass and mitochondrial respiration in the rat soleus muscle. Our findings are consistent with these results. We showed that changes in mitochondrial-related genes and mitochondrial membrane potential do not depend on changes in extracellular pH caused by NaOH or sodium citrate (data not shown). Our findings indicated that NaHCO\(_3\) affects myocyte differentiation via a mechanism other than changes in pH. Therefore, enhanced performance of prolonged exercise after NaHCO\(_3\) ingestion might be associated with changes in mitochondrial mass and function following NaHCO\(_3\) ingestion. Ingestion of NaHCO\(_3\) is thought to enhance performance by increasing extracellular buffering capacity via reducing H\(^{+}\) accumulation (2, 32). We speculate that reducing H\(^{+}\) accumulation following NaHCO\(_3\) ingestion might lead to short-term improvements in exercise performance. However, additional studies are needed to clarify the mechanism that links NaHCO\(_3\) to improvements in athletic performance.

Mitochondrial dysfunction is implicated in many age-related diseases, such as sarcopenia, insulin resistance and type 2 diabetes (13, 20, 37). Regular exercise elicits a rapid increase in oxidative enzyme activities, mitochondrial density, and mitochondrial respiration (11, 34). Prolonged bouts of exercise induce PGC-1\(\alpha\) gene expression in human skeletal muscle (31), and exercise conditioning enhances this induction. Muscle temperatures range from 36 to 39.5°C during aerobic exercise such as soccer and...
cycling (24, 27); therefore, a mild increase in muscle temperature may enhance mitochondria-related gene expression. We showed here that NaHCO₃ and CMHS additively induce a fast-to-slow fiber type shift and the expression of mitochondrial-related genes. Thus the present findings might help to establish novel therapeutic strategies to prevent metabolic diseases through improving mitochondrial function.

GRANTS
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DISCLOSURES
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AUTHOR CONTRIBUTIONS
Author contributions: T.Y. conception and design of research; T.Y., M.O., and N.T. performed experiments; T.Y. analyzed data; T.Y. interpreted results of experiments; T.Y. prepared figures; T.Y. and N.F. drafted manuscript; T.Y. and N.F. edited and revised manuscript; T.Y. approved final version of manuscript.

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