Acute cold exposure-induced down-regulation of CIDEA, cell death-inducing DNA fragmentation factor-α-like effector A, in rat interscapular brown adipose tissue by sympathetically activated β_3-adrenoreceptors

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Abstract

The thermogenic activity of brown adipose tissue (BAT) largely depends on the mitochondrial uncoupling protein 1 (UCP1), which is up-regulated by environmental alterations such as cold. Recently, CIDEA (cell death-inducing DNA fragmentation factor-α-like effector A) has also been shown to be expressed at high levels in the mitochondria of BAT. Here we examined the effect of cold on the mRNA and protein levels of CIDEA in interscapular BAT of conscious rats with regard to the sympathetic nervous system. Cold exposure (4°C for 3 h) elevated the plasma norepinephrine level and increased norepinephrine-turnover in BAT. Cold exposure resulted in down-regulation of the mRNA and protein levels of CIDEA in BAT, accompanied by up-regulation of mRNA and protein levels of UCP1. The cold exposure-induced changes of CIDEA and UCP1 were attenuated by intraperitoneal pretreatment with propranolol (a non-selective β-adrenoreceptor antagonist) (2 mg/animal) or SR59230A (a selective β_3-adrenoreceptor antagonist) (2 mg/animal), respectively. These results suggest that acute cold exposure resulted in down-regulation of CIDEA in interscapular BAT by sympathetically activated β_3-adrenoreceptor-mediated mechanisms in rats.

Keywords: Brown adipose tissue; Uncoupling protein 1; Cold exposure; Cell death-inducing DNA fragmentation factor-α-like effector A; Sympathetic nervous system; β_3-Adrenoreceptor

Introduction

CIDEA (cell death-inducing DNA fragmentation factor-α-like effector A) was originally identified by their sequence similarity to the N-terminal region of the apoptotic DNA fragmentation factor DFF45 [1]. Recently, CIDEA has been shown to have roles in energy homeostasis. Human *CIDEA* is highly expressed in white adipose tissue and CIDEA inhibits basal lipolysis in human adipocyte [2]. *CIDEA* gene V115F polymorphism is also reported to be associated with obesity in humans [3].

Cidea is particularly expressed at high levels in the mitochondria of mice brown adipose tissue (BAT) [4]. BAT is the site of energy dissipation linked to heat production (thermogenesis) in rodents [5,6], human newborns and also adult humans [7] by uncoupling protein 1 (UCP1) expressing in the mitochondria of BAT, which causes conversion of the mitochondrial proton-motive force of ATP synthesis into heat [8]. Interestingly, *Cidea*-null mice exhibit higher energy expenditure with enhanced lipolysis in BAT [4]. This result suggests an important role of CIDEA for thermogenesis in BAT, however it is uncertain whether expression of the *Cidea* gene is changed by cold exposure, which triggers BAT thermogenesis. In the present study, therefore, we aimed to investigate whether cold exposure changes CIDEA expression and the expression mechanisms of CIDEA in the rat interscapular BAT with regard to the sympathetic nervous system densely innervating the BAT.

Materials and methods

Materials. Anti-CIDEA polyclonal antibody (pAb), anti-UCP1 pAb and anti-α-tubulin pAb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). SR59230A (3-(2-ethylphenoxy)-1-[[(1*S*)-1,2,3,4-tetrahydronaphth-1-yl]amino]-(2*S*)-2-propanol oxalate), α-methyl-*p*-tyrosine (AMPT) and all other reagents were purchased from Sigma-Aldrich (St Louis, MO, USA). All reagents for norepinephrine measurement were purchased from Nacalai Tesque (Kyoto, Japan).

Animals. Male Wistar rats weighing about 350 g were maintained in an air-conditioned room at 22-24°C under a constant day-night rhythm for more than 2 weeks and given laboratory chow (CE-2, Clea Japan, Hamamatsu, Japan) and water *ad libitum*. The experiments were conducted in compliance with the guiding principles for the care and use of laboratory animals approved by Kochi University.

Experimental protocols. Experiment-1 (Figs. 1A and 2): Rats were anesthetized with pentobarbital (40 mg/kg, i.p.). The left femoral artery was catheterized with a PE-50 tube for collecting blood samples. The catheter was filled with heparinized saline (100 U/ml) and tunneled subcutaneously to exit at the back of neck [9]. One day after catheterization, conscious rats were transferred from their home cage to a new plastic cage. After a 2-h stabilization period, the first blood sample (250 μl) was collected and the cage was transferred to a cold room (4°C) or maintained in an air-conditioned room (23°C). The second blood sample was withdrawn 3 h after exposure to each temperature. After the second blood sampling, rats were decapitated and bilateral interscapular BAT were immediately removed and stored at -80°C until use for RNA

preparation and Western blotting. All blood samples were preserved on ice during the experiment and plasma samples were prepared immediately after the end of BAT sampling. Experiment-2 (Fig. 1B): Conscious rats not having undergone an operation were transferred from their home cage to a new plastic cage. After a 2-h stabilization period, rats were given an intraperitoneal injection of AMPT (250 mg/kg) and the cage was transferred to a cold room (4 °C) or maintained in an air-conditioned room (23°C). After 3-h exposure to each temperature, rats were decapitated and bilateral interscapular BAT was immediately removed for norepinephrine measurement. To measure norepinephrine content in BAT at 0 h, rats were decapitated immediately after the injection of AMPT. Experiment-3 (Figs. 3 and 4): Conscious rats not having undergone an operation were transferred from their home cage to a new plastic cage. After a 2-h stabilization period, rats were given an intraperitoneal injection of propranolol (2 mg/kg) or SR59230A (2 mg/kg). Thirty min after the injection, the cage was transferred to a cold room (4°C) or maintained in an air-conditioned room (23°C). After 3-h exposure to each temperature, rat was decapitated and bilateral interscapular BAT was immediately removed and stored at -80°C until use for RNA preparation and Western blotting.

Norepinephrine measurement in the plasma and interscapular BAT. The BAT was homogenized in 20 ml of 0.4 M perchloric acid containing 18.6 mg of disodium EDTA, 200 μl of 2% sodium pyrosulfite solution and 500 ng of 3,4-dihydroxybenzylamine (DHBA) as an internal standard. The homogenate was centrifuged for 10 min at 14,000 *g* at 4°C. The supernatant was analyzed to determine the tissue level of norepinephrine [10]. Norepinephrine in the plasma and the supernatant of tissue homogenate was

extracted and electrochemically assayed with high performance liquid chromatography as described previously [10,11].

RT-PCR. Total RNA was isolated from BAT using TRIZOL reagent (Invitrogen, Tokyo, Japan). RT-PCR was performed as described previously [12]. The primers used were as follows: rat *Cidea* upstream primer, 5'-GCTGGTGCTGGAGGAG-3'; rat *Cidea* downstream primer, 5'-CTGTCCCGTCATCTGTGC-3'; rat *Ucp1* upstream primer, 5'-GTCTTAGGGACCATCACCA-3'; rat *Ucp1* downstream primer, 5'-CCAGTGTAGCGGGGTTTG-3'; rat β-*actin* upstream primer, 5'-TTGTAACCACCTGGGACGATATGG-3'; rat β-*actin* downstream primer, 5'-GATCTTGATCTTCATGGTGCTAG-3'. The PCR products were resolved by electrophoresis on a 2% agarose gel in 0.5 x Tris acetate-EDTA. The gels were stained with ethidium bromide and photographed. Band densities were obtained by using the KODAK 1D image analysis software program.

Western blotting. The interscapular BAT was homogenized in a solution containing 10 mM Tris-HCl and 1 mM EDTA (pH 7.4). After centrifugation (1,500 *g* for 5 min, at 4°C), the fat cake was discarded and the middle layer (fat-free extract) was used for Western blotting [13]. The fat-free extract was added to the sodium dodecyl sulfate (SDS) sample buffer. The total lysates were then boiled for 5 min. SDS-polyacrylamide gel electrophoresis and Western blotting were performed as described previously [12].

Statistics. All values were expressed as the means±S.E.M. The data were analyzed by repeated-measure analysis of variance (ANOVA), followed by *post-hoc* analysis with the Bonferroni method for comparisons among all values (Fig. 1B). When only two means were compared, an unpaired Student's *t*-test or Welch's *t*-test was used

(Figs. 1A, 2, 3 and 4). *P* values less than 0.05 were taken to indicate statistical significance.

Results

Effect of acute cold exposure on the plasma norepinephrine level and norepinephrine content in interscapular BAT

In preliminary studies in rats, we monitored the time course of plasma norepinephrine level for 4 h after cold exposure (4°C). The norepinephrine level increased at 0.5 h and reached a plateau at 1.5 h after cold exposure in rats (data not shown). Therefore, we examined at 3 h after cold exposure in the following experiments. Acute cold exposure (4°C for 3 h) significantly increased plasma norepinephrine level (from 290±48 to 893±117 pg/ml), although maintaining at 23°C for 3 h had no effect on the plasma norepinephrine level (261±27 pg/ml at 0 h, 271±21 pg/ml at 3 h) in rats (Fig. 1A).

By blocking norepinephrine biosynthesis by pretreatment with AMPT (a tyrosine hydroxylase inhibitor), norepinephrine contents in interscapular BAT of rats exposed to 4°C for 3 h and maintained at 23°C for 3 h were significantly lower than those in BAT before exposure to each temperature (at 0 h) (1.29±0.02 ng/mg tissue at 0 h, 0.90±0.07 ng/mg tissue at 23°C for 3 h, 0.29±0.01 ng/mg tissue at 4°C for 3 h) (Fig. 1B). However, norepinephrine content in the BAT of rats exposed to 4°C was lower than that of rats maintained at 23°C, at 3 h after exposure to each temperature (Fig. 1B).

Effect of acute cold exposure on CIDEA and UCP1 expression levels of mRNA and protein in interscapular BAT

After the experimental protocol (exposure to 4°C or maintenance at 23°C, for 3 h), mRNA levels of *Cidea* in the BAT of rats exposed to 4°C were significantly lower than those maintained at 23°C, while mRNA levels of *Ucp1* in the BAT of rats exposed to 4°C were significantly higher than those maintained at 23°C (Fig. 2, A and B). These tendencies in mRNA levels of *Cidea* and *Ucp1* were also observed in protein levels of CIDEA and UCP1 in the BAT of rats exposed to 4°C and maintained at 23°C, respectively (Fig. 2C).

Effect of propranolol on the acute cold exposure-induced changes of CIDEA and UCP1 expression levels in the interscapular BAT

Even in the vehicle (saline)-pretreated rats, the levels of *Cidea* and CIDEA in the BAT of rats exposed to 4°C for 3 h were also significantly lower than those maintained at 23°C for 3 h, while the levels of *Ucp1* and UCP1 in the BAT of rats exposed to 4°C for 3 h were significantly higher than those maintained at 23°C for 3 h (Fig. 3, A, B and C).

In the propranolol (a non-selective β-adrenoreceptor antagonist) (2 mg/kg, i.p.)-pretreated rats, CIDEA and UCP1 expression levels of mRNA and protein in the BAT of rats exposed to 4°C for 3 h were almost the same as those maintained at 23°C for 3 h (Fig. 3, A, B and C).

Effect of SR59230A on the acute cold exposure-induced changes of CIDEA and UCP1 expression levels in the interscapular BAT

In the vehicle (10% dimethylsulfoxide in saline)-pretreated rats, the levels of *Cidea* and CIDEA in the BAT of rats exposed to 4°C for 3 h were also significantly lower than those maintained at 23°C for 3 h, while the levels of *Ucp1* and UCP1 in the BAT of rats exposed to 4°C for 3 h were significantly higher than those maintained at 23°C for 3 h (Fig. 4, A, B and C).

In the SR59230A (a selective β_3-adrenoreceptor antagonist [14]) (2 mg/kg, i.p.)-pretreated rats, CIDEA and UCP1 expression levels of mRNA and protein in the BAT of rats exposed to 4°C for 3 h were almost the same as those maintained at 23°C for 3 h (Fig. 4, A, B and C).

Discussion

Sympathetic innervations to the rat interscapular BAT are responsible for the regulation of thermogenesis in response to cold exposure [15]. It has been reported that cold exposure increases plasma norepinephrine level by activating exclusively the sympathetic nervous system [16]. In the present study, an increase in plasma norepinephrine was also observed by acute cold exposure in conscious rats. However, plasma norepinephrine can reflect not only the release from sympathetic nerves but also the secretion from norepinephrine-containing cells in the adrenal medulla [17]. Therefore, we further tried to determine the sympathetic outflow by assessing norepinephrine turnover after blockage of norepinephrine biosynthesis with tyrosine hydroxylase inhibitor AMPT [18]. In the AMPT-treated rats, acute cold exposure markedly decreased the norepinephrine content in BAT, indicating the activation of sympathetic nerves innervating the rat BAT.

The sympathetic activation in BAT has been shown to promote thermogenesis, differentiation and cell proliferation of brown adipocytes [6]. The thermogenic activity of BAT largely depends on UCP1, since *Ucp1*-deficient mice undergo a rapid decrease in body temperature during cold exposure [19]. In the present study, mRNA and protein levels of UCP1 were up-regulated in interscapular BAT exposed to acute cold in rats, as previously described [20]. It has been well shown that UCP1 up-regulation induced by cold exposure is mediated by activation of β-adrenoreceptors, and systemic pretreatment with antagonists of the receptors has been reported to reduce BAT thermogenesis [21]. Brown adipocytes express a combination of adrenoreceptor

isoforms (α_1, β_1, β_2 and β_3) with β_3-adrenoreceptors being the most abundant [22], and the β_3-adrenoreceptors have been show to be involved in UCP1 up-regulation and BAT thermogenesis [23,24]. In the present study, systemic pretreatments with propranolol and SR59230A effectively attenuated the UCP1 up-regulation induced by acute cold exposure in interscapular BAT in rats. These results suggest that the acute cold exposure-induced up-regulation of UCP1 expression in BAT is mediated by β_3-adrenoreceptors in rats.

In contrast to UCP1, acute cold exposure induced down-regulation of the mRNA and protein levels of CIDEA in interscapular BAT in the present study. The down-regulation of CIDEA was also effectively attenuated by propranolol and SR59230A. These results suggest that the acute cold exposure-induced down-regulation of CIDEA expression in BAT is also mediated by β_3-adrenoreceptors in rats. Recently, several expression mechanisms of the *CIDEA* gene have been reported. In differentiated brown adipocytes, CIDEA protein can be degraded by the ubiquitin-proteasome system [25], which seems to be inactivated by cold exposure in BAT [26], thereby suggesting a possibility that cold exposure up-regulates CIDEA protein levels. However, in the present experiment, CIDEA in both levels of mRNA and protein was down-regulated by acute cold exposure, the down-regulation of CIDEA occurred by a mechanism other than the ubiquitin-proteasome system. The expression and promoter activity of *CIDEA* have been shown to be induced by peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) [27], which can also stimulate *Ucp1* expression [6,20]. Since CIDEA and UCP1 expression levels were inversely changed by acute cold exposure in BAT in the present study, a mechanism other than PGC-1α may be involved in the

acute cold exposure-induced down-regulation of CIDEA in BAT. Tumor necrosis factor-α (TNF-α) has been shown to reduce the transcriptional activity of the human *CIDEA* promoter [28]. Actually, TNF-α treatment with human adipocytes decreased *CIDEA* gene expression [2]. In addition, norepinephrine induces the release of TNF-α in mouse macrophages [29]. These findings suggest a possibility that TNF-α released by activation of the sympathetic nervous system induces down-regulation of CIDEA expression in response to acute cold exposure in rat BAT. However, the precise inhibitory mechanisms of rat *Cidea* gene expression in BAT are largely undefined. Further studies are required to clarify the mechanisms of cold exposure-induced down-regulation of CIDEA expression in BAT.

Both CIDEA and UCP1 are highly expressed in mice BAT. Interestingly, co-expression of *Cidea* and *Ucp1* in yeast resulted in inhibition of UCP1 activity of conversing the proton-motive force into heat and *Cidea*-null mice had higher body temperature when exposed to cold than wild-type mice [4]. Furthermore, treatment with a very low calorie diet induced an increase in *CIDEA* gene expression in human white adipose tissue [30]. These findings suggest that CIDEA plays an inhibitory role in thermogenesis and energy expenditure in adipose tissue by negatively modulating the activity of UCP1. As shown in the present study, CIDEA and UCP1 expression levels in BAT are inversely changed by acute cold exposure, indicating a reasonable mechanism in adaptation to cold environments by activation of BAT thermogenesis through not only increasing UCP1 but also decreasing CIDEA.

In summary, we demonstrated here for the first time that CIDEA is down-regulated in response to acute cold exposure in rat interscapular BAT by sympathetically activated β_3-adrenoreceptors.

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Legends to figures

Fig. 1. Effect of acute cold exposure on the plasma norepinephrine level and norepinephrine content in interscapular brown adipose tissue (BAT). (A) Plasma level of norepinephrine (NE). Conscious rats were either exposed to 4°C or maintained at 23°C for 3 h, and blood samples were collected twice at 0 h (before) and 3 h through an arterial catheter. Each value represents the mean±S.E.M. (n=6/group). *Significantly different from the values at 0 h with an unpaired Student's *t*-test or Welch's *t*-test ($P<0.05$). (B) NE content in interscapular BAT. Conscious rats were either exposed to 4°C or maintained at 23°C for 3 h after administration of AMPT (a tyrosine hydroxylase inhibitor) (250 mg/kg, i.p.) and the BAT were collected. NE content at 0 h was obtained immediately after the injection of AMPT. Each value represents the mean±S.E.M (n=4/group). Significant differences among all values were determined with Bonferroni method (*$P<0.05$ compared with the values at 0 h, $^{\#}P<0.05$ compared with the values at 23°C).

Fig. 2. Effect of acute cold exposure on CIDEA and UCP1 expression levels of mRNA and protein in interscapular BAT. Conscious rats were either exposed to 4°C or maintained at 23°C for 3 h and interscapular BAT were isolated. (A) RT-PCR analysis of *Cidea* and *Ucp1* expression in the BAT. (B) Quantitation of mRNA signal intensity relative to the internal standard, β-*actin*. Each value represents the mean±S.E.M (n=6/group). *Significantly different from the group maintained at 23°C with an unpaired Student's *t*-test or Welch's *t*-test ($P<0.05$). (C) CIDEA and UCP1 levels in the BAT were detected by Western blotting.

Fig. 3. Effect of propranolol on the acute cold exposure-induced changes of CIDEA and UCP1 expression levels in interscapular BAT. Conscious rats pretreated with propranolol (a non-selective antagonist of β-adrenoreceptors) (2 mg/kg, i.p.) or vehicle (1 ml/kg of saline, i.p.) were either exposed to 4°C or maintained at 23°C for 3 h, and BAT was isolated. (A) RT-PCR analysis of *Cidea* and *Ucp1* expression in BAT. (B) Quantitation of mRNA signal intensity relative to the internal standard, β-*actin*. Each value represents the mean±S.E.M (n=6/group). *Significantly different from the group maintained at 23°C with an unpaired Student's *t*-test or Welch's *t*-test ($P<0.05$). (C) CIDEA and UCP1 levels in BAT were detected by Western blotting.

Fig. 4. Effect of SR59230A on the acute cold exposure-induced changes of CIDEA and UCP1 expression levels in the interscapular BAT. Conscious rats pretreated with SR59230A (a selective antagonist of β_3-adrenoreceptors) (2 mg/kg, i.p.) or vehicle (1 ml/kg of 10% dimethylsulfoxide in saline, i.p.) were either exposed to 4°C or maintained at 23°C for 3 h, and the BAT were isolated. (A) RT-PCR analysis of *Cidea* and *Ucp1* expression in BAT. (B) Quantitation of mRNA signal intensity relative to the internal standard, β-*actin*. Each value represents the mean±S.E.M (n=6/group). *Significantly different from the group maintained at 23°C with an unpaired Student's *t*-test or Welch's *t*-test ($P<0.05$). (C) CIDEA and UCP1 levels in BAT were detected by Western blotting.

Figure-1

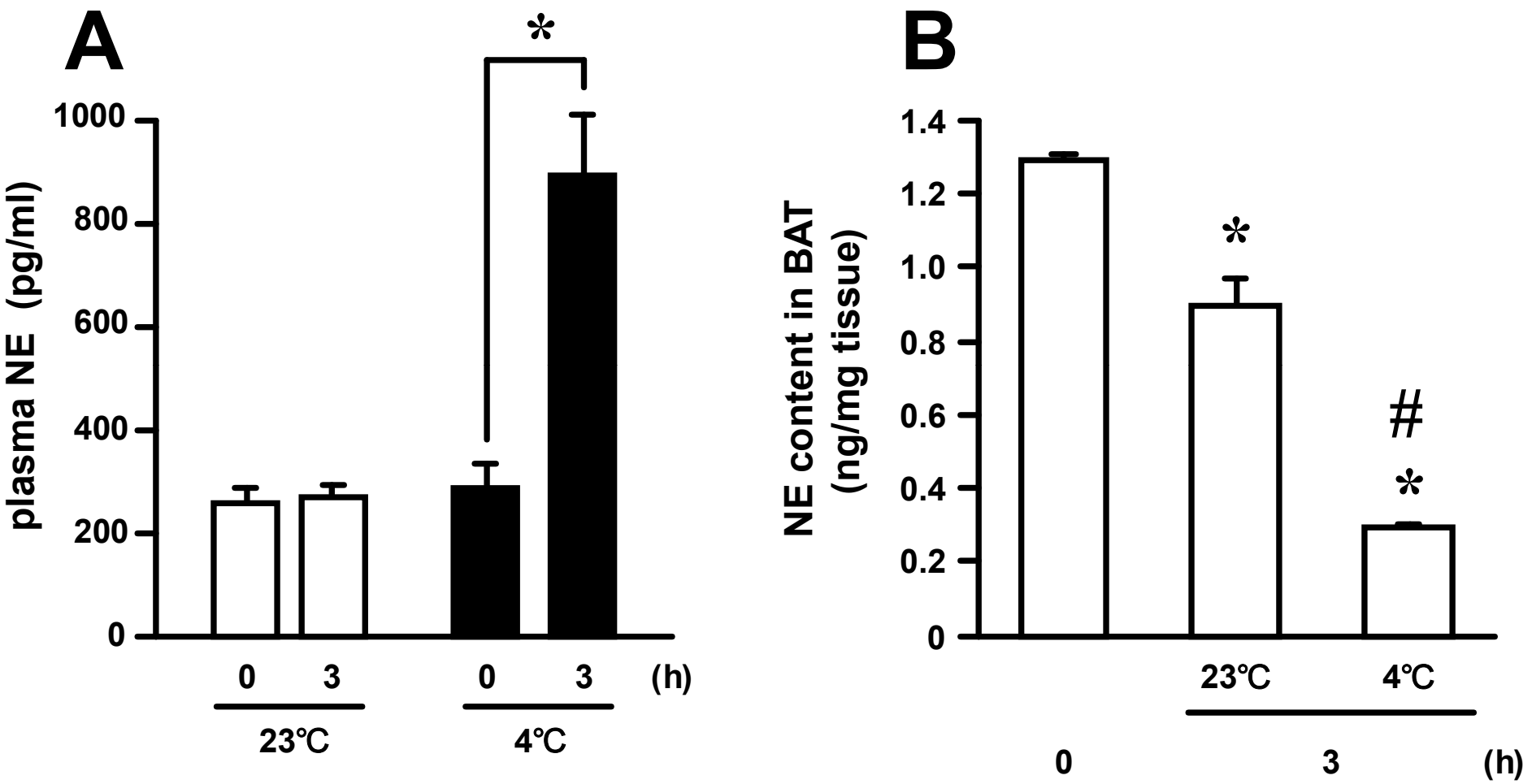
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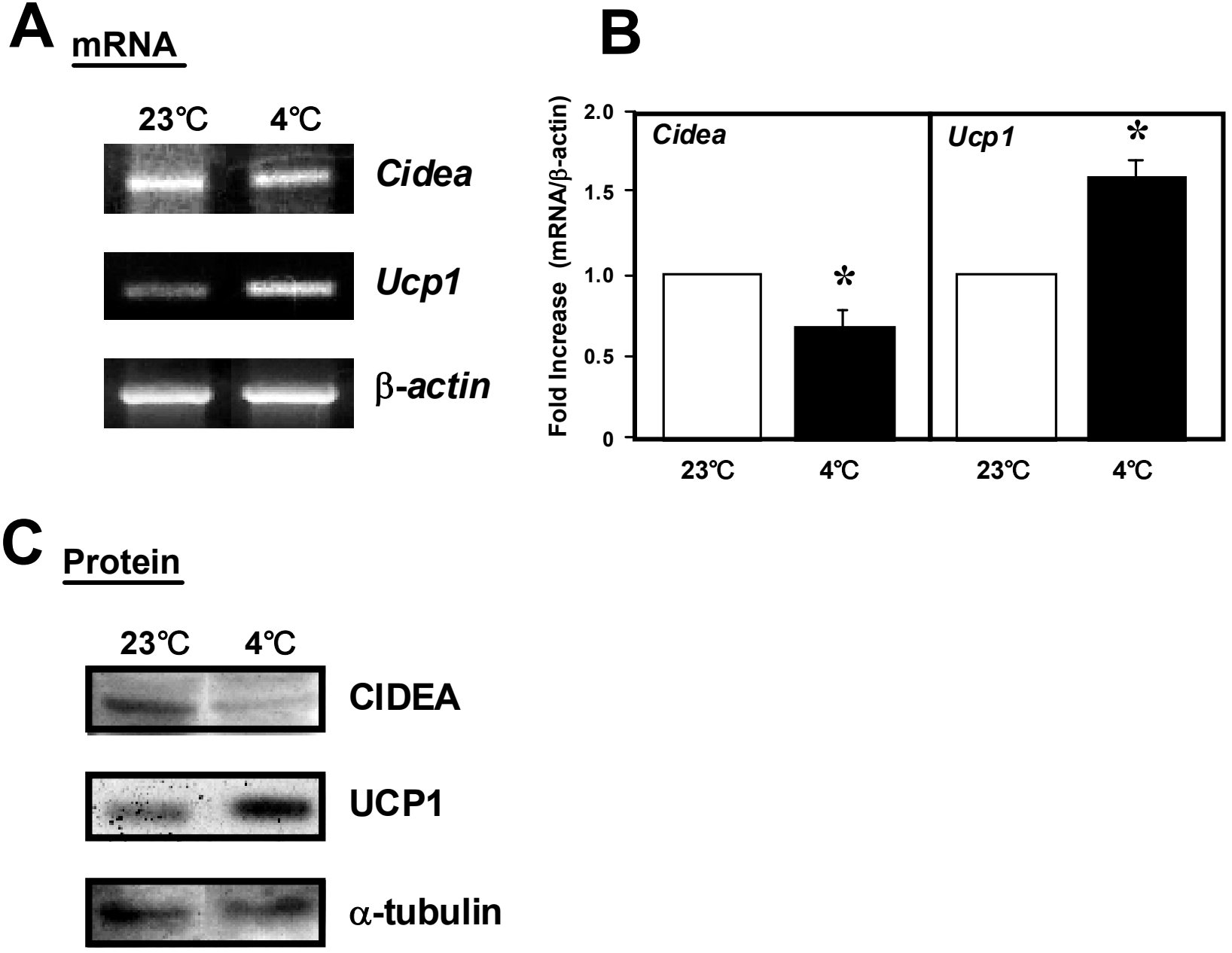
Figure-2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Cidea
Ucp1
*
*
23℃
4℃
23℃
4℃
C
Protein
23℃
4℃
CIDEA
UCP1
α-tubulin

Figure-3

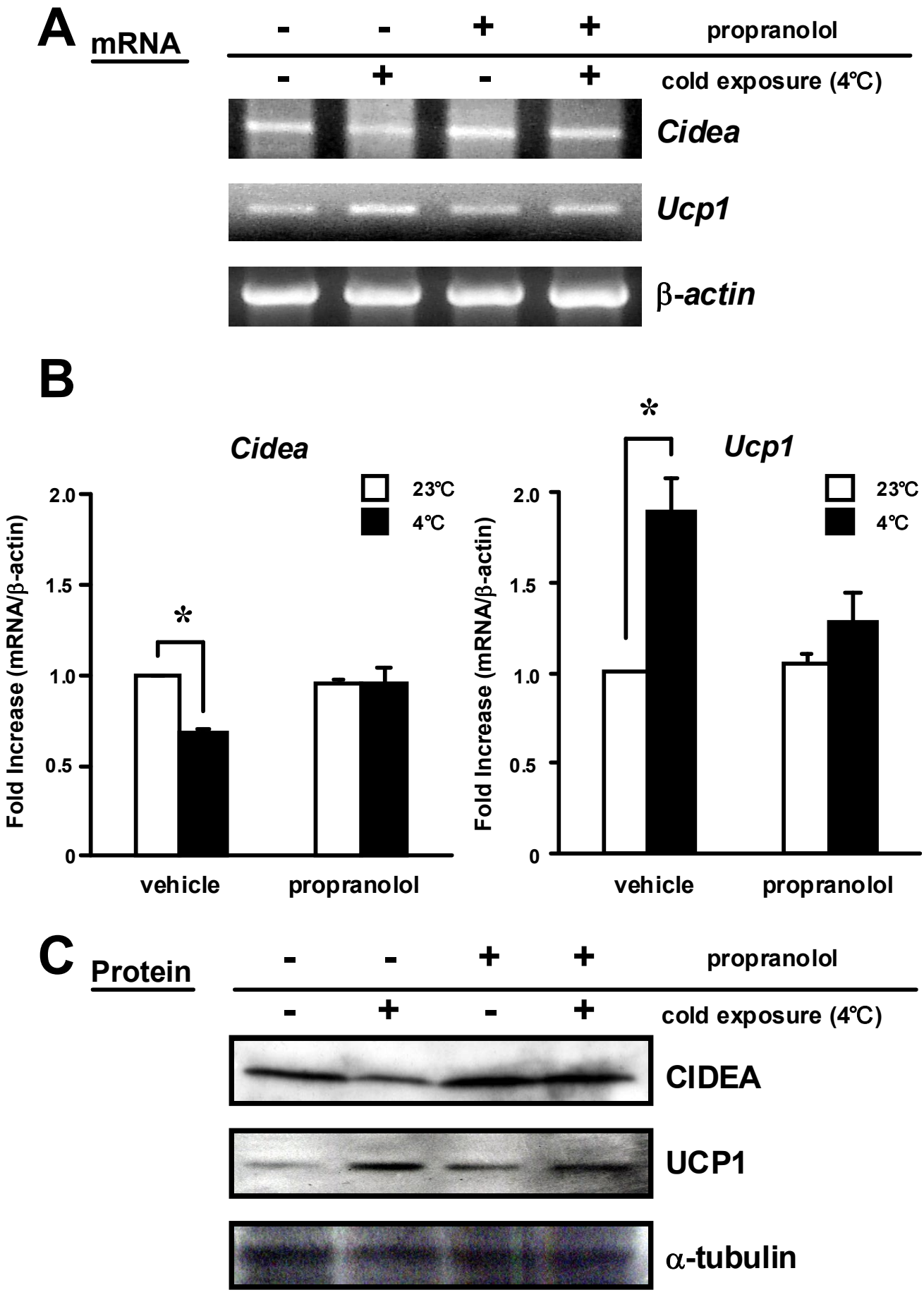
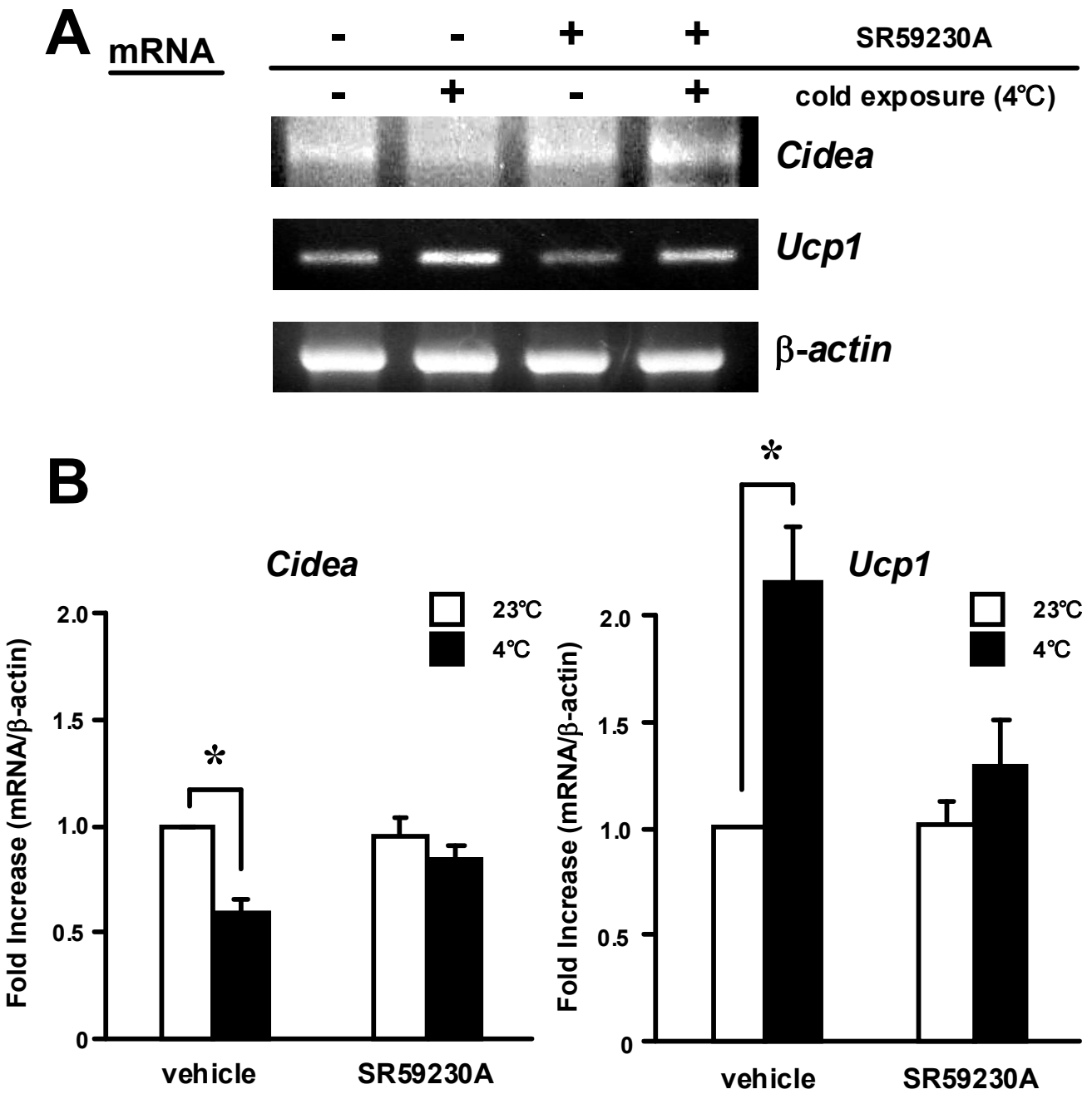

A
mRNA
- - + + propranolol
- + - + cold exposure (4℃)
Cidea
Ucp1
β-actin
B
Cidea
Ucp1
23℃
4℃
Fold Increase (mRNA/β-actin)
2.0
1.5
1.0
0.5
0
*
vehicle
propranolol
C
Protein
- - + + propranolol
- + - + cold exposure (4℃)
CIDEA
UCP1
α-tubulin

Figure-4


A
mRNA
- - + + SR59230A
- + - + cold exposure (4℃)
Cidea
Ucp1
β-actin
B
Cidea
Ucp1
23℃
4℃
Fold Increase (mRNA/β-actin)
2.0
1.5
1.0
0.5
0
*
vehicle
SR59230A
C
Protein
- - + + SR59230A
- + - + cold exposure (4℃)
CIDEA
UCP1
α-tubulin