AKADÉMIAI KIADÓ

Intermittent cold exposure upregulates regulators of cardiac mitochondrial biogenesis and function in mice

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ABSTRACT

Hypothermic conditions enhance the incidence of cardiovascular diseases due to increased blood pressure. Cold-induced adaptive thermogenesis increased mitochondrial biogenesis and function in skeletal muscles and adipocytes. Here, we studied the effect of intermittent cold exposure on the regulators of cardiac mitochondrial biogenesis, function, and its regulation by SIRT-3. Intermittent cold exposed mice hearts showed normal histopathology with increased mitochondrial antioxidant and metabolic function, as evidenced by an increase in the activity and expression of MnSOD and SDH. A substantial increase in mitochondrial DNA copy number and increase in the expression of PGC-1α and its downstream targets NRF-1 and Tfam indicated the possibility of enhanced cardiac mitochondrial biogenesis and function on intermittent cold exposure. Increased mitochondrial SIRT-3 level and decreased total protein lysine acetylation indicate increased sirtuin activity in cold exposed mice hearts. *Ex vivo* cold mimic using norepinephrine showed a significant increase in PGC-1α, NRF-1, and Tfam levels. AGK-7, a SIRT-3 inhibitor, reversed the norepinephrine-induced upregulation of PGC-1α and NRF-1, indicating the role

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of SIRT-3 on the production of PGC-1α and NRF-1. Inhibition of PKA with KT5720 in norepinephrine treated cardiac tissue slices indicates the role of PKA in regulating the production of PGC-1α and NRF-1. In conclusion, intermittent cold exposure upregulated the regulators of mitochondrial biogenesis and function through PKA and SIRT-3 mediated pathway. Our results emphasize the role of intermittent cold-induced adaptive thermogenesis in overcoming chronic cold-induced cardiac damage.

INTRODUCTION

Prolonged exposure to low temperatures causes mortality and morbidity [1]. Mammals respond to cold through behavioral and physiological changes, which helps minimize heat dissipation [2]. Chronic exposure to cold is reported to develop significant elevation in blood pressure, tachycardia, and cardiac hypertrophy [1]. Though elevation in blood pressure is an adaptive response to cold, constantly elevated blood pressure could result in cardiovascular damage [1]. The mechanism to maintain body temperature in endotherms is called adaptive thermogenesis, in which the body produces additional heat above the basal metabolic rate [3]. Although brown adipose tissue is the major site where adaptive thermogenesis occurs [4], changes to maintain core body temperatures also affect the cardiac system [5]. Mitochondrial biogenesis and metabolism play an important role in cardiac pathophysiology, as cardiac system depends more on mitochondria for their energy needs [6]. Both physiological and pathological stimuli enhance mitochondrial biogenesis to maintain cardiac metabolism and function [6]. Factors like environmental stresses, exercise, caloric restriction, oxidative stress, cell division, renewal, and differentiation can trigger mitochondrial biogenesis [7]. Replication and transcription of mitochondrial DNA are mainly regulated by mitochondrial transcription factor-A (Tfam) [8], which in turn is transcriptionally regulated via NRF-1 & 2 [9]. PGC 1-α, the master regulator of mitochondrial biogenesis and function, regulates the induction of NRF-1&2 [10–12]. According to previous reports exercise and cold exposure promoted mitochondrial biogenesis in the soleus muscles of experimental mice [13].

Sirtuins are highly conserved NAD^+ dependent class III histone deacetylases that act on acetylated proteins involved in cellular metabolism. SIRT-3 is highly expressed in the heart and has a cardiac protective role [14]. It is a mitochondrial matrix protein which regulates oxidative phosphorylation and ATP synthesis [15–17]. It has also been reported that during pathological cardiac hypertrophy, SIRT-3 influences oxidative metabolism by regulating mitochondrial proteins [18]. In C2C12 myotubes, SIRT-3 regulates mitochondrial biogenesis and ROS production [19]. Another study suggested a positive feedback regulation of PGC-1 α and SIRT-3 expression [19]. Cold exposure enhances SIRT-3 expression in BAT. Overexpression of SIRT-3 in HIB1B brown adipocytes upregulated a series of mitochondrial-related genes [20]. Following the previous reports, adaptive conditions like intermittent cold exposure may affect the regulation of cardiac mitochondrial biogenesis and function. Mild cold exposure induces a cold adaptive state, which could be a promising strategy against chronic cold-induced deleterious effects. In this context, we have studied the effect of intermittent cold exposure on the regulators of cardiac

mitochondrial biogenesis, function, and its mechanism, including the role of SIRT-3. Our results showed that intermittent cold exposure leading to increase in mitochondrial biogenesis and function. Norepinephrine released on cold exposure binds to β-adrenergic receptors and activate cAMP-PKA pathway leading to PGC-1α activation. PGC-1α in turn activates its downstream targets NRF-1 and Tfam leading to increase in mitochondrial DNA copy number.

METHODS

Animals and cold exposure

All animal experiments were reviewed and approved by the Institutional animal ethics committee (IAEC1-KU-12b/2014-15-BC-SK (4b)). Female *Mus musculus* (swiss albino) mice aged 3–4 months were used for the experiment. A total of 24 animals were used for the experiments included in this study. Animals were divided into two groups of 6 animals each and were housed under a 12 h light-dark cycle. Feed and water were given *ad libitum*. Group 1 was maintained at room temperature (28 °C), and Group 2 was exposed to intermittent cold at 2–8 °C for an hour for three weeks. After the treatment, animals were sacrificed, and cardiac tissue and interscapular BAT were collected for the study.

Histochemical analysis

Cardiac tissue was collected, fixed in 10% formaldehyde, and preserved at room temperature. Histochemical analysis was done by hematoxylin and eosin stain and observed under an optical microscope.

Enzyme-linked immunosorbent assay (ELISA)

Protein equivalent volumes of cardiac tissue lysates were coated on elisa plates and incubated overnight. After washing each well, they were incubated with primary antibodies of UCP-1 (U6382; Sigma Aldrich), PGC-1α (SAB4200209; Sigma Aldrich), NRF-1 (#12381; Cell Signaling Technology), SIRT-3 (#5490; Cell Signaling Technology), MnSOD (#13141; Cell Signaling Technology), and HRP conjugated secondary antibody (#7074; Cell Signaling Technology) with 1: 1,000 dilution were used for indirect ELISA. O-phenylenediamine dihydrochloride (P8787; Sigma Aldrich) dissolved in 0.05 M citrate phosphate buffer pH 5, and H_2O_2 was used as substrate. The intensity of the colour was read at 490 nm [21].

RNA isolation and quantitative real-time PCR (qRT-PCR)

500 ng of total RNA from each sample was reverse transcribed into cDNA using Bio-rad iScript cDNA synthesis kit according to the manufacturer's protocol. Quantitative RT-PCR was carried out using Bio-rad Sybr Green master mix. Details of the primers are β-actin, forward 5′CAAGATCATTGCTCCTCCTG3′ and reverse 5′TCATCGTACTCCTGCTTGCT3′; MnSOD, forward 5′TTACGCGCAGATCATGCA3′ and reverse 5′GGTGGCGTTGAGATTGTTCA3′, SDH, forward 5′GTTGCTGTGTGGCTGATCG3′ and reverse 5′GCACAGTGCAATGACACC AC3′; Tfam, forward 5′GAGCGTGCTAAAAGCACTGG3′ and reverse 5′GCTACCCATGCTG GAAAAACA3′; SIRT-3, forward 5′GCTGCTTCTGCGGCTCTATAC3′ and reverse 5′GCTACC CATGCTGGAAAAACA3′; PGC-1α, forward 5′AAACTTGCTAGCGGTCCTCA3′ and

5′TGGCTGGTGCCAGTAAGAG3'; NRF-1, forward 5′CCACATTACAGGGCGGTGAA3′ and reverse 5′AGTGGCTCCCTGTTGCATCT3′.

Zymographic assay of SOD

Cardiac tissue lysate was prepared using extraction buffer (0.05 M Tris-HCl pH 8.5) and centrifuged at 12,000 rpm for 20 min. Protein equivalent volumes of samples were separated using 10% native acrylamide gel electrophoresis at 4 °C using Tris-glycine buffer pH 8.5. The gel was immersed in a SOD staining solution containing NBT, riboflavin 5 phosphate, and TEMED for visualizing the bands [22].

Western blotting

Cardiac tissue lysate were prepared using RIPA buffer. Protein concentration was measured by Lowry's method [23]. 10% polyacrylamide gel electrophoresis [24] was done to separate the proteins and were transferred onto the nitrocellulose membrane. The membrane was blocked using 5% skimmed milk powder and incubated with primary antibodies of Acetyl lysine (#9441; Cell Signaling Technology), PGC-1α (SAB4200209; Sigma Aldrich) and SIRT-3 (#5490; Cell Signaling Technology) (1:1,000) overnight at 4 °C. Then the membrane was washed and incubated with appropriate secondary antibodies (#7074; Cell signaling Technology) for 1 h at room temperature [25]. The immunoblotting images were captured with Chemidoc XRS+ System (Bio-Rad) by developing the membranes in Clarity Western ECL Substrate (170-5060; Bio-Rad).

Succinate dehydrogenase assay

SDH activity was estimated by the method of Slater and Bonner [26]. The reaction mixture contained 1 ml phosphate buffer (0.3 M), 0.1 ml EDTA (0.03 M), 1 ml potassium ferricyanide (0.075 M) and 0.3 ml sodium succinate (0.4 M) made up to 2.9 ml with water. The reaction was started by adding the enzyme, and a change in absorbance was noted during the first 2 min. Absorbances were measured at 420 nm at 15-s intervals.

Mitochondrial DNA copy number

Phenol-chloroform method was used to isolate total DNA from cardiac tissues. Mitochondrial DNA copy number was measured by qRT-PCR. The mitochondrial DNA copy number was assessed as COX-II (mitochondrial encoded gene)/Cyclophilin A (nuclear-encoded gene) ratio [27]. The primers are Cyclophilin A forward 5′TTCCTCCTTTCACAGAATTATTCCA3′ and reverse 5′ CCGCCAGTGCCATTATGG3′; forward COX-II 5′CAGGCCGACTAAATCAAGCA AC3′ and reverse 5′ CTAGGACAAT GGGCATAAAGCT3'.

Tissue slice culture

Cardiac tissue was collected from female *Mus musculus* mice, which were washed and removed blood and adipose tissue [28]. Tissues were sliced about 1 mm in thickness and were maintained in DMEM (D7777; Sigma Aldrich) with 10% FBS and 1% antibiotic and antimycotic solution with and without norepinephrine (69815-49-2; Calbiochem) for 8 h at 37° C in a CO_2 incubator.

Statistical analysis

Statistical analysis was done using Graph Pad Prism 9.4.1. All the data were expressed in Mean $\pm$ SEM. The significance of difference between two groups were analyzed by Student's t-test and more than two groups were analyzed by One way ANOVA.

RESULTS

Effect of intermittent cold exposure on body weight, organ weight, cardiac hypertrophic index, and the induction of adaptive thermogenesis

We have selected mice subjected to intermittent cold exposure as the model system for studying the regulators of cardiac mitochondrial biogenesis and function during adaptive thermogenesis. Female mice of 3–4 months were subjected to cold exposure at 2–8 °C, 1 h daily for 3 weeks, and cardiac tissues were harvested. Daily food intake and changes in the body weight of animals were observed during the experimental period. There was no significant change in food intake and gain in body weight between the control and cold exposed animals (Table 1). Intermittent cold exposure showed normal cardiac hypertrophic index indicating that the heart adapted to cold exposure without inducing hypertrophy. An increase in brown adipose tissue UCP-1 (Fig. 1A) indicates induction of adaptive thermogenesis in animals. Intermittent cold exposure significantly upregulated UCP-1 production in interscapular BAT.

Effect of intermittent cold exposure on cardiac histopathology

Histopathological analysis of both control and cold exposed mice hearts was done to study the myocardial tissue damage on intermittent cold exposure. Results showed normal myocardial structural features. Myocytes appeared normal, and there was no evidence of hypertrophy, inflammation/necrosis, or edema (Fig. 1B).

Effect of intermittent cold exposure on mitochondrial antioxidant function and metabolism

One of the major functions of mitochondria is to protect the cell from free radicals. The activity of the antioxidant system indicates the proper functioning of mitochondria. MnSOD and Succinate dehydrogenase represent crucial anti-oxidant and metabolic marker of mitochondria. The effect of intermittent cold exposure on MnSOD and Succinate dehydrogenase activity and expression were analyzed. Zymography with cardiac tissue homogenate showed increased activity of MnSOD in intermittent cold exposed mice (Fig. 2A). Protein and mRNA expression of MnSOD also showed a significant increase on cold exposure compared to control (Fig. 2B, C).

Table 1. Body weight gain, Internal organ weight, Heart weight/Body weight ratio on cold exposure

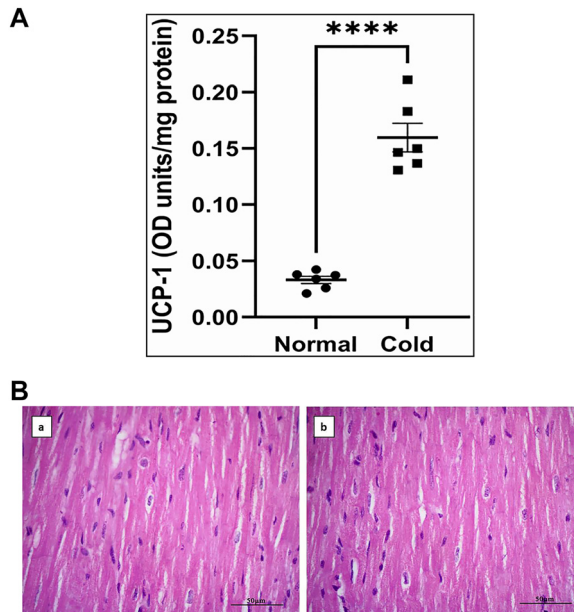|	Body weight gain (g)	Heart weight (g)	Liver weight (g)	Kidney weight (g)	Heart weight/ Bodyweight ratio
Normal	4.75 $\pm$ 0.96	0.169 $\pm$ 0.014	1.245 $\pm$ 0.072	0.199 $\pm$ 0.022	0.0055 $\pm$ 0.000424
Cold	5.25 $\pm$ 0.5	0.151 $\pm$ 0.016	1.283 $\pm$ 0.030	0.194 $\pm$ 0.022	0.0048 $\pm$ 0.000443

Fig. 1. A) Effect of intermittent cold exposure on BAT UCP-1. Animals were exposed to cold 1 h/day for 3 weeks, and control groups were maintained at room temperature. Animals were sacrificed after the experimental period, brown adipose tissue was collected, and the tissue lysate was used to analyze the level of UCP-1 by ELISA. The results given are the average of triplicate experiments ± SEM. Statistically significant when compared control with cold exposed animals. ****$P < 0.0001$. B) Hematoxylin and Eosin (H&E) staining. a) Control group, b) Intermittent cold exposed group

Upregulation of mRNA expression and activity of succinate dehydrogenase showed increased mitochondrial metabolism on intermittent cold exposure (Fig. 2D, E).

Effect of intermittent cold exposure on the regulators of mitochondrial biogenesis

Increased mitochondrial biogenesis may be the possible mechanism for the cold-induced upregulation of mitochondrial anti-oxidant function and metabolism. DNA copy number and regulators of mitochondrial biogenesis such as PGC-1α, NRF-1, and Tfam were analyzed by ELISA and qRT-PCR. Our results indicated a significant increase in DNA copy number, PGC-1α, NRF-1, and Tfam expression in cold exposed mice hearts compared to control (Fig. 3A, B, C, D, E, F).

Effect of intermittent cold exposure on SIRT-3 and total acetylome

SIRT-3, a major mitochondrial sirtuin, plays an important role in regulating mitochondrial biogenesis and antioxidant function. As intermittent cold exposure has significantly upregulated regulators of mitochondrial biogenesis and function, we further examined the production of SIRT-3 on intermittent cold exposure by qRT-PCR and immunoblot analysis. Intermittent cold exposure significantly upregulated mRNA expression and protein level of mitochondrial SIRT-3
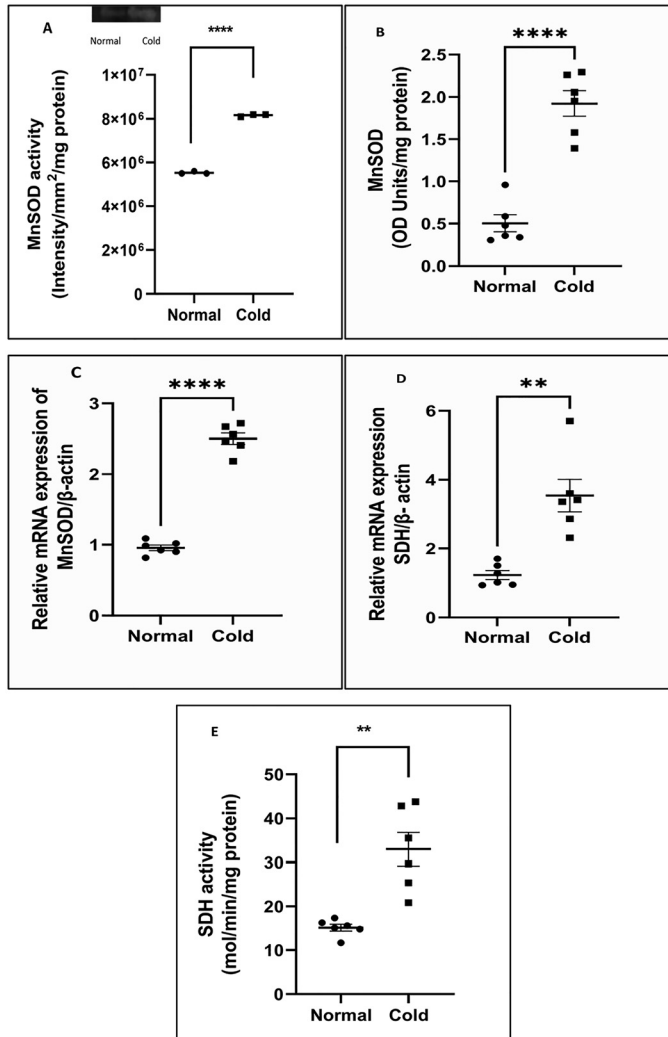
Fig. 2. Effect of intermittent cold exposure on mitochondrial antioxidant and metabolic function. Mice were exposed to 2–8 °C 1 h/day for 3 weeks, and the control mice were maintained at room temperature. Cardiac tissue extract was used for zymography, and bands were quantified. A representative zymogram is given (A). Cardiac tissue homogenates were analyzed for MnSOD by ELISA using a specific antibody (B). Total RNA was isolated from cardiac tissue for qRT-PCR analysis of MnSOD (C) and SDH (D). SDH activity assay was conducted in cardiac tissue lysate. The amount of succinate oxidized per $min \, mg^{-1}$ protein was used for calculating SDH activity (E). The scatter plot represents individual data points with the central value as the mean drawn in Graph pad prism. The mean ± SEM for $n = 6$ were compared control with cold exposed animals. $^{****}P < 0.0001$, $^{***}P < 0.001$, $^{**}P < 0.01$.
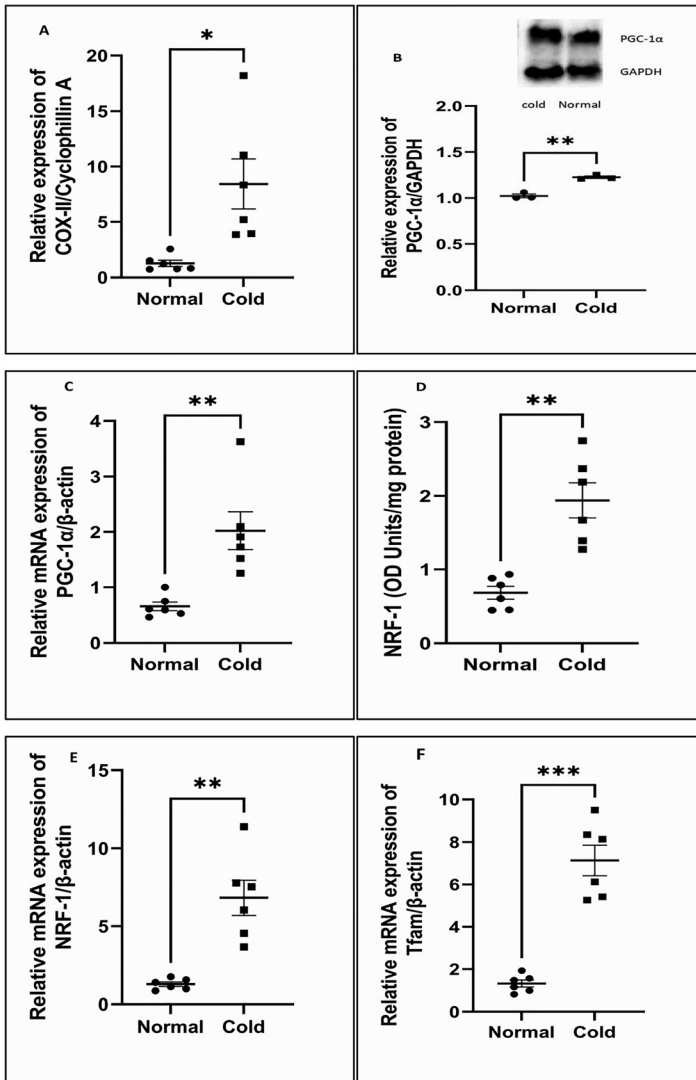
Fig. 3. Effect of intermittent cold exposure on the regulators of mitochondrial biogenesis. Mice were exposed to 2–8° C 1 h/day for 3 weeks. The control mice were maintained at room temperature. Total DNA was isolated from cardiac tissue for qRT-PCR analysis for COX-II and Cyclophilin A ratio, indicating the mitochondrial DNA copy number (A). Cardiac tissue homogenates were analyzed for PGC-1α by immuno blot (B) and NRF-1 (D) by ELISA using specific antibodies. Total RNA was isolated from the cardiac tissue for qRT-PCR analysis of PGC-1α, NRF-1, and Tfam (C, E, F). The scatter plot represents individual data points with the central value as the mean drawn in Graph pad prism. The mean ± SEM for $n = 6$ were compared control with cold exposed animals. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

(Fig. 4A, B). To verify the effect of sirtuins on total protein acetylation, immunoblot analysis using acetylated lysine antibodies was performed. The results indicated a decrease in total protein acetylation in cold-induced mice hearts, which confirmed the increased activity of sirtuins (Fig. 4C).

Effect of norepinephrine on the regulators of mitochondrial biogenesis

Cold exposure activates the sympathoadrenal system and releases norepinephrine [29]; hence we mimicked cold exposure *ex vivo* using norepinephrine treated cardiac tissue slices. Cardiac tissue
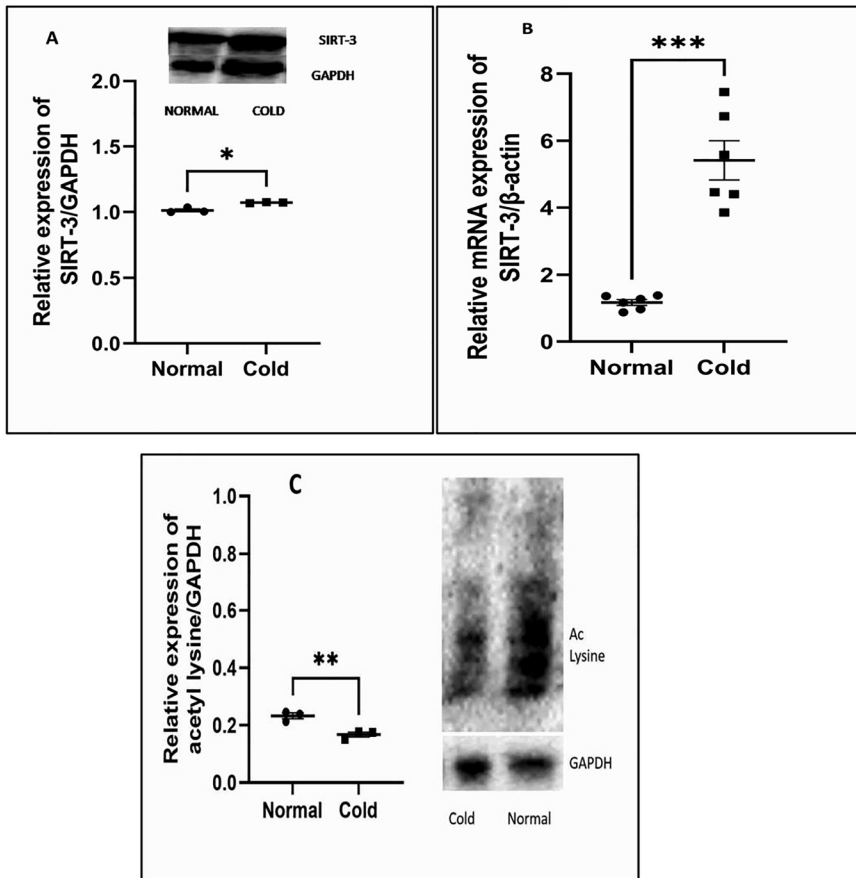

Fig. 4. Effect of intermittent cold exposure on sirtuins and total acetylome. Mice were exposed to 2–8° C 1 h/day for 3 weeks. Control mice were maintained at room temperature. Cardiac tissue homogenates were analyzed for SIRT-3 (A) and acetylated proteins (C) by Immunoblot using specific antibodies against SIRT-3 and acetyl-lysine. Representative immunoblot images are given. Total RNA was isolated from the heart of cold exposed animals for qRT-PCR analysis using SIRT-3 specific primers (B). The scatter plot represents individual data points with the central value as the mean drawn in Graph pad prism. The mean $\pm$ SEM for $n = 6$ were compared control with cold exposed animals. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

slices were maintained in culture in the presence and absence of norepinephrine (20 µM) for 8 h. Our results in *in vivo* study indicated that intermittent cold exposure significantly upregulated the expression of regulators of mitochondrial biogenesis. Hence we studied the effect of norepinephrine on mitochondrial biogenesis regulatory factors such as PGC-1α, NRF-1, and Tfam. These factors were significantly upregulated on treatment with norepinephrine compared to untreated control (Fig. 5A, B, C, D, E).
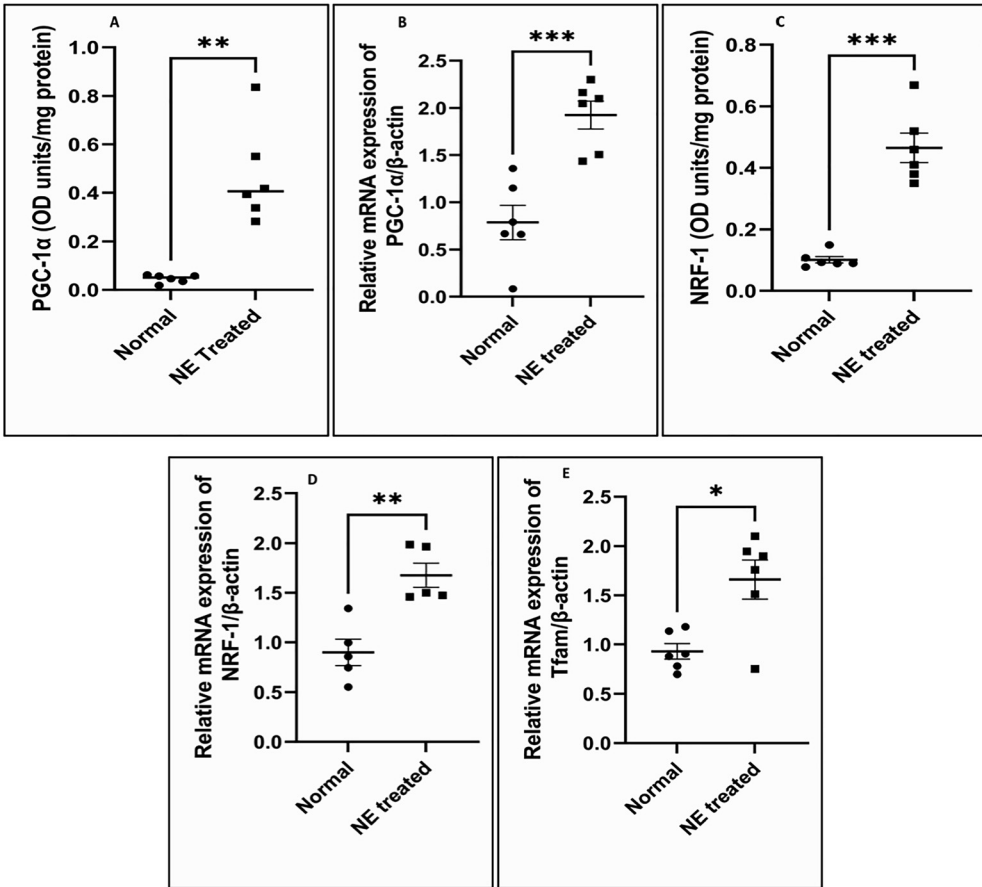

Fig. 5. Effect of norepinephrine on the regulators of mitochondrial biogenesis. Cardiac tissue slices were maintained in culture with norepinephrine (20 µM) for 8 h. Untreated tissue slices served as control. Cardiac tissue homogenates were analyzed by ELISA using specific antibodies against PGC-1α and NRF-1(A, C). Total RNA was isolated from the cardiac tissue slices for qRT-PCR analysis using specific primers of PGC-1α, NRF-1, and Tfam (B, D, E). The scatter plot represents individual data points with the central value as the mean drawn in Graph pad prism. The mean ± SEM for $n = 6$ were compared control with cold exposed animals $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

Role of SIRT-3 on the regulators of mitochondrial biogenesis in cold mimic

We tried to elucidate the role of SIRT-3 on the regulators of mitochondrial biogenesis during cold exposure using *ex vivo* cold mimic. For this, we studied the effect of specific SIRT-3 inhibitor AGK-7 on the production of PGC-1α and its downstream target NRF-1. AGK-7 partially reversed the effect of norepinephrine on the protein expression of PGC-1α and NRF-1 (Fig. 6A, B).

Role of PKA on the regulators of mitochondrial biogenesis in cold mimic

Activating beta-adrenergic receptors through norepinephrine is reported to mediate its effect through PKA activation. To study the role of PKA on the regulators of mitochondrial biogenesis, cardiac tissue slices were pretreated with KT5720, a specific inhibitor of PKA, and maintained in the presence of norepinephrine for 8 h. PGC-1α and its downstream target NRF-1 showed a significant downregulation on treatment with KT5720 in norepinephrine treated cardiac tissue slices, indicating the role of PKA on the regulators of mitochondrial biogenesis (Fig. 7A, B).
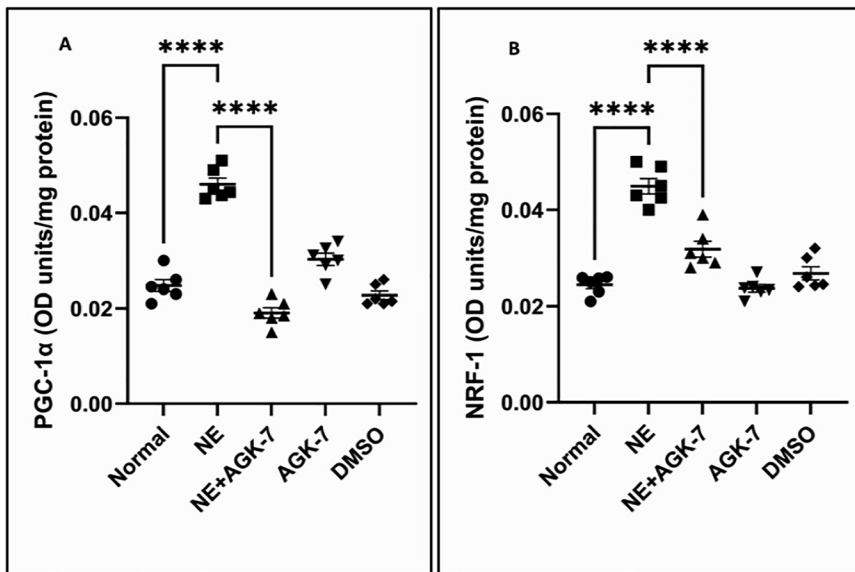

Fig. 6. Role of SIRT-3 on the regulators of mitochondrial biogenesis in cold mimic. Cardiac tissue slices were pretreated with AGK-7 (30 μM) for 24 h and maintained in culture in the presence and absence of norepinephrine (20 μM) for 8 h. Cardiac tissue homogenates were analyzed for PGC-1α (A) and NRF-1 (B) by ELISA using specific antibodies. The scatter plot represents individual data points with the central value as the mean drawn in Graph pad prism. The mean ± SEM for $n = 6$, were compared norepinephrine with control and norepinephrine with norepinephrine + AGK-7. ###$P < 0.001$ and ***$P < 0.001$.
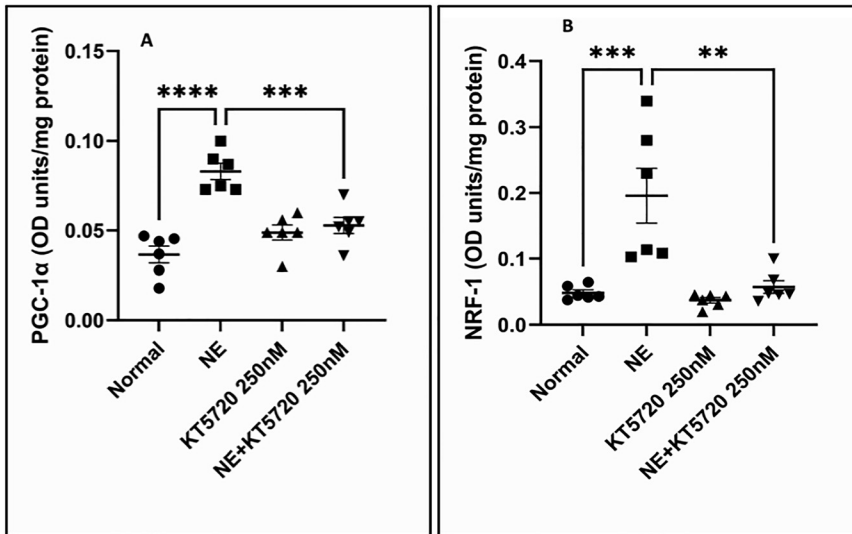
Fig. 7. Role of PKA on the regulators of mitochondrial biogenesis in cold mimic. Cardiac tissue slices were pretreated with KT5720 (250 nM) for 24 h and maintained in the presence and absence of norepinephrine (20 µM) for 8 h. Cardiac tissue homogenates were analyzed for PGC-1α (A) and NRF-1(B) by ELISA using specific antibodies. The scatter plot represents individual data points with the central value as the mean drawn in Graph pad prism. The mean $\pm$ SEM for $n = 6$, were compared norepinephrine with control and norepinephrine with norepinephrine $+$ KT5720. ###$P < 0.001$ and ***$P < 0.001$.

DISCUSSION

The heart is a metabolically active organ where mitochondria are the major energy source. Mitochondrial dysfunction during heart failure is considered to be an important target for therapy to improve cardiac function [30]. It has been previously reported that severe cold exposure leads to reduction in cardiac function due to the over activation of the SNS. It leads to increased blood pressure and decreased cardiac output [1]. Repeated mild cold stress induces cold adaptation, a protective mechanism against cold stress-induced damage. Hence we studied the effect of intermittent cold exposure on the regulators of mitochondrial biogenesis, function, and its mechanism. Our results suggest that intermittent cold exposure significantly increased the regulators of mitochondrial biogenesis and function through PGC-1α mediated activation of SIRT-3. Evidence in support of this are the following; a) Increased activity and expression of MnSOD and SDH in *in vivo* intermittent cold exposed mice. b) Increased mitochondrial DNA copy number along with PGC-1 α and its downstream targets NRF-1 and Tfam, indicating increased mitochondrial biogenesis and function. c) Increased SIRT-3 expression and decreased protein-lysine acetylation indicates increased sirtuin activity. d) In *ex vivo* cold mimic, downregulation of PGC-1α and NRF-1 on PKA and SIRT-3 inhibition reveals their role in the regulation of PGC-1α and NRF-1.

The activity and expression of MnSOD, the key marker of mitochondrial antioxidant function, were significantly upregulated on intermittent cold exposure. MnSOD is activated at low

temperatures and acts as a catalytic thermoreceptor in humans to microorganisms [31]. Elevated antioxidant enzyme may be due to increase in oxidative stress. Neri et al. reported that norepinephrine administration significantly increased antioxidant defence mechanisms such as GPS activity and SOD activity in rat heart [32]. The effect of cold exposure on mitochondrial metabolic status was studied by checking the activity and expression of succinate dehydrogenase. Succinate dehydrogenase supports mitochondrial metabolism via the TCA cycle and electron transport chain. Increased activity and mRNA expression of succinate dehydrogenase in our study indicate the increased mitochondrial metabolic status during cold exposure. Whereas the activity of Glucose-6-phosphatase, a key gluconeogenic enzyme, and a key lysosomal enzyme, β-D-glucuronidase, didn't show any significant change on cold exposure (data not shown). It has been previously reported that prolonged cold exposure enhances succinate dehydrogenase, malate dehydrogenase, and cytochrome oxidase activities in rat liver and muscles [33].

Oxidative metabolism is the main energy source for cardiac muscles. Increasing the mitochondrial content is one of the mechanism for increasing mitochondrial oxidative metabolism and energy production. Mitochondrial biogenesis controls mitochondrial content and thus correlates with energy production which can sustain cardiac contractility [6]. The change in mitochondrial metabolism and antioxidant function during intermittent cold exposure may be due to increased mitochondrial biogenesis. In our study, intermittent cold exposure significantly upregulated the key regulators of mitochondrial biogenesis, such as PGC-1 α, NRF-1, and Tfam, along with mitochondrial DNA copy number, indicating increased mitochondrial biogenesis. Previous reports suggest that exercise and cold exposure significantly upregulated the expression of PGC-1α, NRF-1, and Tfam in soleus muscles and adipose tissue [13]. Chronic intermittent cold exposure-induced PGC-1α and Tfam expression in mice beige adipocytes [34]. Another study indicated that intermittent cold exposure significantly upregulated PGC-1α, NRF-1, and NRF-2 expressions in rat liver. When this cold acclimatized rat was further subjected to chronic cold exposure, these parameters did not show any significant change, indicating that cold training attenuates the effects of continuous cold exposure [35]. Transcription and replication of mitochondrial DNA are regulated by nuclear-encoded mitochondrial transcription factor A [8]. Previous studies reported that Nuclear respiratory factor-1 (NRF-1) has a role in mitochondrial biogenesis through the activation of Tfam, which contains recognition sites for NRF-1 [8, 9, 11]. Mitochondrial biogenesis and respiration are stimulated by the induction of NRFs expression [8].

On intermittent cold exposure, increased cardiac SIRT-3 production and decreased total protein lysine acetylation were observed. SIRT-3 deacetylates and regulates mitochondrial proteins involved in metabolism and homeostasis. Previous studies showed that PGC-1α stimulates SIRT-3 expression in C2C12 myotubes and mouse primary hepatocytes [19], and SIRT-3 regulates mitochondrial biogenesis-related genes such as PGC-1α, NRF-1, Tfam, mtSSB in colon cancer cells [36]. Cold exposure, calorie restriction, and exercise upregulated SIRT-3 in BAT and skeletal muscles [37].

Norepinephrine treatment of *ex vivo* cardiac tissue slice upregulated PGC-1α, NRF-1, and Tfam, indicating similar results with *in vivo* cold treatment. This indicates *ex vivo* norepinephrine treated cardiac slices mimic *in vivo* intermittent cold exposure and activates mitochondrial biogenesis regulators such as PGC-1α, NRF-1, and Tfam.

AGK-7 significantly downregulated the production of PGC-1α and NRF-1 in *ex vivo* cold mimic. SIRT-3 and PGC-1α act synergistically to maintain mitochondrial biogenesis
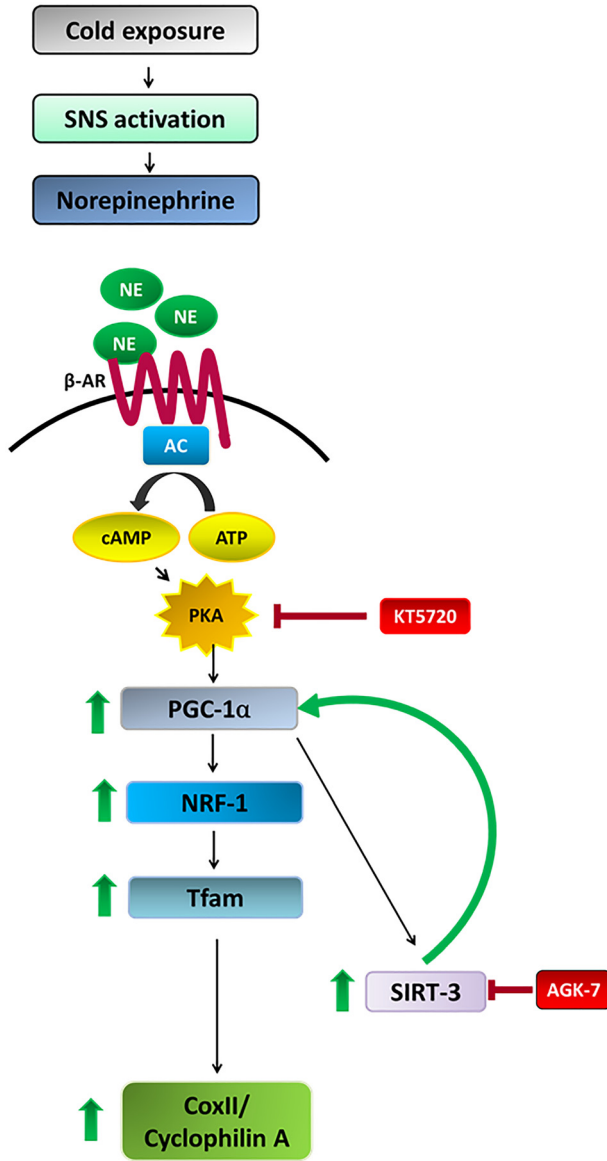
Fig. 8. Intermittent cold exposure leads to the activation of the SNS, which releases NE. NE binds to β-adrenergic receptors and activates the cAMP-PKA pathway leading to the activation of PGC-1α, NRF-1, and Tfam. Increase in COX-II/Cyclophilin indicate increase in mitochondrial DNA copy number. PGC-1α may activate SIRT-3. PKA inhibition using KT5720 inhibits PGC-1α production. AGK-7, inhibit SIRT-3, which can downregulate PGC-1α. (SNS–Sympathetic nervous system, NE–norepinephrine, β-AR– β-adrenergic receptor, AC–Adenylate Cyclase)

in C2C12 myotubes [19]. Cold exposure activates PGC-1α in brown fat and muscle cells [38]. The sympathetic nervous system is activated through the β₃ adrenergic system during cold exposure, leading to enhanced cAMP signaling and activation of PKA [39]. CREB, a major target of PKA, and the PGC-1α promoter has a CREB binding region; PKA activation leads to increased PGC-1α expression [40]. In our study, inhibition of PKA with KT5720 showed a partial reversal of the effect of norepinephrine on PGC-1α and its downstream target NRF-1, which indicate the role of PKA in the regulation of PGC-1α in *ex vivo* cold mimic.

In summary, intermittent cold exposure activates sympathetic nervous system leading to the release of norepinephrine which binds to β-adrenergic receptors. Activation of adenylate cyclase, downstream of β-adrenergic receptors activates cAMP-PKA pathway which in turn activate PGC-1α. PGC-1α enhances other regulators of mitochondrial biogenesis such as NRF-1, Tfam, mitochondrial DNA copy number, and SIRT-3. PKA may regulate PGC-1α as PKA inhibition showed a decrease in PGC-1α production. Also, SIRT-3 may have a role in regulating PGC-1α in *ex vivo* cold mimic. The inhibition of SIRT-3 decreased the production of PGC-1α (Fig. 8). Intermittent cold exposure induces only adaptive changes and does not lead to mortality. These changes due to adaptation occur via modulating mitochondrial function. In this context, cold adaptation could be a promising strategy to protect against cold-induced cardiac dysfunction. More studies are required to extrapolate the data to humans. However, a limitation of the study is the lack of data showing direct evidence for increased mitochondrial biogenesis. Further studies are needed in this direction.

Competing interests: The authors have no competing interests to declare that are relevant to the content of this article.

Author contributions: Mithra. S. Mohan, P. R Sudhakaran and Saja. K conceptualized the study and planned the experiments, and discussed the results. Mithra. S. Mohan wrote the draft of the manuscript. Saja. K, P.R Sudhakaran and P.T Boban reviewed and edited the manuscript. Mithra. S. Mohan, Aswani. S. S. and Aparna N.S carried out the experiments. All authors participated in the design of the study and interpretation and analysis of the data, and reviewed the manuscript.

Data availability: All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Ethics approval: Experiments in this research were reviewed and approved by the animal ethical committee, University of Kerala (IAEC1-KU-12b/2014-15-BC-SK(4b)).

ABBREVIATIONS

BAT   Brown adipose tissue
NE   Norepinephrine
SNS   Sympathetic nervous system
Tfam   Mitochondrial transcription factor A
UCP-1 Uncoupling Protein1
SOD   Superoxide dismutase
mtSSB mitochondrial single-stranded DNA binding protein

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