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Influence of intermittent cold stimulations on CREB and its targeting genes in muscle: investigations into molecular mechanisms of local cryotherapy

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Abstract

Local cryotherapy is widely used as a treatment for sports-related skeletal muscle injury. However, its molecular mechanisms are unknown. To clarify these mechanisms, in this study, we applied one to three 15-min cold stimulations at 4 °C to various cell lines (in vitro), the tibialis anterior (TA) muscle (ex vivo), and mouse limbs (in vivo). In the in vitro assay, cAMP response element-binding protein 1 (CREB1) was markedly phosphorylated (as pCREB1) and CREB-binding protein (CBP) was recruited to pCREB-1 in response to two or three cold stimulations. In a reporter assay with the cAMP-responsive element, the signals significantly increased after two to three cold stimulations at 4 °C. In the ex vivo study, CREB-targeting genes were significantly upregulated following two or three cold stimulations. The in vivo experiment disclosed that cold stimulation of a mouse limb for 9 days significantly increased mitochondrial DNA copy number and upregulated genes such as *Pgc-1α* involved in mitochondrial biogenesis. The foregoing results suggest that local cryotherapy increases CREB transcription and upregulates CREB-targeting genes in a manner dependent on cold stimulation frequency and duration. This information may serve as an impetus for further investigations into local cryotherapy as a treatment for sports-related skeletal muscle trauma.

Keywords: CREB, cryotherapy, gene expression, icing, mitochondria, *Pgc-1α*, transcription

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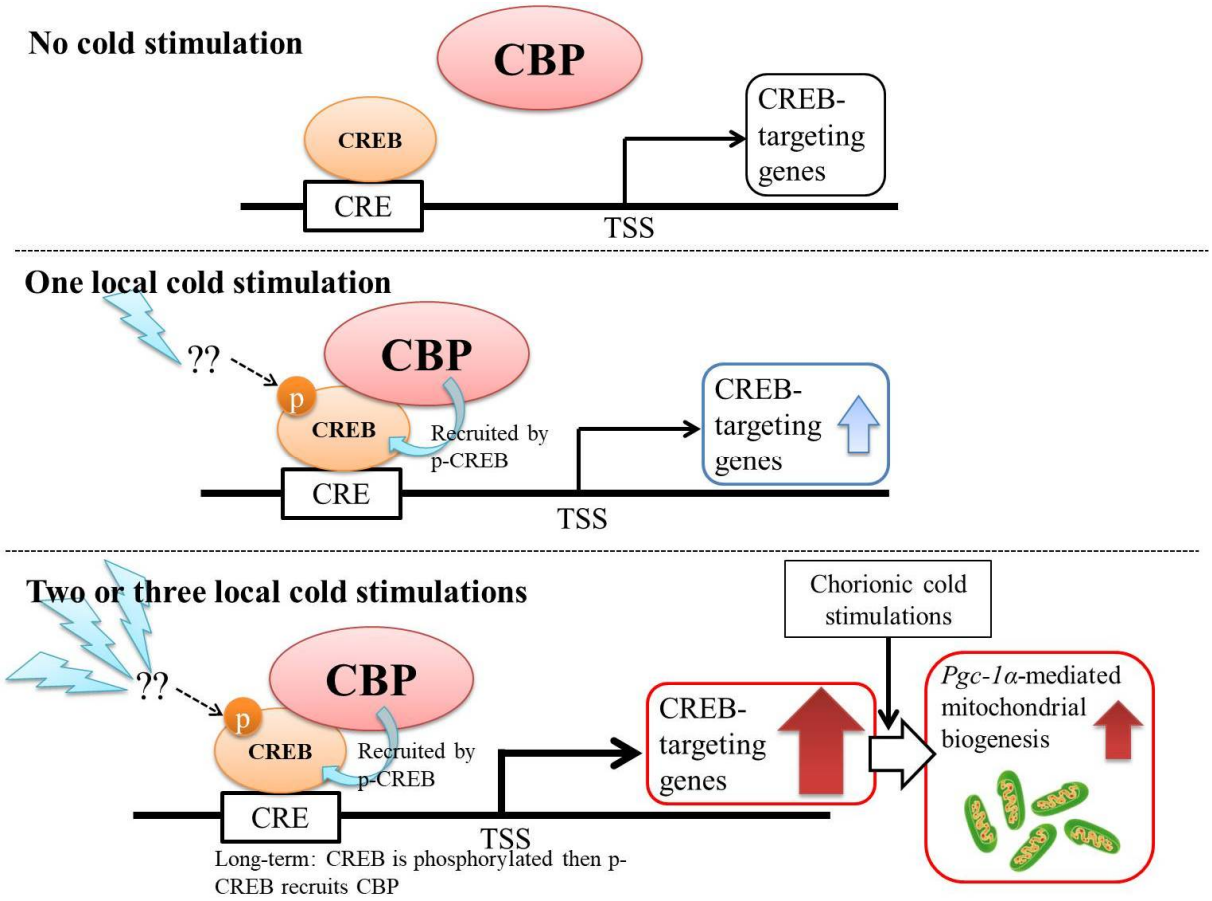

Graphical abstract

This model proposes the molecular mechanisms of local cryotherapy. CREB: cAMP response element-binding protein; CBP: CREB-binding protein; CRE: cAMP-responsive element; TSS: transcription start site.

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1. Introduction

Local cryotherapy has been widely used in the acute treatment of traumatic injuries such as fractures, dislocations, sprains, and pulled muscles. It has also been applied for recovery after daily training and sports competitions. At all levels of athletic performance, it is common to see ice bags applied to knees, shoulder joints, thigh muscles, etc. The use of cryotherapy in the management of sports injuries was first reported in Greece in the 1950s. Knight proposed that cryotherapy retards cellular metabolism and mitigates impairment caused by secondary hypoxia in the injured area [1]. This theory is accepted as the standard explanation for the mechanism underlying local cryotherapy in sports injury management. However, there are numerous uncertainties regarding the efficacy and basic mechanism of local cryotherapy. For example, previous in vivo studies reported that cryotherapy was both effective [2-4] and ineffective [5,6] in the treatment of soft tissue injuries. Two systematic reviews on cryotherapy for soft tissue injuries concluded that the efficacy of cryotherapy remains unclear and further study is required to elucidate its mechanism [7,8]. Hohenauer et al. [9] presented a meta-analysis of the effects of post-exercise cryotherapy on recovery. Cold water immersions after exercise significantly improved delayed-onset muscle soreness (DOMS) and ratings of perceived exertion (RPE) relative to the untreated control. In view of these earlier reports, local cryotherapy may have certain beneficial, adverse, or invalid effects on soft tissue injury and influence cellular-level molecular dynamics such as transcription and gene expression. Elucidation of the basic molecular mechanisms of local human cryotherapy may help advance its practical application. In a previous study, we conducted in vitro experiments and found that three to four intermittent cold stimulations at 17 °C increase mitochondrial number and activity. As this response may be temperature-dependent, we acknowledge the importance of determining the optimum temperature required for the induction of cellular response to cold stimulation [10]. However, the preceding studies were not performed at the in vivo level. The relative influence of recurring cold stimulations must also be clarified as the actual numbers of cold stimulations differed among trainers and experiments. In our previous study, we used only three cold stimulations. In the present study, we varied the numbers of cold stimulations and conducted in vitro, ex vivo, and in vivo experiments on myoblasts, muscle fibroblasts, whole mouse tibialis anterior muscle, and mouse limbs. The aim was to elucidate the basic molecular mechanisms of local cryotherapy (especially transcription and gene expression). We also investigated cAMP response element binding protein 1 (CREB1) as a transcriptional factor because earlier reports showed that CREB1 is associated with mitochondrial biogenesis via the upregulation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC-1α*) as a CREB-targeting gene [11-13]. Our previous study revealed that cold stimulation at the optimal temperature increases mitochondrial number and activity [10]. Thus, it was hypothesized that multiple cold stimulations at the optimal temperature enhance mitochondrial biogenesis through transcriptional CREB activation and *Pgc-1α* upregulation.

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2. Methods

2.1 In vitro experiments

2.1.1 Cell lines

Five cell lines were used in the present study. These included mouse myoblasts (C2C12 cells: RCB0987, Riken BRC Cell Bank) mouse embryonic fibroblasts (3T3-L1 cells: JCRB9014, JCRB Cell Bank; original developers: Green et al.), human embryonic fibroblasts (HF cells: JCRB 1006.7, JCRB Cell Bank; original developers: Kouchi and Namba), rat skeletal muscle myoblasts (L6 cells: JCRB 9081, JCRB Cell Bank; original developer: Yaffe), and human embryonic kidney cells (HEK 293 cells). All cell lines were cultured in Dulbecco modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FetalClone™ III serum (GE Healthcare, Chicago, IL, USA), penicillin, and streptomycin and incubated at 37 °C, 100% RH, and 5% CO_2.

2.1.2 Cold stimulations for cells

Cold stimulations of each cell line were performed according to the method of a previous study [10] with minor modifications. In brief, the C2C12, 3T3-L, HF, and L6 lines were seeded at a density of 2 x 10^5 cells in six-well culture plates containing 2 mL medium per cell and incubated at 37 ℃. After 24 h, the culture plates were wrapped in polyethylene bags and placed for 15 min in a shaded container holding water cooled to 4 ℃. This chilling process was performed once, twice, or thrice with 15-min intervals between coolings. During the intervals, the cells were maintained in the incubator at 37 °C. After each cold stimulation, cells were harvested and subjected to western blotting. An overview of these experimental procedures is presented in Fig. 1a.

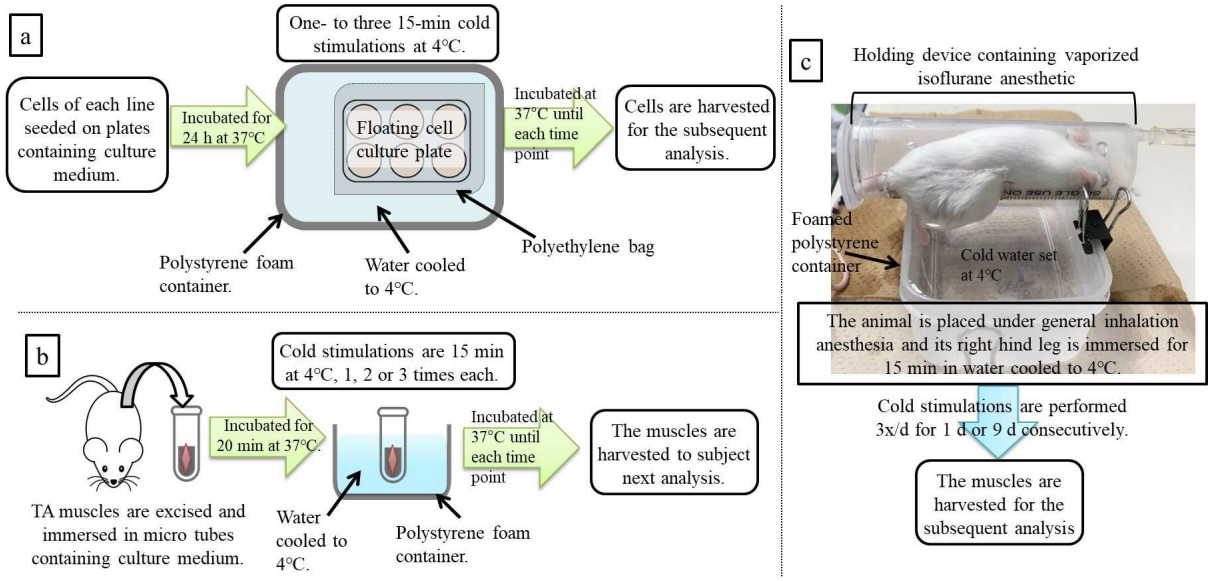

Fig. 1. Overview of cold stimulation methods. a: In vitro experiment; b: ex vivo experiment; c: in vivo experiment.

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2.1.3 Western blotting (WB)

Cells subjected to cold stimulations were dissolved in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, 1 mM EDTA, 10 mM NaF, and 2 mM Na_3VO_4) with protease inhibitor cocktail (Nakalai Tesque, Kyoto, Japan). The cell lysates were centrifuged at 12,000 × g for 15 min at 4 °C and total protein in the supernatants was measured with a bicinchoninic acid (BCA) protein assay kit (TaKaRa Bio, Kusatsu, Shiga, Japan). The protein concentrations were adjusted to 2 mg mL^{-1} with SDS-PAGE buffer and the samples were heated to 95 ℃ for 5 min. Ten-microgram protein samples were subjected to SDS-PAGE on 10% gel at 140 V for 80 min. The gel-bound proteins were applied to polyvinylidene fluoride (PVDF) membranes by the wet transfer method and run overnight at 40 V. Target protein bands ware detected using the primary antibodies anti-p-CREB (ser133) (CST: #9198, Santa Cruz Biotechnology, Dallas, TX, USA: sc-81486), anti-CREB (CST: No. 4820), and anti-GAPDH (Santa Cruz Biotechnology, Dallas, TX, USA: sc-32233) and the secondary HRP-linked antibodies anti-mouse IgG (CST: No. 7076) and anti-rabbit IgG (CST: No. 7074).

2.1.4 Cell damage assessments

Cell damage caused by cold stimulation was evaluated for C2C12, 3T3-L, HF, and L6 cells. Twenty-four hours after the final cold stimulation, cell morphology was examined and a trypan blue assay was performed according to a previously described method [10].

2.1.5 Immunoprecipitation (IP)

To analyze the interaction between p-CREB and CREB-binding protein (CBP; a CREB coactivator), HEK 293 cells were seeded at a density of 2 x 10^5 in a six-well plate containing 2 mL culture medium per cell and incubated at 37 ℃. After 24 h, the cells were transfected with 6 μg of pcDNA3β-FLAG-CBP-HA plasmids (source: Tso-Pang Yao; Addgene plasmid No. 32908; http://n2t.net/addgene:32908; RRID:Addgene_32908) using Polyethylenimine Max (Polysciences, Inc., Warrington, PA, USA) and the existing medium was replaced with DMEM containing 1% FetalClone™ III serum and antibiotics (serum reduction medium) to enhance transfection efficacy. After 12 h, the serum reduction medium was replaced with normal medium and the cells were incubated at 37 ℃ for 24 h. The cells were then subjected to cold stimulations as previously described. Ten minutes after the final cold stimulations, the cells were harvested and lysed and their protein concentrations were measured as previously described. Five hundred micrograms of total protein was mixed with 25 μL of Pierce Anti-HA Magnetic Beads (Thermo Fisher Scientific, Waltham, MA, USA). The samples were incubated with agitation at 4 ℃ overnight then rinsed with a buffer consisting of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP40, and 1 mM EDTA. The beads were boiled in SDS sample buffer to separate the antibody and antigen. In an independent experiment, a positive control sample to enhance the recruitment of CBP to p-CREB was made by applying 50 μM forskolin (FSK; Tokyo Chemical Industries, Chuo-ku, Tokyo, Japan) for 30 min. Interactions between p-CREB and CBP were analyzed by western blotting as previously described. Anti-CBP (Santa Cruz Biotechnology, Dallas, TX, USA: sc-7300) and anti-human influenza hemagglutinin (HA) tag antibody (Wako Pure Chemical Industries Ltd., Chuo-ku, Osaka, Japan: 041-21881) were also used in the western blotting analysis.

2.1.6 Reporter assay using cAMP response elements

To analyze the transcriptional activity of CREB in response to the cold stimulations, a reporter assay was performed. The repeat sequences of the cAMP response elements (CRE; 5'-TGACGTCA-3') were cloned to pGL3-Basic Vector (Promega, Madison, WI, USA). HEK 293 cells were seeded at a density of 0.25 x 10^5 in a twenty-four-well plate containing 500 μL culture medium per cell and incubated at 37 ℃. After 24 h, the cells were transfected with CRE-pGL3-Basic vector plasmids (750 μg) using Polyethylenimine Max. The existing medium was replaced by serum reduction medium consisting of DMEM containing 1% FetalClone™ III and antibiotics to enhance transfection efficacy. After 12 h, the

serum reduction medium was replaced with normal medium and the cells were incubated at 37 °C for 24 h. The cells were then subjected to cold stimulations as previously described. Ten minutes after the final cold stimulation, a reporter assay was performed using a dual-luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Total protein content was also measured with a Pierce Coomassie Plus (Bradford) assay kit (Thermo Fisher Scientific, Waltham, MA, USA) to normalize the luminescence intensity.

2.2 Ex vivo and in vivo experiments

2.2.1 Animals

All animal experiments were approved by the Animal Care Committee, University of Tsukuba (No. 18-071). Forty-eight male CBA/J mouse (4 wks; average weight 14.7 ± 1.2 g) and 12 ICR mice (10–11 wks; average weight 41.1 ± 1.7 g) were used in this study. All animals were maintained in a temperature-controlled environment with a 12-h light/dark cycle and given free access to standard laboratory food and water.

2.2.2 Cold stimulations for ex vivo experiments

An overview of the experimental procedures is shown in Fig. 1b. Both tibialis anterior (TA) muscles were harvested from 24 euthanized CBA/J mice, immersed in a 2-mL micro tube containing DMEM with 10 mM HEPES (pH 7.4), 10% FetalClone™ III, and antibiotics, and pre-incubated at 37 ℃ for 20 min. The micro tubes containing the right TA muscles were immersed for 15 min in a shaded container containing water cooled to 4 ℃. This step was performed once, twice, or thrice. There were 15-min intervals between coolings during which the muscle tissue was kept in an incubator at 37 ℃. The left TA muscles were incubated at 37 ℃ as a control. Ten minutes after the final cold stimulation, the TA muscles were homogenized in lysis buffer containing a protease inhibitor cocktail. The protein concentrations were measured and 10 μg protein was subjected to western blotting to detect p-CREB, CREB, and CBP as previously described. In an independent assay, Sepasol®-RNA I Super G (Nakalai Tesque, Kyoto, Japan) was used according to the manufacturer's instructions to extract total RNA from each TA muscle sample at 1 h after the final cold stimulations. Reverse transcription and cDNA generation were performed with 500 ng total RNA and PrimeScript™ RT Master Mix (Takara Bio, Kusatsu, Shiga, Japan). The cDNA was diluted 10× with nuclease-free water. Quantitative PCR (qPCR) was performed with a KAPA SYBR Fast qPCR kit (Nippon Genetics Co. Ltd., Bungyo-ku, Japan) to evaluate the expression levels of the genes targeting CREB. The 18s ribosomal RNA expression was also measured and gene expressions were normalized by the $2^{-\Delta\Delta Ct}$ method. Primer sequences are presented in Table 1. CREB-targeting genes involved in mitochondrial biogenesis and metabolic regulators such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC-1α*), cAMP responsive element binding protein 1 (*Creb1*), glucose transporter type 4 (*Glut4*), and carnitine palmitoyltransferase 1b (*Cpt1b*) were selected on the basis of earlier studies [14-17].

202 **Table 1. Primer sequences for qPCR.**

No.	Gene	Primer sequences		Reference	No.	Gene	Primer sequences		Reference
1	*18s rRNA*	F:	AACTTTCGATGGTAGTCGCCG		14	*Tomm20*	F:	AGCTGGGCTTTCCAAGTTACC	
		R:	CCTTGGATGTGGTAGCCGTTT				R:	TGGTCCACACCCTTCTCGTA	
2	*Pgc1α*	F:	TCTCAGTAAGGGGCTGGTTG		15	*Tomm70a*	F:	CGGCCAAGCCTGACTTAGAT	
		R:	AGCAGCACACTCTATGTCACTC				R:	TTTTCAGCTGTCCTCGGTGG	
3	*Creb1*	F:	CCCCAGCACTTCCTACACAG		16	*Ucp3*	F:	ACAAAGGATTTGTGCCCTCC	
		R:	CTTGCTGCCTCCCTGTTCTT				R:	TCAAAACGGAGATTCCCGCA	
4	*Glut4*	F:	GCCCCACAGAAGGTGATTGA		17	*Ndufs3*	F:	CTGACTTGACGGCAGTGGAT	18
		R:	GAAGATGGCCACGGAGAGAG				R:	CATACCAATTGGCCGCGATG	
5	*Cpt1b*	F:	TGGCTACGGGGTCTCTTACA		18	*Sdhb*	F:	TGGTGGAACGGAGACAAGTA	18
		R:	GGGCGTTCGTCTCTGAACTT				R:	TGGCAGCGGTAGACAGAGAA	
6	*Tfam*	F:	TAGGCACCGTATTGCGTGAG		19	*Sdhd*	F:	GATCCCTGCTGGGTACTTGA	18
		R:	GACAAGACTGATAGACGAGGGG				R:	AAGTAGCAAAGCCCAGCAAA	
7	*Tfb1*	F:	GGAAGCAAACAGCACAGTCG	18	20	*Uqcr11*	F:	TGCTGAGCAGGTTTCTAGGC	18
		R:	GCTGCTTGATCTTGGGCTCT				R:	TCCTTCTTAAACTTGCCGTTG	
8	*Tfb2*	F:	CCCGTGGACATCCAGGAATC	18	21	*Cox4i1*	F:	TTCAGTTGTACCGCATCCAG	18
		R:	CCACTCTGGCACCAGCTTTA				R:	GGATGGGGCCATACACATAG	
9	*Nrf1*	F:	TGCTTCAGAACTGCCAACCA		22	*Cox5b*	F:	CGTCCATCAGCAACAAGAGA	18
		R:	GTTCCACCTCTCCATCAGCC				R:	AGATAACACAGGGGCTCAGT	
10	*Sod2*	F:	ACAACAGGCCTTATTCCGCT		23	*Atp5l*	F:	CCCCTGCTGAAATCCCTACA	18
		R:	TAGTAAGCGTGCTCCCACAC				R:	TAAAACCACATCCACACCTC	
11	*Cat*	F:	CTCGCAGAGACCTGATGTCC		24	*Atp5po*	F:	GCAACACCCAGGGTATCATC	18
		R:	GACCCCGCGGTCATGATATT				R:	TTGGTTTGGACTCAGGAAGC	
12	*Hmox1*	F:	GAGCAGAACCAGCCTGAACT		25	mtDNA	F:	CTAGAAACCCCGAAACCAAA	19
		R:	AAATCCTGGGGCATGCTGTC				R:	CCAGCTATCACCAAGCTCGT	
13	*Cytc*	F:	AGAAGGGAGAAAGGGCAGAC	18	26	nDNA	F:	ATGGGAAGCCGAACATACTG	19
		R:	TGATCTGAATTTGGTGTGTGAA				R:	CAGTCTCAGTGGGGGTGAAT	
		F: Forward primer. R: reverse primer.							

203

204 2.2.3 Cold stimulations for in vivo experiments

205 An overview of the experimental procedures is shown in Fig. 1c. ICR mice were placed in an in-house-
206 designed holding device and anesthetized by isoflurane inhalation. The right limb of each mouse was
207 immersed to the knee for 15 min in water cooled to 4 °C . This treatment was repeated twice more with
208 15-min intervals between cold stimulations during which the mice were awakened and kept in a
209 breeding cage. The right limbs of the control mice were placed in empty containers. For the acute cold
210 exposure experiment, the treatments were applied for 1 d. For the chronic cold stimulation experiment,
211 the treatments were applied once daily for nine consecutive days. Twelve hours after the final cold
212 stimulation, the animals were euthanized by cervical dislocation under anesthesia induced using
213 inhaled isoflurane, and the limb muscles, TA, extensor digitorum longus (EDL), lateral head of
214 gastrocnemius (LG), and medial head of gastrocnemius (MG) were individually harvested (n =
215 12/group). Total RNA was extracted and qPCR was performed as previously described. Based on earlier
216 studies [14,20-22], *Pgc1a*-regulated, mitochondrial complex, and component genes were selected and
217 measured in this experiment. Biceps femoris and gracilis on the surface of the lateral and medial heads
218 of the gastrocnemius muscle were also harvested and their total DNA was extracted with
219 phenol/chloroform/isoamyl alcohol (25:24:1; Nakalai Tesque, Kyoto, Japan) according to the

manufacturer`s instructions. The total DNA of each sample was adjusted to 10 ng μL^{-1}. Mitochondrial DNA (mtDNA) copy numbers were measured by qPCR as previously described. Primers specific for mtDNA and nuclear DNA (nDNA) were used and gene expressions were normalized by the $2^{-\Delta\Delta Ct}$ method. The right feet and total muscle protein were harvested to assess any adverse effects associated with the cold stimulations. The mouse feet were sectioned at a thickness of 3 μm, embedded in paraffin, stained with hematoxylin and eosin (H&E), and their muscle, bone, and skin components were examined under an optical microscope(Keyence; BZ-X710). The total protein of 100 μg muscle tissue (n = 12/group) was subjected to a thiobarbituric acid-reactive substances (TBARS) assay to measure lipid peroxidation. The TBARS were measured according to the method of Kikugawa et al. [23] with certain modifications. One hundred microliters of total protein (1 mg mL^{-1}) was added to a screw-cap tube containing a mixture of 325 μL thiobarbituric acid (TBA) with 2 mL of 5.2% (w/v) sodium dodecyl sulfate (SDS) in distilled water (DW), 500 μL of 0.8% (w/v) butylated hydroxytoluene (BHT) in glacial acetic acid, 15 mL of 0.8% (w/v) thiobarbituric acid (TBA) in DW, and 17 mL of DW alone. Then 75 μL acetate buffer (pH 3.5) was added to the tube to make up a final volume of 500 μL. The tube was stored at 4 °C for 60 min then heated at 95 °C for 60 min. Then 500 μL of 15:1 v/v 1-butanol and pyridine was added to the chilled tube and the mixture was centrifuged at 3,000 rpm for 10 min at 4 °C. Fluorescence of the supernatant was measured at 540 nm excitation and 590 nm emission. The TBARS concentrations were interpolated from a regression equation plotted with a 1,1,3,3-tetraethoxypropane standard (100 to 0.4 nmol/ml)

2.3 Statistical analysis

Data are presented as means ±SD. Distribution normality was verified with the Shapiro-Wilk test. Normally distributed data of four groups were subjected to one-way ANOVA in GraphPad Prism v. 7.04 (GraphPad Software, La Jolla, CA, USA). Normally distributed data of two groups were subjected to a paired *t*-test or Welch's *t*-test in Excel 2010 (Microsoft Corp., Redmond, WA, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1 Cold stimulation induced CREB1 phosphorylation in a frequency- and duration-dependent manner

Two bands appeared on the C2C12, 3T3-L1, and HF cells in response to anti p-CREP1 antibody. The upper band was p-CREB1 and the lower band was phosphorylated cyclic AMP-dependent transcription factor 1 (p-ATF1). Both of these have the same function. In four cell lines, cold stimulation induced CREB1 phosphorylation in a frequency- and duration-dependent manner. A single cold stimulation slightly induced CREB phosphorylation whereas two and three cold stimulations markedly induced CREB phosphorylation. The strongest and weakest CREB1 phosphorylation was measured in the C2C12 and L6 cell lines, respectively. Thirty minutes after the final cold stimulation, the p-CREB1 bands in all cell lines returned to the same intensity as those of the control (Figs. 2a-d).

3.2 Cold stimulation did not cause cell damage

Total cell numbers, viability, and morphology served as indices of damage in the four cell lines. No changes relative to the control were detected even after three cold stimulations (Figs. 3a-e).

3.3 CBP was recruited to p-CREB in response to cold stimulation

Two bands appeared in response to anti p-CREP1 antibody. The upper band was p-CREB and the lower band was phosphorylated cyclic AMP-dependent transcription factor 1 (p-ATF1). An interaction between CBP and p-CREB1 was observed in the control (Con.) lane. The interaction was also strong in response to the cold stimulations and induced an increase in CREB1 phosphorylation (Fig. 4; lane: 1–3 cold stimulations). Therefore, CBP recruitment to p-CREB1 increased in response to cold stimulation. The p-CREB1:CREB1 ratios in whole cell lysates increased with the number of cold stimulations.

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Nevertheless, the p-CREB1:HA (CBP) ratios (indices of post immunoprecipitation (IP) binding intensity) increased equally for all cold stimulation repetitions (Fig. 4).

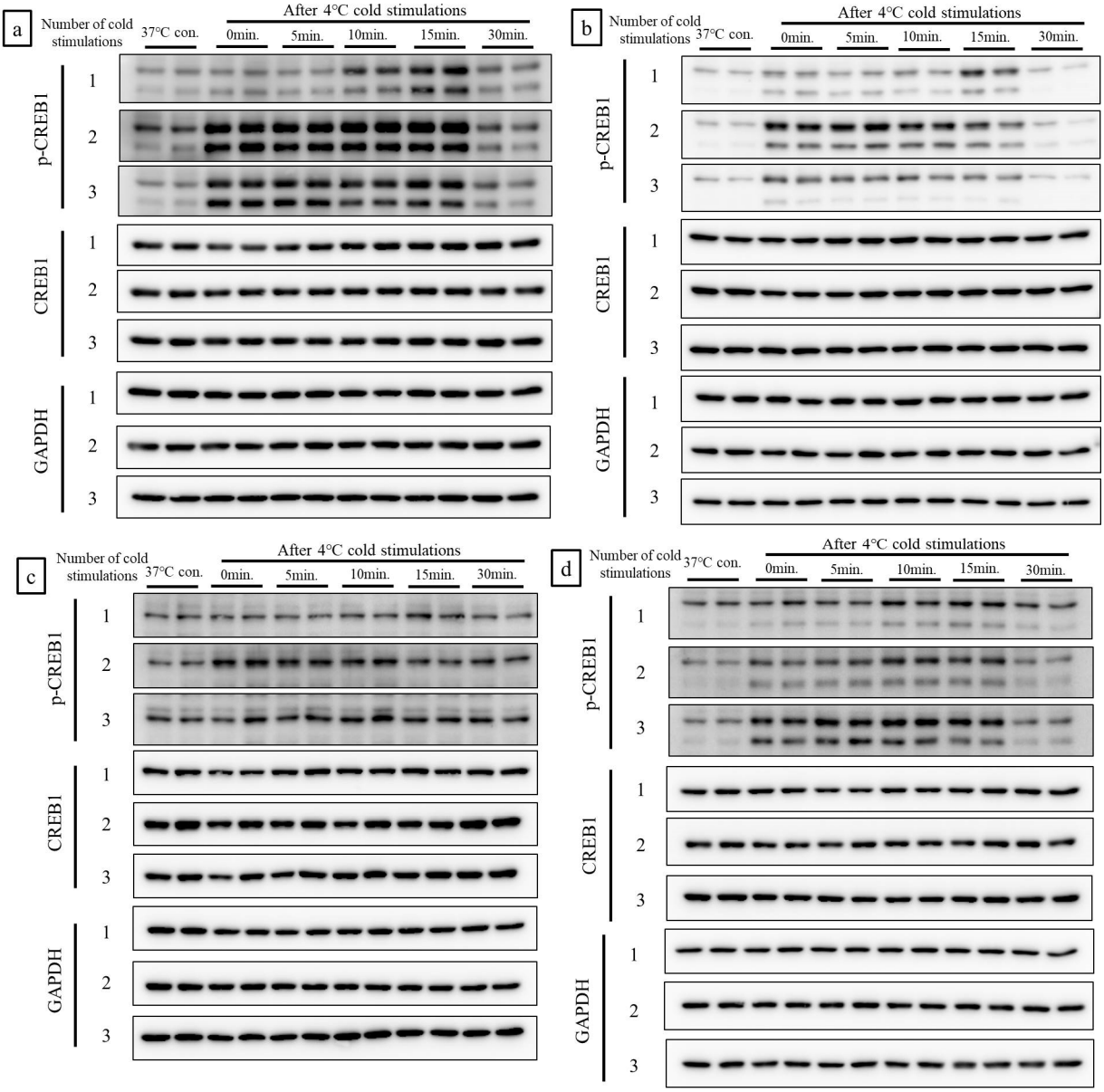

Fig. 2. Cold stimulation induced CREB1 phosphorylation in a frequency- and duration-dependent manner. a: C2C12 cells; b: 3T3-L1 cells; c: L6 cells; d: HF cells. Con.: control (no cold stimulation).

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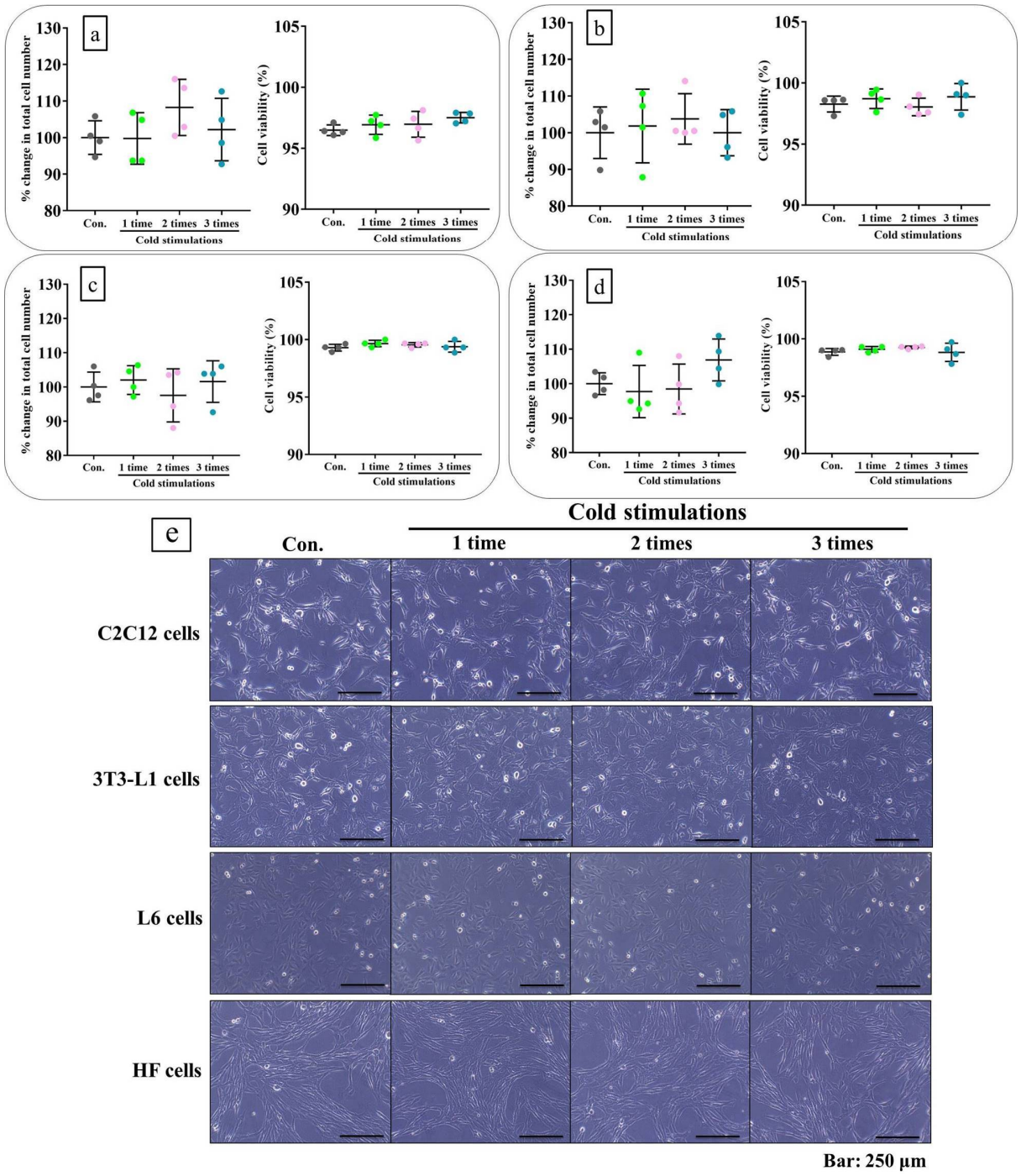

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273 **Fig. 3. Cold stimulation did not cause cell damage.** Percent change in total cell number and viability. a:
274 C2C12 cells; b: 3T3-L1 cells; c: L6 cells; d: HF cells. e: cell morphology. Con: control (no cold stimulation).

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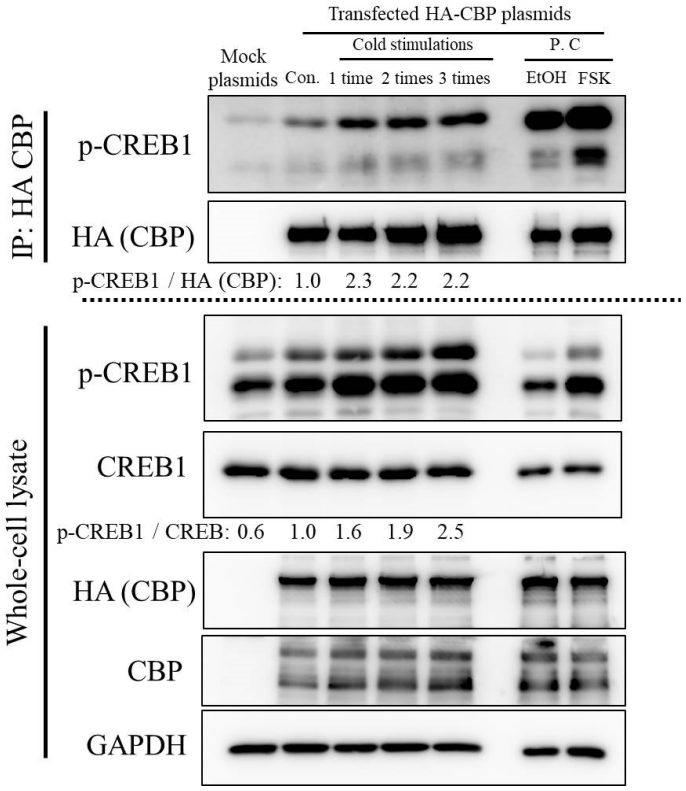

Fig. 4. Cold stimulation induced the recruitment of CBP to p-CREB1. P.C lane: positive control as check with forskolin (FSK) or ethanol (EtOH) for increases in interactions between CBP and p-CREB. Mock plasmids were used as negative controls. Con.: control (no cold stimulation).

3.4 Cold stimulation activated CREB transcription

Cold stimulation significantly activated CREB transcription in an exposure frequency-dependent manner. A single cold stimulation did not activate CREB transcription whereas two or three cold stimulations did so. Three cold stimulations induced the strongest transcriptional activity (Fig. 5).

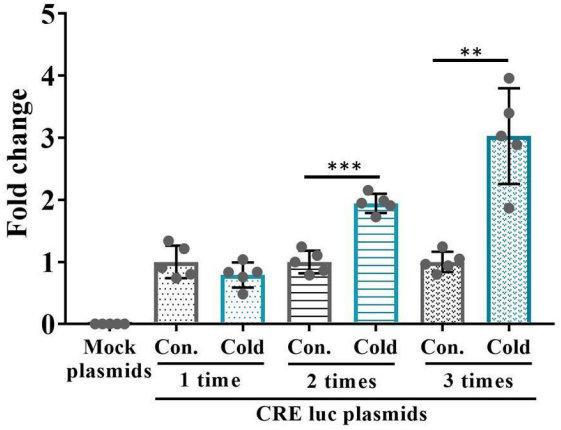

Fig. 5. Cold stimulations activated CREB transcription in an exposure frequency-dependent manner. Mock plasmids were used as negative controls with values ~0. Con: control (no cold stimulation); Cold: 15-min cold stimulations. N = 5 per group. ** $P < 0.01$, ***$P < 0.001$ according to Welch's *t*-test.

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3.5 Cold stimulations upregulated the CREB-targeting gene and induced CREB1 phosphorylation

One- to three cold stimulations significantly induced CREB1 phosphorylation in TA muscle. However, the intensities of the p-CREB1 bands in response to one, two, or three cold stimulations were nearly equal (Figs. 6a, b). Conversely, the expressions levels of the CREB1-targeting genes significantly increased with the number of cold stimulations. A single cold stimulation had almost no effect at enhancing gene expression whereas two or three cold stimulations (especially the latter) significantly upregulate the genes (Fig. 7).

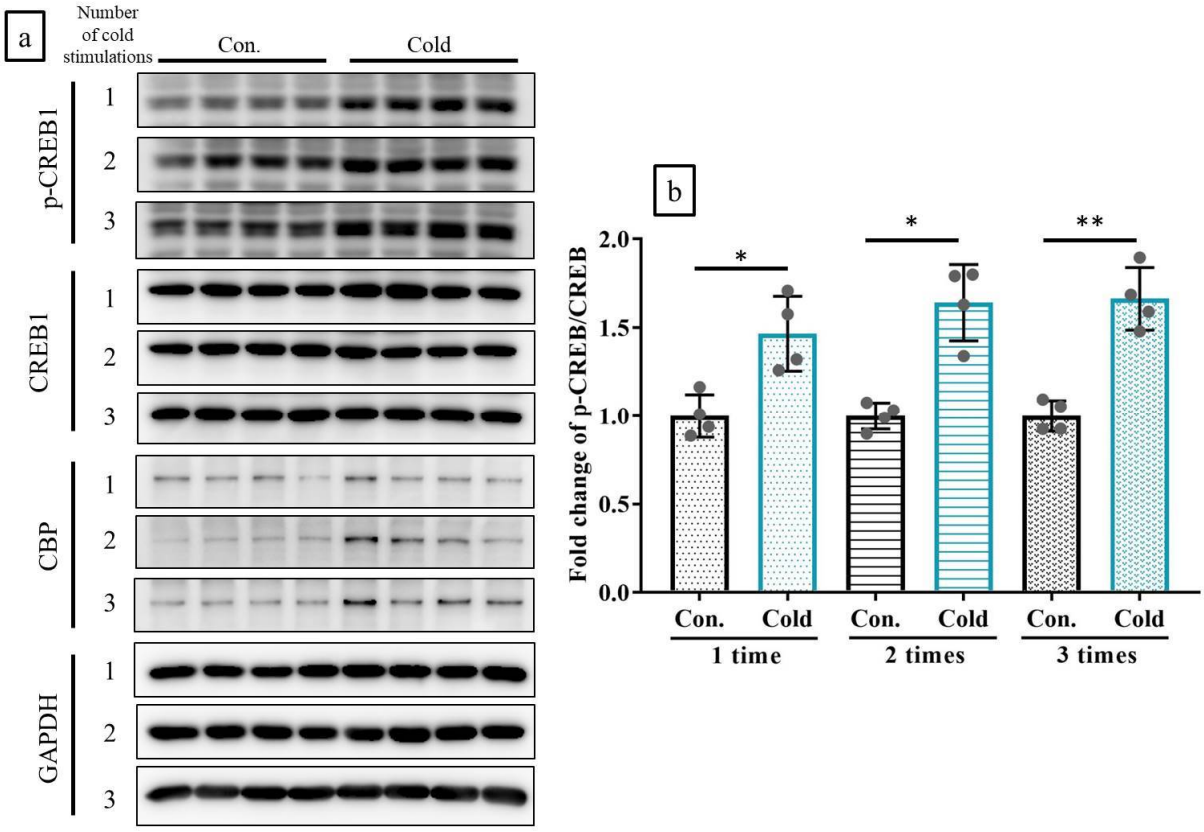

Fig. 6. Cold stimulations significantly induced ex vivo CREB1 phosphorylation. a: WB analysis of p-CREB1 and other factors; b: Quantification of band intensities of p-CREB1/CREB1. Con: control (no cold stimulation); Cold: 15-min cold stimulations. N = 4 per group. * $P < 0.05$, ** $P < 0.01$ according to a paired *t*-test.

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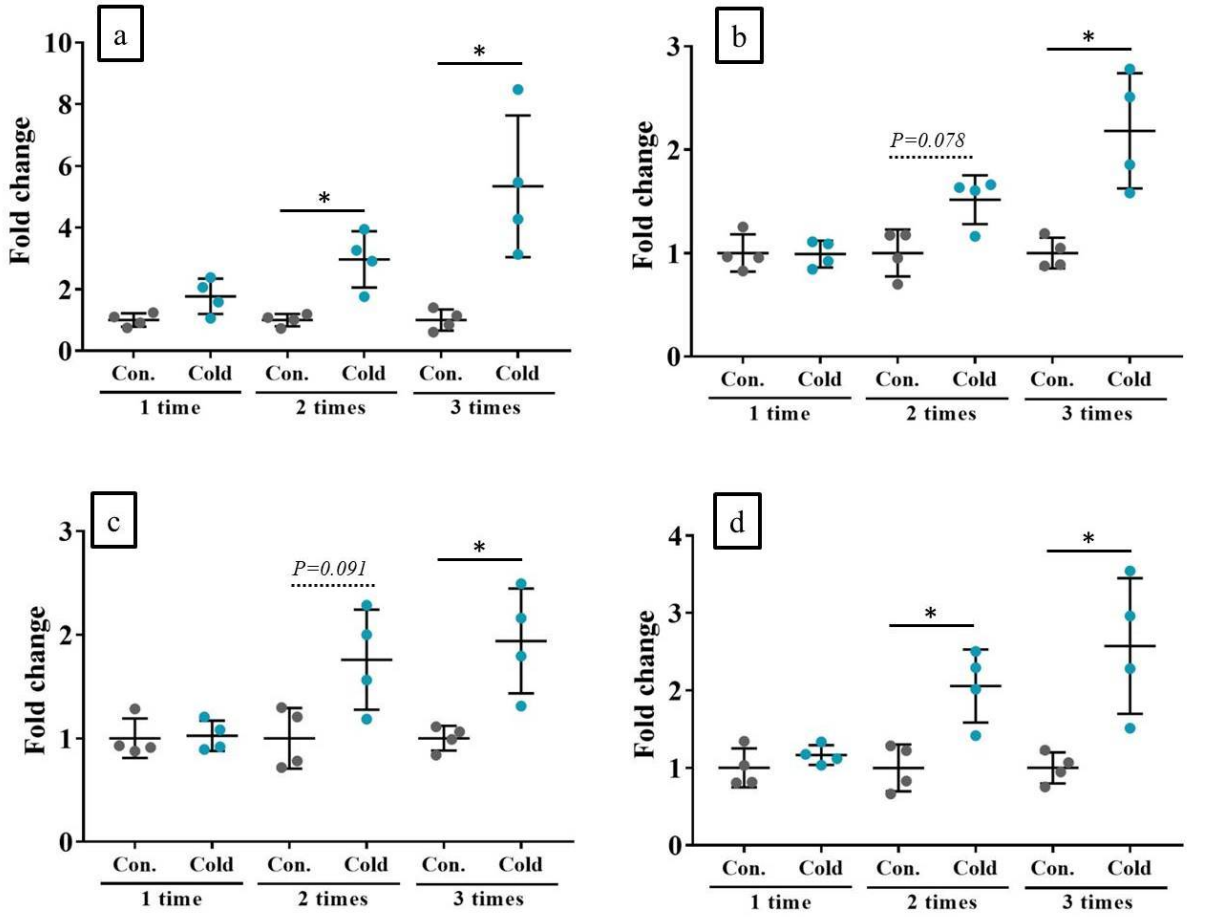

Fig. 7. Cold stimulations significantly upregulated CREB1-targeting genes in an exposure frequency-dependent manner. a: *Pgc-1α*; b: *Creb1*; c: *Glut4*; d: *Cpt1b*. Con: control (no cold stimulation); Cold: 15-min cold stimulation. N = 4 per group. * $P < 0.05$ according to a paired *t*-test.

3.6 Chronic cold stimulation increased mtDNA copy number, mitochondrial biogenesis, and its component gene expression levels whereas acute cold stimulation had no such effects

Chronic cold stimulations of mouse limb significantly increased mtDNA copy number in the muscles. Mitochondrial components, complexes, and biogenesis and the expression levels of CREB-targeting genes were also increased. The average rate of increase was ~1.5–2× (Figs. 8a-e). However, acute cold stimulations did not have any of these effects. *Ndufs1* (a complex 1 gene) was upregulated by only ~4× (Figs. 9a-e).

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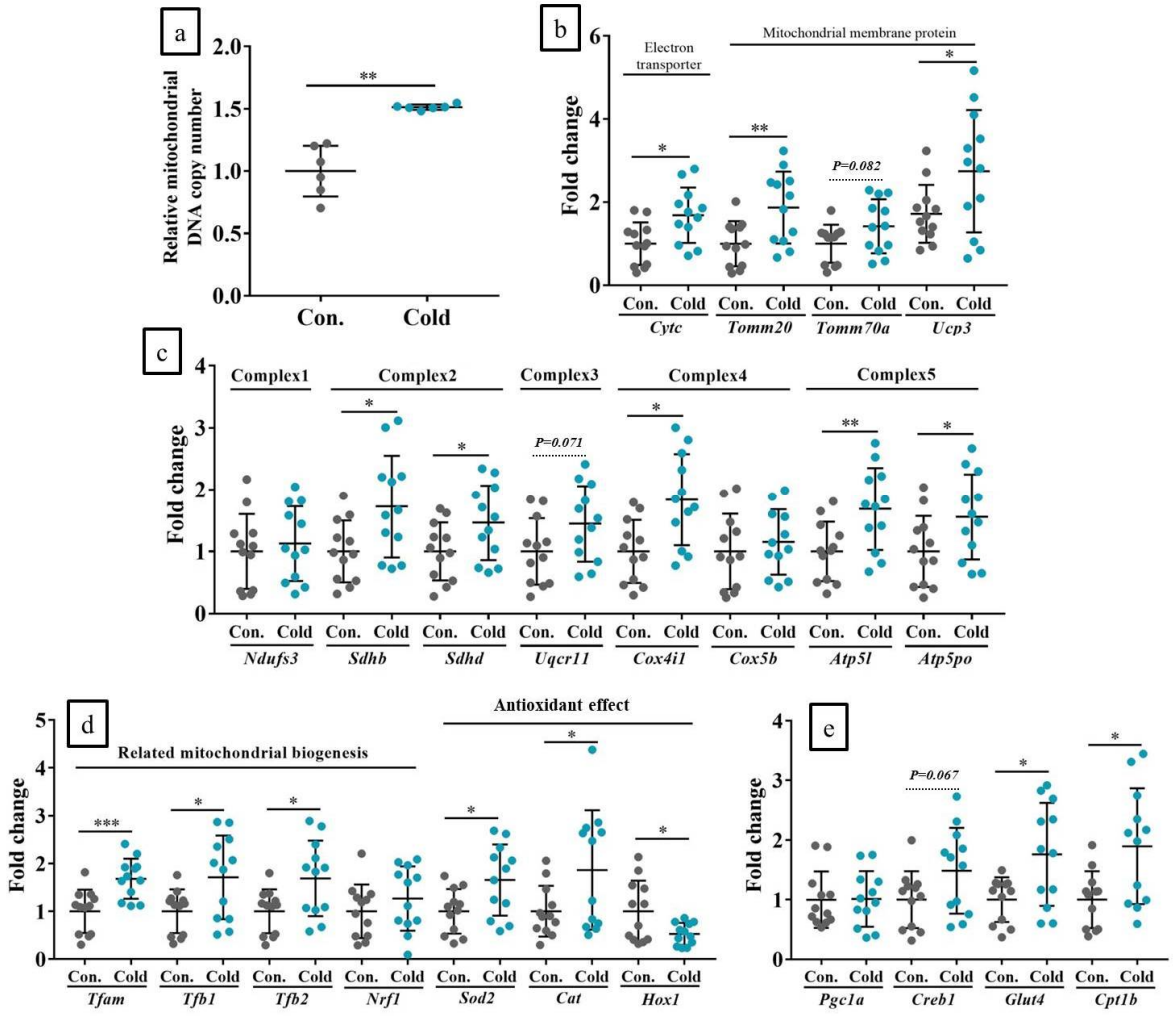

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312 **Fig. 8. Chronic cold stimulation increased mtDNA copy number and mitochondrial biogenesis.** a:
313 Mitochondrial DNA copy number; b: mitochondrial component genes; c: mitochondrial complex genes;
314 d: *Pgc1-α* regulated genes; e: CREB-targeting genes in response to chronic in vivo cold stimulation. Con:
315 control (no cold stimulation); Cold: 15-min cold stimulations. N = 12 per group. * $P < 0.05$, ** $P < 0.01$
316 according to Welch's *t*-test.

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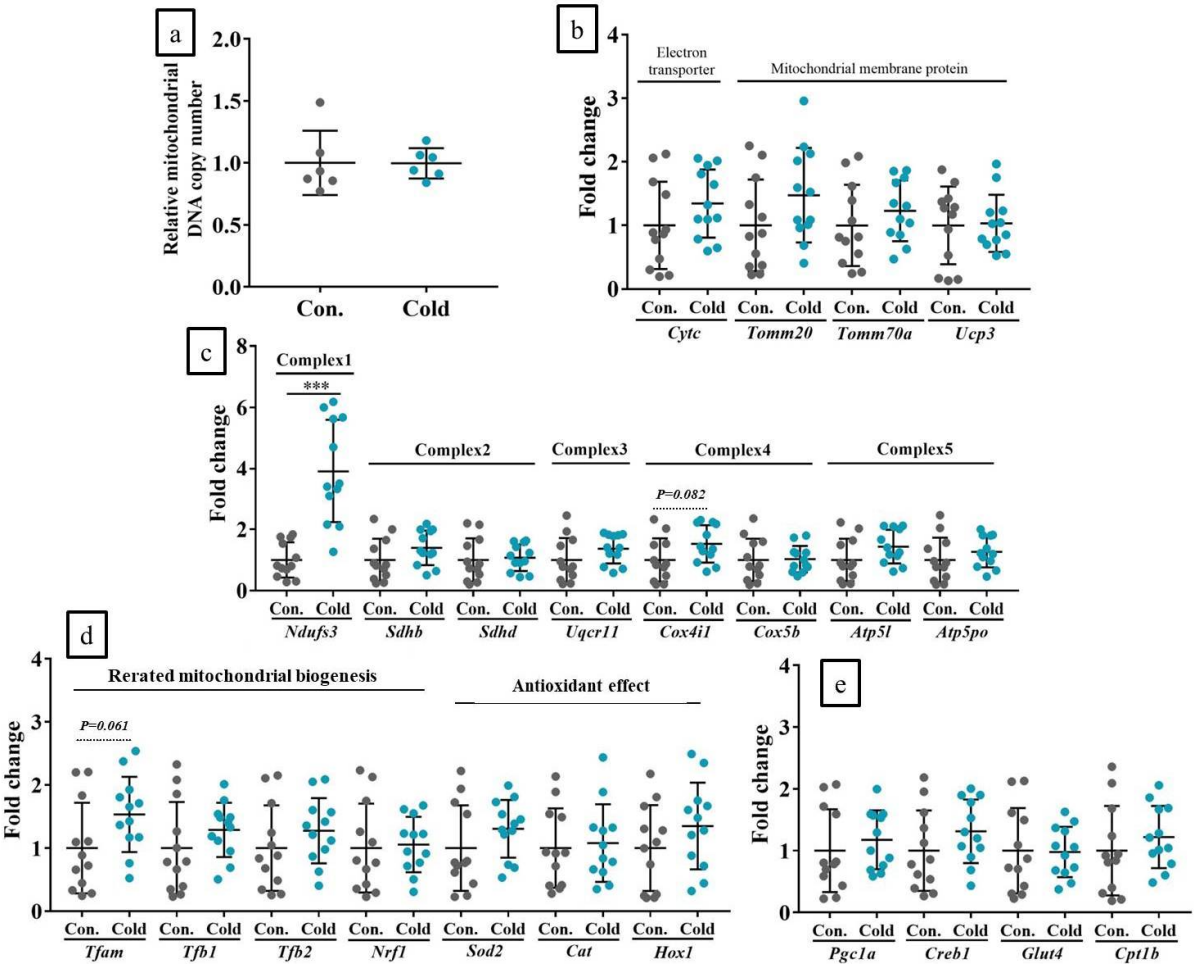

Fig. 9. Acute cold stimulation had almost no effect on mitochondrial biogenesis or other factors. a: Mitochondrial DNA copy number; b: mitochondrial components genes, c: mitochondrial complex genes, d: *Pgc1-α* regulated genes; e: CREB targeting genes in acute cold stimulation in vivo. Con: control (no cold stimulation); Cold: 15-min cold stimulation. N = 12 per group. ***$P < 0.001$ according to Welch's *t*-test.

3.7 Cold stimulation had no adverse effect

In H&E-stained specimens of mouse feet subjected to acute and chronic cold stimulation, there was no evidence of any adverse effect such as tissue inflammation or degeneration of muscle, bone, or skin tissue. Neither treatment altered body weight or muscle TBARS levels (Figs. 10a-c).

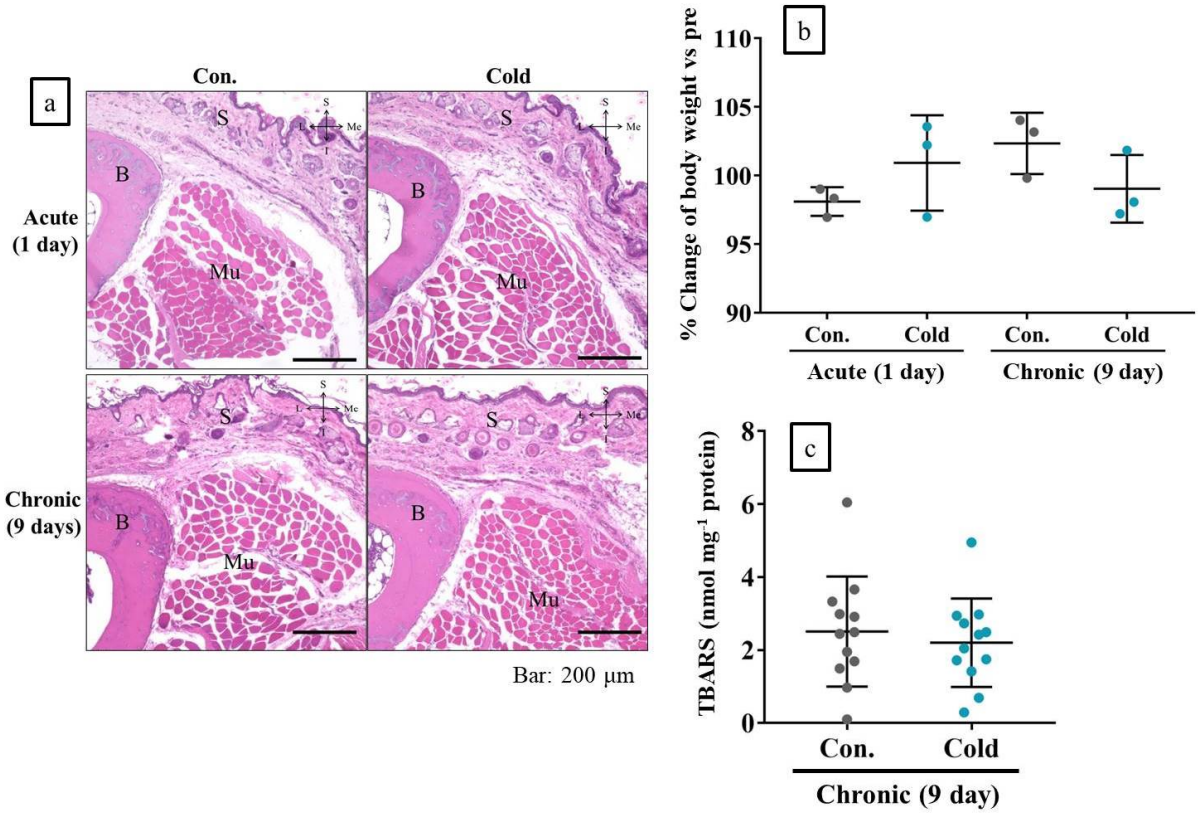

Fig. 10. Acute and chronic cold stimulations did not induce adverse effects in muscle, bone, or skin tissue and did not alter body weight. a: Representative H&E-stained tissue sample; b: % change in body weight relative to pre-experiment; c: TBARS levels in limb muscle in response to chronic cold stimulation. B: bone (second metatarsal); S: skin; Mu: muscle (first dorsal interosseous muscles of foot); S: superior; I: inferior; L: lateral; Me: medial. Con: control (no cold stimulation); Cold: 15-min cold stimulation. N = 3 for body weight. N = 12 for TBARS levels.

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4. Discussion

In both the in vitro and in vivo experiments, cold stimulation induced CREB1 phosphorylation, activated CREB1 transcription, and upregulated CREB-targeting genes. Sympathetic nerves were not stimulated. In response to cold exposure, the sensory nerves in peripheral tissues transduce the signal to the hypothalamus which regulates the sympathetic nervous system (SNS) and triggers the release of noradrenalin (NE) from the nerve terminals whence it enters the target tissue [24]. NE binds to the β-adrenergic receptor (β-AR) and activates downstream signals including the production of cyclic AMP (cAMP), activation of protein kinase A (PKA), phosphorylation of CREB, upregulation of *Pgc1-α*, uncoupling of protein 1 (*Ucp1*), etc. [24]. In the present study, however, the SNS had no apparent influence on the release of NE to the β-AR because the in vitro and ex vivo experiments conducted here did not consider the nervous systems. Cryotherapy induces CREB1 phosphorylation and CREB-targeting gene upregulation in human skeletal muscle. In the present study, however, we did not identify the upstream signals of p-CREB1 which could help predict other CREB1 phosphorylation pathways such as cAMP-PKA or Ca^{2+}/calmodulin (CaM)-Ca^{2+}/calmodulin-dependent protein kinase (CaMK).

In vitro analysis of the interaction between p-CREB1 and CBP indicated that cold stimulation induced the recruitment of CBP to p-CREB. In this process, the transcription and induction of CREB-targeting genes increase [25,26]. CBP is a transcriptional coactivator and an acetyltransferase serving as a bridge for DNA-bound transcription factors (activators). It forms a basal transcription machinery through direct interactions, acts as a histone acetyl transferase to relax histone, and is an epigenetic regulator enhancing transcription [27]. Therefore, downstream responses to optimal cold stimulation may link epigenetic regulation to gene expression in muscle tissues. The ex vivo experiment confirmed that CBP and CREB1 protein were expressed in the muscle tissue.

Sarver et al. [28] performed a detailed investigation of the effects of local cryotherapy using metabolome and transcriptome analyses. A single 15-min ice cup massage of the anterolateral thigh in normal human subjects had virtually no effect on muscle metabolomics or transcriptomics. Therefore, the authors suggested that acute local cryotherapy has nearly zero biological effect on muscle. In the present study, however, CREB transcription increased with CREB-targeting gene upregulation. The number of cold stimulations applied differed between the present study and that of Sarver et al. [28]. Our previous study demonstrated that temperature significantly influences the extent to which cold stimulation elicits biological effects. The optimal cold temperature range was 4–17 °C [10]. Therefore, the best results of local cryotherapy of human muscle are probably obtained when cold stimulation is repeated twice or thrice at 4–17 °C.

Two or three 15-min cold stimulations markedly enhanced CREB1 phosphorylation especially in C2C12 myoblasts and 3T3-L1 fibroblasts. One to three 15-min cold stimulations substantially increased CREB1 phosphorylation in TA muscle. In the ex vivo experiment, however, the extent of CREB1 phosphorylation was independent of the number of cold stimulations applied. In the in vivo experiment, the CREB-targeting genes *Pgc1-α*, *Glut4*, *Creb1* and *Cpt1b* were dramatically upregulated when cold stimulations were applied only two or three times for 15 min each time. Taken together, the foregoing findings indicate that the number of cold stimulations significantly affects CREB1 activation in local cryotherapy.

In vivo experiments revealed that the mitochondrial DNA copy number, the genes associated with mitochondrial components, complexes, and biogenesis, and the CREB-targeting genes were considerably increased in response to three 15-min cold stimulations per day for 9 d. In contrast, single and short-term cold stimulations had virtually no effect on any of the aforementioned parameters. Therefore, repetition of cold stimulation over > 1 d was also necessary to elicit CREB1 activation and

mitochondrial biogenesis. For acute, short-term local cryotherapy in vivo, only the expression of *Ndufs1* was markedly altered. This gene encodes a major protein in mitochondrial complex 1.

In a wound healing experiment, it was found that mitochondrial dysfunction induced by embelin hindered tissue repair [29]. CREB enhanced tissue repair by inducing the secretion of WNT1-inducible signaling protein 1 (WISP-1) [30]. Activated CREB also enhanced cell migration [31]. Excessive ROS production or impaired ROS detoxication resulted in oxidative damage which impedes chronic wound healing [32]. In the present study, we discovered that optimal cold stimulation activates CREB1 and mitochondrial biogenesis and upregulates antioxidant genes. Therefore, optimal cryotherapy may stimulate tissue repair following sprains, pulled muscle, and bruises.

It was reported that cryotherapy for tissue injury [6] and cold stimulation for cellular repair [33] applied for > 24 h hinder tissue and cell recovery, respectively. Reactive oxygen species (ROS) produced in response to DNA damage and cell death increased when the temperature of the cell culture was lowered to 25 °C, maintained there for 5 d, then raised to 37 °C [34]. In the present study, however, 15-min cold stimulations repeated several times did not induce cell damage in vitro or tissue degradation in vivo. Moreover, they did not alter the levels of TBARS which indicate chronic oxidative stress in muscle. Therefore, both short-term and longer-term cold stimulations were safe and effective in vivo and in vitro.

There are some limitations to this study in that we used only cell lines and animals. Therefore, the knowledge gained here cannot be directly applied to human subjects. To clarify the true effect of local cryotherapy on human muscles, a randomized controlled trial (RCT) involving cryotherapy as a treatment for muscle trauma or fatigue is needed. If an RCT is performed in human subjects, the cold stimulation temperature and applied number of stimulations may be important factors for eliciting an effect from local cryotherapy. This is because in this study, we discovered that the expression of CREB-targeting genes and transcriptional activation of CREB were dependent on repeated numbers of cold stimulation both in vitro and in vivo. Moreover, in our previous study, we discovered that cold stimulation increased mitochondrial activities dependent on cold temperature. In addition, it could be hypothesized that because the temperature and stimulation numbers differed among previous in vivo studies, consistent results have not been obtained [2-8]. In order to solve these problems, the consistent management of cooling temperatures and application methods may be essential for a human study of local cryotherapy.

5. Conclusions

The present study showed that optimal local cold stimulations activate CREB transcription and increase its downstream reactions such as mitochondrial biogenesis in local muscle without SNS involvement. In addition, the magnitudes of these effects were commensurate with the frequency and duration of the cold stimulations. Our model is presented in the form of a graphical abstract. The discoveries reported in the present study could accelerate the refinement and practical application of local cryotherapy.

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Author Contributions

The first author performed most of the experiments in the present study and wrote the paper. The second and fourth authors prepared the tissue specimens shown in Fig. 10a. The third author made the CRE reporter vector

and assisted the first author in setting up the experiments. The fifth to ninth authors inclusive assisted the first author in the WB and IP analyses. The tenth and eleventh authors reviewed the article.

Conflicts of Interest

The authors declare no conflicts of interest in the present study.

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