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Nrf2 induces Ucp1 expression in adipocytes in response to β 3-AR stimulation and enhances oxygen consumption in high-fat diet-fed obese mice

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Running title: A Role of Nrf2 in β 3-AR signaling

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Abstract

Cold-induced norepinephrine activates β 3-adrenergic receptors (β 3-AR) to stimulate the kinase cascade and cAMP-response element-binding protein, leading to the induction of thermogenic gene expression including uncoupling protein 1 (Ucp1). Here, we showed that stimulation of the β 3-AR by its agonists isoproterenol and CL316,243 in adipocytes increased the expression of *Ucp1* and Heme Oxygenase 1 (*Hmox1*), the principal Nrf2 target gene, suggesting the functional interaction of Nrf2 with β 3-AR signaling. The activation of Nrf2 by tert-butylhydroquinone and reactive oxygen species (ROS) production by glucose oxidase induced both *Ucp1* and *Hmox1* expression. The increased expression of *Ucp1* and *Hmox1* was significantly reduced in the presence of a Nrf2 chemical inhibitor or in Nrf2-deleted (knockout) adipocytes. Furthermore, Nrf2 directly activated the Ucp1 promoter, and this required DNA regions located at -3.7 and -2.0 kb of the transcription start site. The CL316,243-induced Ucp1 expression in adipocytes and oxygen consumption in obese mice were partly compromised in the absence of Nrf2 expression. These data provide additional insight into the role of Nrf2 in β 3-AR-mediated *Ucp1* expression and energy expenditure, further highlighting the utility of Nrf2-mediated thermogenic stimulation as a therapeutic approach to diet-induced obesity.

Key words: β 3-adrenergic receptor, Uncoupling protein 1, Nrf2, obesity, energy expenditure
(187 words)

INTRODUCTION

The uncoupling of respiration and ATP synthesis contribute to non-shivering thermogenesis in adipose tissues during chronic cold exposure (1, 2). The respiratory chain in mitochondria accumulates protons (H^+) in the intermembrane space that fall into ATP synthase to generate ATP. During cold exposure, the accumulated protons in the intermembrane space are dissipated by uncoupling protein 1 (Ucp1) and produce heat instead of ATP (3, 4). Thermogenic adipocytes expressing Ucp1 mainly found in brown adipose tissue (BAT) and subcutaneous white adipose tissue (WAT) is stimulated by β -adrenergic receptor (β 3-AR) activation (2, 5-8).

Cold stress induces the secretion of norepinephrine, leading to the activation of β 3-ARs in adipose tissue (9, 10). β 3-AR regulates whole-body thermogenesis in part through Ucp1 stimulation in adipose tissue (11). BAT in Ucp1 knockout (KO) mice has been reported to be unresponsive to norepinephrine (12, 13), suggesting the importance of Ucp1 in β 3-AR signaling-mediated thermogenesis. Stimulation of β 3-ARs by agonists induces the G_s -dependent activation of adenylate cyclase, increases intracellular cAMP levels, and activates protein kinase A (PKA) (10, 14). Activated PKA increases the transcription of various target genes through transcription factor cAMP-responsive element-binding protein (Creb). Creb binds to the promoter region of target genes containing the cAMP-responsive element (CRE) (15).

Nuclear factor erythroid 2-related factor 2 (Nrf2), a master regulator of the anti-oxidant response, removes excess reactive oxygen species (ROS) by inducing antioxidant-related genes under oxidative stress conditions (16, 17). Nrf2 binds to the antioxidant response element (ARE) of the target genes to induce the transcription of cytoprotective antioxidation-related genes such as Heme Oxygenase-1 (Hmox1), NAD(P)H quinone oxidoreductase 1 (Nqo1), and glutamate-cysteine ligase modifier subunit (Gclm), and glutathione reductase (16). Previous studies

showed that Nrf2 interact with other signaling pathways including the arylhydrocarbon receptor, NF- κ B, NFATc1, and p53 (18, 19). Thus, it is possible that Nrf2 acts cooperatively with intracellular signaling pathways responding to various cellular stresses.

In this study, we examined the potential roles of Nrf2 in β 3-AR-mediated *Ucp1* expression in adipocytes. We showed that the β 3-AR-induced *Ucp1* expression and stimulated oxygen consumption was partly impaired in Nrf2 KO obese mice. These studies indicate the novel role of Nrf2 in β 3-AR activation in adipocytes.

RESULTS

β 3-AR activation induces both *Ucp1* and *Hmox1* expression in adipocytes.

To examine the possible interactions between Nrf2 and β 3-AR signaling in adipocytes, we stimulated adipocytes with β 3-AR agonists and measured the expression of *Hmox1*, the principal Nrf2 target gene in adipocytes. Strong *Ucp1* mRNA induction was observed within 1 h and reached maximal levels of ~50-fold at 3 h. Similarly, time-dependent increases in *Hmox1* mRNA levels were observed in C3H10T1/2 adipocytes (Fig 1A). Treatment of primary adipocytes isolated from inguinal white adipose tissue with CL316,243 also induced *Ucp1* and *Hmox1* expression (Fig. 1B). We also confirmed that the treatment of C3H10T1/2 adipocytes with another β -AR agonist isoproterenol stimulated *Hmox1* and *Ucp1* expression (Fig 1C). Adipocytes exposed to cool temperatures at 30 °C similarly induced both *Ucp1* and *Hmox1* expression (Fig. 1D). These data suggest an interaction between Nrf2 and β 3-AR signaling in adipocytes.

Nrf2 activation mimics β 3-AR stimulated-*Ucp1* expression in adipocytes.

Given the *Ucp1* and *Hmox1* induction by β 3-AR activation in adipocytes, we investigated whether Nrf2 activation would promote *Ucp1* expression similar to that of β 3-AR activators. We found that pharmacological Nrf2 activation by tert-butylhydroquinone (tBHQ) also stimulated *Hmox1* and *Ucp1* expression (Fig. 2A-2B).

An increase in oxidative stress due to the generation of ROS leads to the activation of Nrf2 (20). To determine whether ROS-induced Nrf2 activation affected *Ucp1* and *Hmox1* expression, we treated cells with glucose oxidase to generate ROS (21) and measured *Hmox1* and *Ucp1* expression. Consistently, treatment with glucose oxidase increased *Ucp1* and *Hmox1* levels similar to those from tBHQ treatment (Fig. 2C-2D).

Because Nrf2 activation induced *Ucp1* expression, we further investigated the effect of Nrf2 on human *Ucp1* promoter activity. Toward this, we cloned the proximal 3.7 kb of the human *Ucp1* promoter into the PGL-3 vector to obtain *Ucp1* promoter-driven luciferase reporter constructs. Transient Nrf2 expression stimulated -3.7kb *Ucp1* promoter driven luciferase activity, whereas Nrf2 did not promote luciferase activity in the -2.0 kb and -1.0 kb *Ucp1* proximal promoter (Fig. 2E). These data indicate that Nrf2 increases *Ucp1* transcription and that this stimulatory effect required proximal promoter regions located at -3.7 and -2.0 kb of the transcription start site.

Nrf2 is essential for the tBHQ (Nrf2 activator) induced *Ucp1* expression in adipocytes.

The effects of Nrf2 activation in adipocytes suggested that Nrf2 was essential for the effects of tBHQ on adipocytes. To test this, we treated the cells with N-acetylcysteine (NAC), a known ROS-scavenging chemical, to inhibit Nrf2 activity, and assessed its effects. Treatment with NAC has been shown to decrease Nrf2 activity (22). Consistently, NAC impaired the tBHQ - induced expression of *Ucp1* and Nrf2 target genes (Fig 3A).

To further show the role of Nrf2 in *Ucp1* induction, we treated primary adipocytes isolated from the inguinal fat pads of wild-type (Nrf2 WT) and Nrf2 knockout (Nrf2 KO) mice with tBHQ. Nrf2 activation strongly induced the *Ucp1* and Nrf2 target gene expression of *Hmox1*, *Nqo1*, *Srxn1*, and *Gclc* in Nrf2 WT primary adipocytes. However, these effects were significantly impaired in the Nrf2 KO adipocytes (Fig. 3B). Together, these data show that Nrf2 activation can induce *Ucp1* expression in adipocytes.

β 3-AR induced oxygen consumption is partly dependent on Nrf2.

The ability of β 3-AR to increase *Hmox1* and *Ucp1* expression suggested that Nrf2 might be involved in β 3-AR-induced *Ucp1* expression in adipocytes. To investigate this possibility, we

treated Nrf2 WT and Nrf2 KO adipocytes with CL316,243. β 3-AR activation strongly induced the expression of *Ucp1* and Nrf2 target genes in Nrf2 WT adipocytes. However, these effects were significantly blunted in Nrf2 KO adipocytes (Fig. 4A).

To further assess the role of Nrf2 in β 3-AR activation in mice, we measured the CL316,243-induced O₂ consumption rates of aged WT and Nrf2 KO female mice. CL316,243 treatment of 14-month-old mice increased oxygen consumption by about 123% compared to the basal levels in WT mice, whereas CL316,243 exhibited 110% increases in Nrf2 KO aged female mice (Fig. 4B). We also measured β 3-AR-induced oxygen consumption in obese C57BL/6J male mice fed high-fat diet (HFD) for 16 weeks. The WT obese mice stimulated with CL316,243 showed increased rates of oxygen consumption up to 120% but its stimulatory effects were less (113%) in the Nrf2 KO obese mice (Fig. 4C). These findings suggest the role of Nrf2 in β 3-AR-mediated oxygen consumption. Increased metabolic activity by β 3-AR stimulation increases body temperature. Thus, we further investigated rectal temperatures for 24 h in the Nrf2 WT and Nrf2 KO obese mice. Consistent with the oxygen consumption, the nighttime (at 4 A.M) rectal temperature in Nrf2 WT mice was significantly higher than that in the Nrf2 KO mice (Fig. 4D). Together, these data strongly suggest that Nrf2 was partly involved in the β 3-AR-*Ucp1* axis in certain conditions and further suggest that adipose Nrf2 activation can be a regulator of the energy expenditure of obese or aged mice.

DISCUSSION

In oxidative stress conditions, Nrf2 is activated to induce the expression of cytoprotective genes (16). It has become evident that Nrf2 is interconnected with other signaling pathways such as NF- κ B and p53 (18). Furthermore, Nrf2 may also exert its effects through interactions with additional pathways such as the PKA/Creb signaling pathway. Nrf2 in fasting conditions becomes activated by the cAMP/PKA pathway and regulates its downstream target genes in hepatocytes (23). CREB signaling was also shown to increase Hmox1 in mouse adrenal cells and human umbilical vein endothelial cells (24-26). Therefore, these studies raise the possibility of crosstalk between Nrf2 and cAMP/PKA signaling in adipocytes.

β 3-AR stimulation induces cAMP mediated PKA activity and subsequent Creb phosphorylation, leading to transcriptional regulation of target genes containing cAMP-responsive element (CRE) (15). (10, 14). β 3-AR can also relay the signaling through other cellular pathways including p38, PKC, and ERK1/2 in adipocytes (27) (28-30). Interestingly, adipocytes exposed to cool temperatures induce Ucp1 expression independent of the PKA/Creb pathway (31), indicating the existence of alternative or parallel pathways for β -AR mediated Ucp1 induction in adipocytes. Our data show that β 3-AR stimulation induces Nrf2 activation and the Nrf2 can contribute to the increases of Ucp1 expression in adipocytes. Since the adrenergic signaling is often associated with increased production of ROS (32), it is also reasonable that the increased ROS levels by β 3-AR activation can induce Nrf2 mediated gene expression including Ucp1 and Nrf2 target genes in adipocytes. Therefore, the dissection on the β 3-AR/cAMP/PKA/CREB and the β 3-AR/Nrf2 signaling cascades for functional relationships should be investigated in future.

We found that β 3-AR stimulation increased the expression of conventional Nrf2 downstream target genes in an Nrf2-dependent manner. We also presented evidence that the degree of Ucp1 induction by β 3-AR agonists was partly blunted in the absence of Nrf2 expression in adipocytes. Additionally, the CL316,243-induced oxygen consumption was partly impaired in Nrf2 KO obese mice, indicating a role of Nrf2 in β 3-AR-induced Ucp1 expression in adipocytes and energy expenditure in obese mice. Consistent with our findings, it has been reported that C3H10T1/2 undifferentiated mesenchymal stem cells and bone marrow cells expressed β 2-AR and its activation by adrenaline protected cells from oxidative stress through the Nrf2-mediated induction of glutathione synthesis (33). It is thus possible that adrenergic stimulation-induced Nrf2 activation may not be β -AR subtype-specific.

At present, it is not clear how the β 3-AR signaling pathway regulates Nrf2 activity. Under basal conditions, the Nrf2-Keap1 complex present in the cytosol constantly degrades Nrf2. When Keap1 senses cellular oxidative and electrophilic stress, Keap1 and Nrf2 complexes are disrupted, leading to the stabilization and activation of Nrf2. Oxidative/electrophilic stress results in kinase mediated phosphorylation on Nrf2 or Nrf2 negative regulators (i.e. Keap1 and Bach1) for increasing Nrf2 activities. Thus, it is tempting to speculate that β 3-AR signaling-induced kinase including PKA may affect Nrf2 or its negative regulators to stabilize Nrf2. Future studies are necessary to dissect the exact molecular connection between Nrf2 and β 3-AR signaling in adipocytes.

In conclusion, we provide data for new insight into the role of Nrf2 in β 3-AR-mediated effects and further suggest the utility of Nrf2-mediated thermogenic stimulation as a therapeutic approach for diet-induced obesity.

Figures

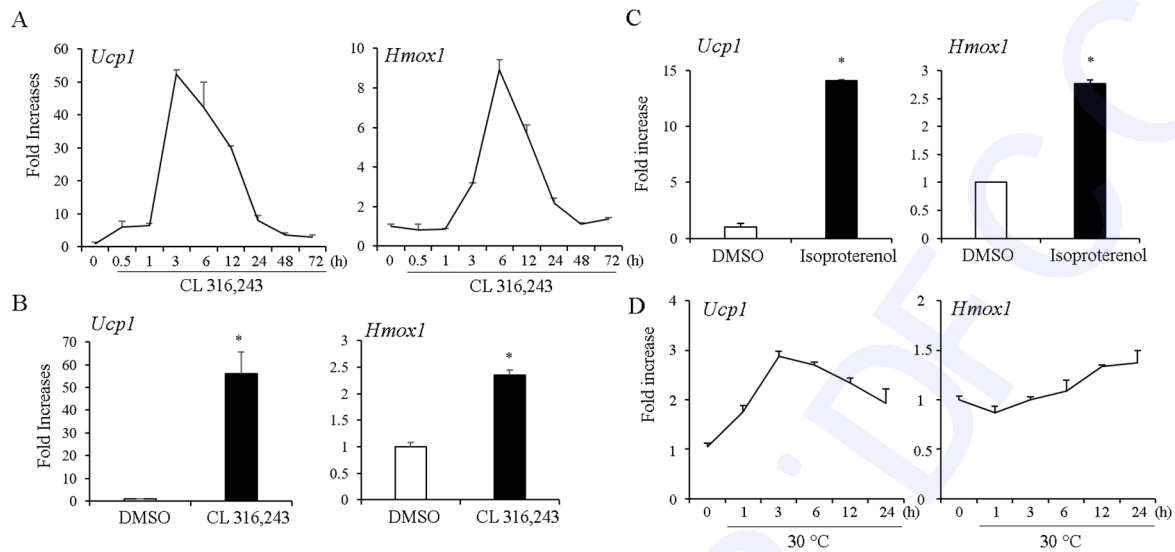


Fig. 1. β 3-AR activation induces *Ucp1* and *Hmox1* expression in adipocytes. (A) Time-dependent increases in *Ucp1* and *Hmox1* mRNA levels by treatment with CL316,243 (5 μ M) expressed as fold-increases relative to levels in dimethyl sulfoxide (DMSO)-treated C3H10T1/2 adipocytes. (B) Treatment of primary adipocytes isolated from inguinal adipose tissues with CL316,243 (5 μ M) for 6 h. (C) Treatment of C3H10T1/2 adipocytes with isoproterenol at 10 nM for 6 h. (D) Differentiated C3H10T1/2 adipocytes were exposed to 37 °C or 30 °C and the expression of *Ucp1* and *Hmox1* was measured. The data represent means \pm s.e.m. and are representative of three independent experiments. Statistical significance was determined relative to controls using the Student's *t*-test (* $P < 0.05$).

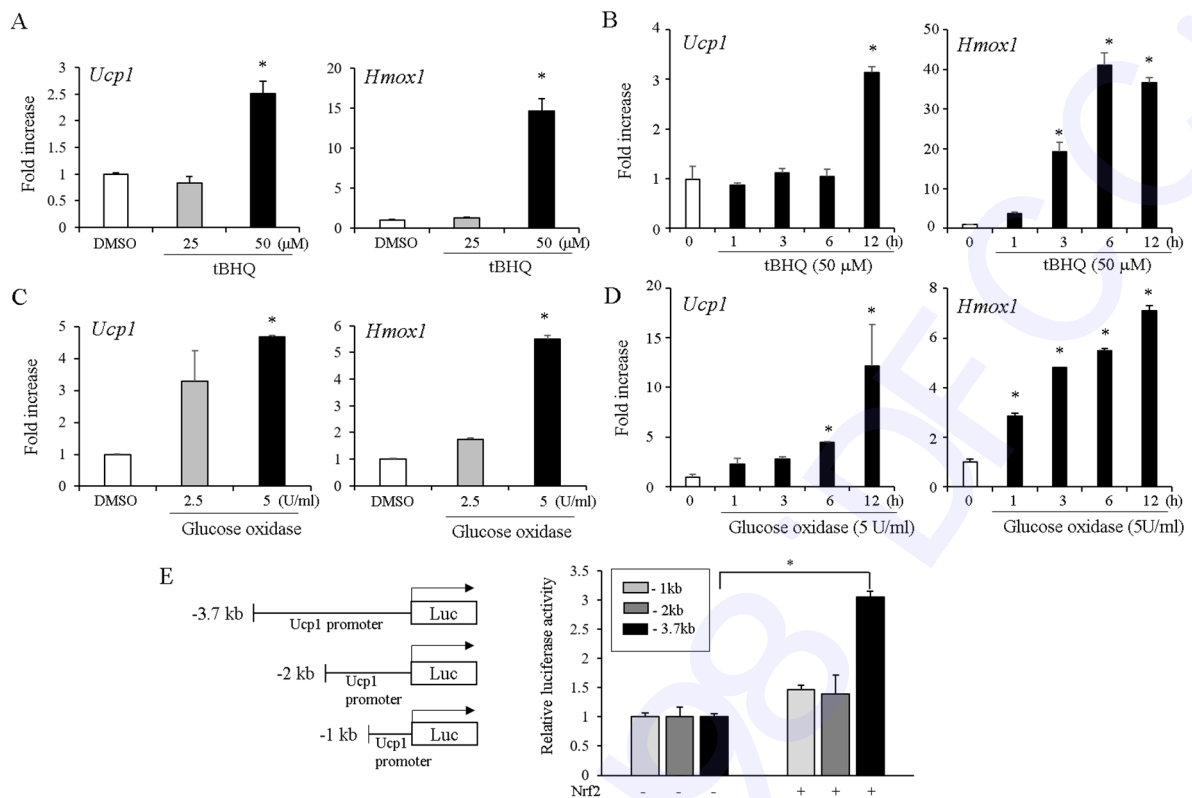


Fig. 2. Nrf2 activation increases *Ucp1* expression in adipocytes. (A) tert-Butylhydroquinone (tBHQ) at 25 and 50 μ M increases *Ucp1* and *Hmox1* expression in C3H10T1/2 adipocytes. (B) Time-dependent increases in *Ucp1* and *Hmox1* mRNA levels by treatment with 50 μ M tBHQ expressed as fold-increases relative to the levels in DMSO-treated C3H10T1/2 adipocytes. (C) Treatment with glucose oxidase (2.5 U or 5 U/ml) increases *Ucp1* and *Hmox1* expression in C3H10T1/2 adipocytes. (D) Time-dependent increases in *Ucp1* and *Hmox1* mRNA levels by 5 U/ml glucose oxidase expressed as fold-increases relative to levels in DMSO-treated C3H10T1/2 adipocytes. (E) Nrf2 activates the *Ucp1* promoter in the reporter assay. 293FT cells were transiently transfected with *Ucp1* promoter-luciferase with or without the Nrf2-expressing vector. The proximal 3.7 kb, 2 kb, or 1 kb of the human *Ucp1* promoter sequences were fused to express the *Ucp1* promoter-driven luciferase reporter. The data represent averages \pm s.e.m. of triplicates and are expressed as fold-increases relative to empty vector-transfected cells. Statistical significance was determined relative to controls using the Student's *t*-test (* $P < 0.05$).

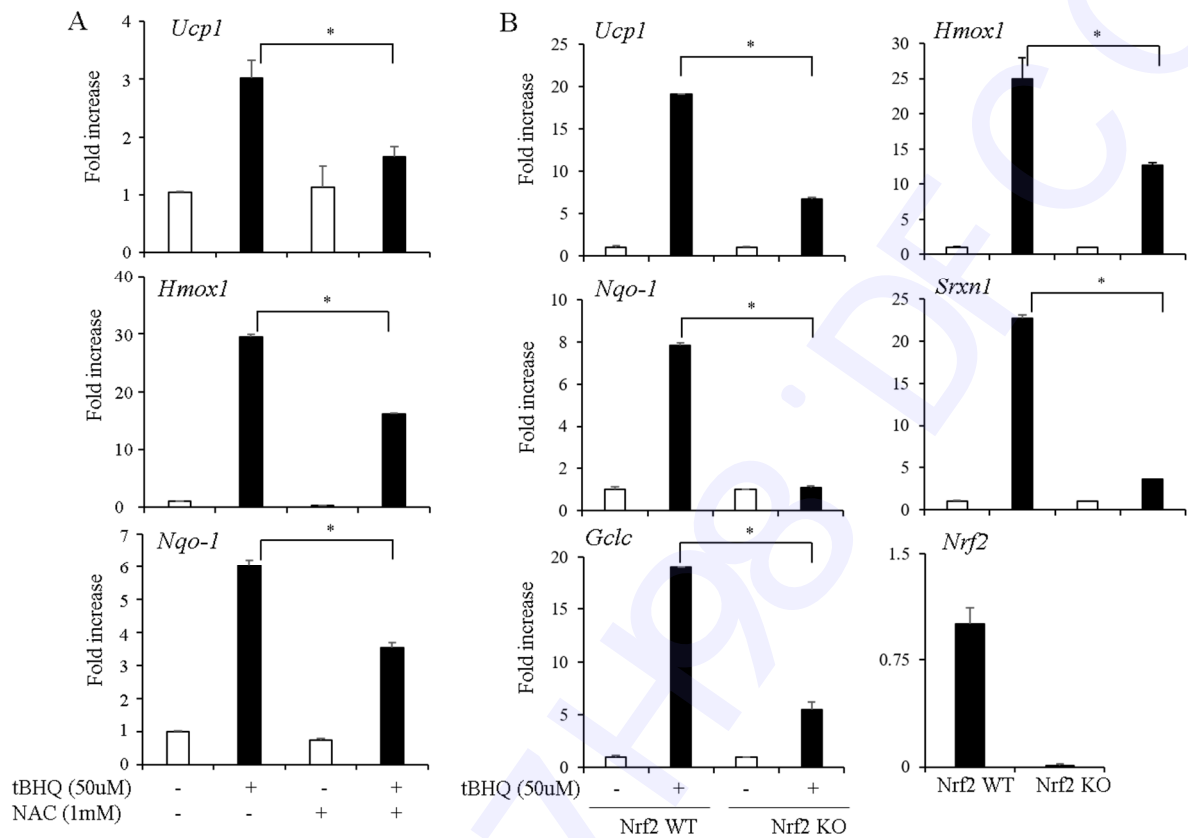


Fig. 3. Nrf2 is essential for tert-butylhydroquinone (tBHQ)-mediated *Ucp1* induction in adipocytes. (A) C3H10T1/2 adipocytes were treated with tBHQ (50 μ M) and NAC (1mM) for 12 h and the expression of *Ucp1*, *Hmox1*, and *Nqo1* was measured. (B) Primary adipocytes isolated from the inguinal fat pads of Nrf2 wild-type (Nrf2 WT) and Nrf2 knockout mice (Nrf2 KO) were treated with tBHQ (50 μ M) for 12 h and the expression levels of *Ucp1* and Nrf2 target genes (*Hmox1*, *Nqo1*, *Srxn1*, and *Gclc*) were measured. The data represent means \pm s.e.m. and are representative of three independent experiments. Statistical significance was determined relative to controls using the Student's *t*-test (* $P < 0.05$).

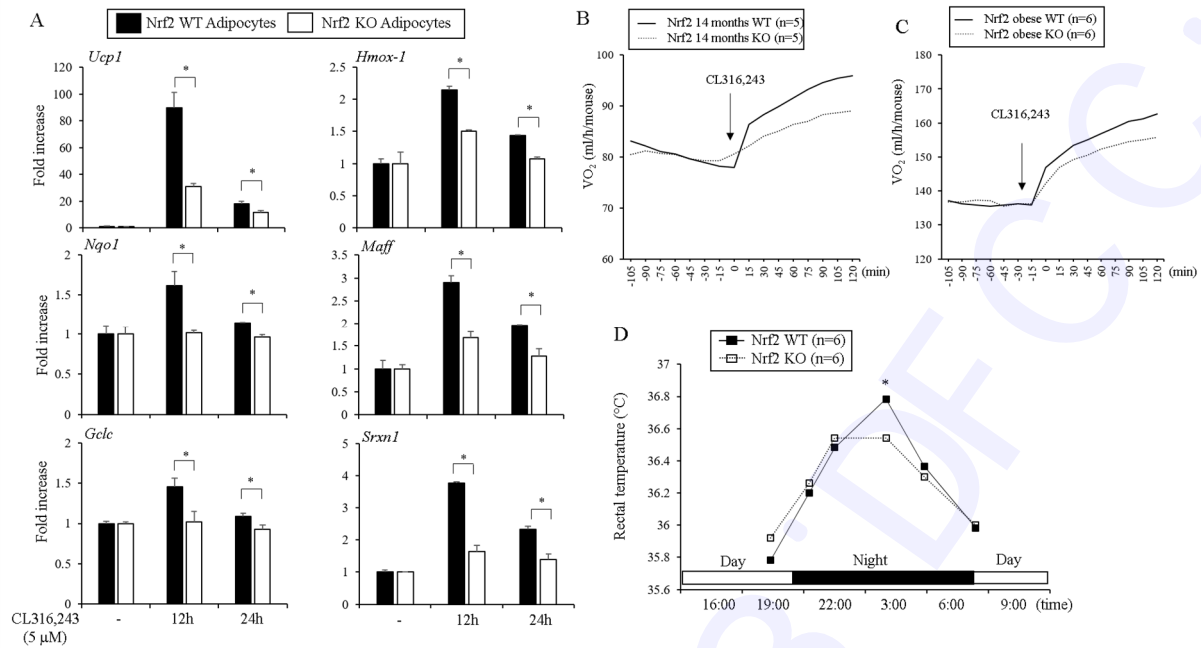


Fig. 4. β 3-AR-stimulated *Ucp1* induction and oxygen consumption are partly dependent upon Nrf2. (A) Primary adipocytes isolated from the inguinal fat pads of Nrf2 WT and Nrf2 KO mice were treated with CL316,243 (5 μ M) for 12 or 24 h and the expression levels of *Ucp1* and Nrf2 target genes (*Hmox1*, *Nqo1*, *Maff*, *Gclc*, and *Srxn1*) were measured. (B) Fourteen-month-old Nrf2 WT and Nrf2 KO female mice were stimulated with β 3-AR agonist (CL316,243, 2 mg/kg) and the O_2 consumption rates were measured. (C) The O_2 consumption rates of Nrf2 WT and Nrf2 KO obese male mice fed high-fat diet for 16 weeks were measured. (D) Rectal temperature was measured for 24 h in WT and Nrf2 KO obese mice. The data represent mean \pm s.e.m. and statistically significant differences between WT and Nrf2 KO mice were determined by the Student's *t*-test * $P < 0.05$).

MATERIALS AND METHODS

Cell culture and expression analysis

C3H10T1/2 cells and primary adipocytes were maintained and differentiated as previously reported (34). The adipocytes were treated with 5 μ M CL316,243 (Sigma, St. Louis, MO, USA) or 10nM isoproterenol (Sigma) and total RNA was isolated for expression analysis. To measure gene expression levels, total RNA was isolated using TRIzol reagent (Invitrogen, Waltham, MA, USA) and reverse-transcribed using ReverTra Ace[®] qPCR RT Master Mix (Toyobo, Japan). Real-time PCR was performed using THUNDERBIRD[®] SYBR[®] qPCR Mix (Toyobo). PCR primers were described previously (34, 35). Expression was normalized to the level of ribosomal 36B4 protein as previously described.

Animal studies

All animal studies were carried out in accordance with the guidelines of the Animal Research Committee (SKKUIACUC2020-08-21-1) of Sungkyunkwan University. Seven weeks old male C57BL/6J mice were purchased from Central Lab Animal Inc (Seoul, Korea) and housed in rooms at ambient temperature. The Nrf2 KO mice were previously described (35). Whole-body energy metabolism was measured using the Columbus Instruments Oxymax Lab Animal Monitoring System. The mice were placed in metabolic cages and acclimated in the metabolic chambers for one day before measuring O₂ consumption.

Statistical analysis

The data are presented as the mean \pm standard error of the mean (s.e.m). Comparisons between the control and experimental groups were analyzed using unpaired Student's t-tests. Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA).

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CONFLICTS OF INTERESTS

The authors declare no competing financial interest.

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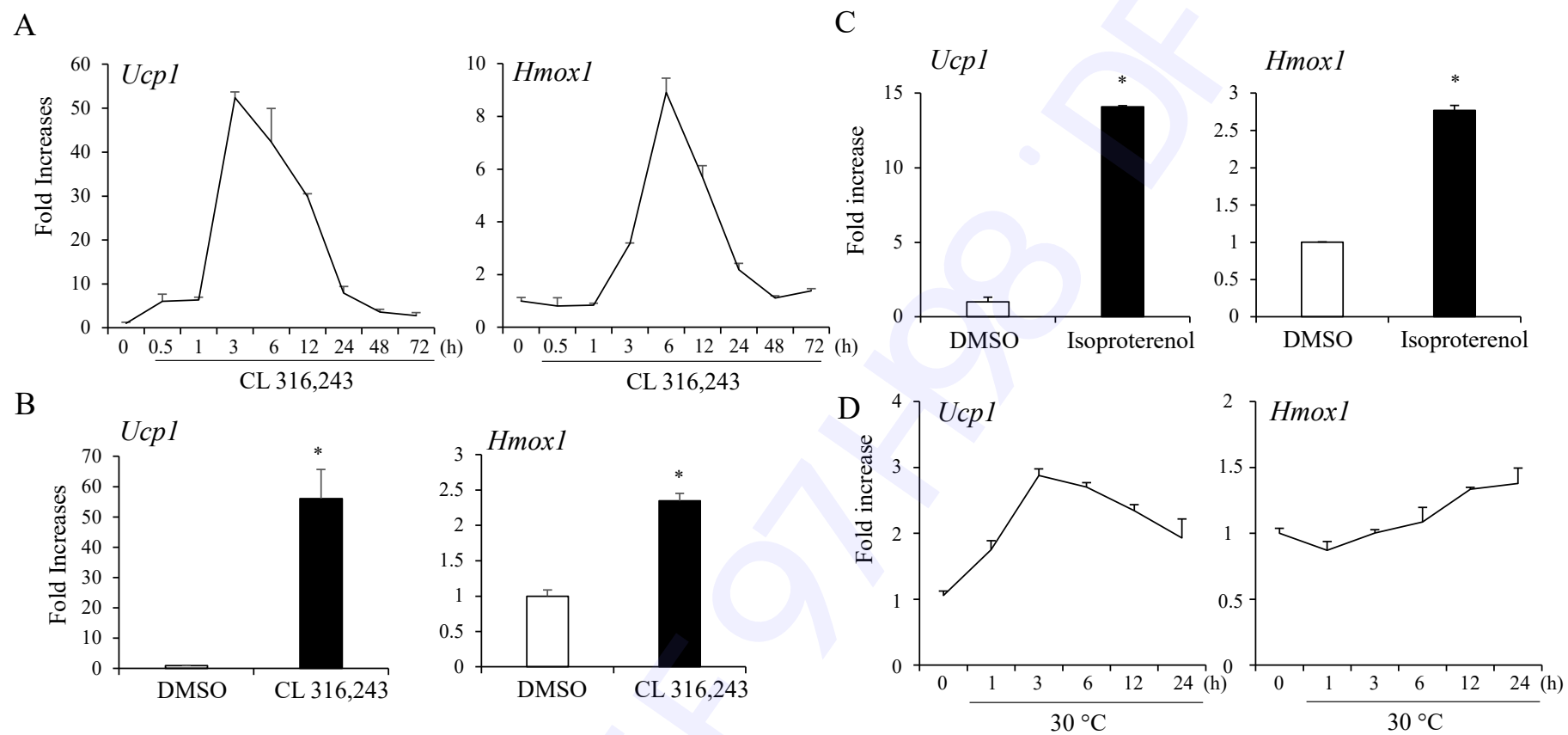


Fig. 1

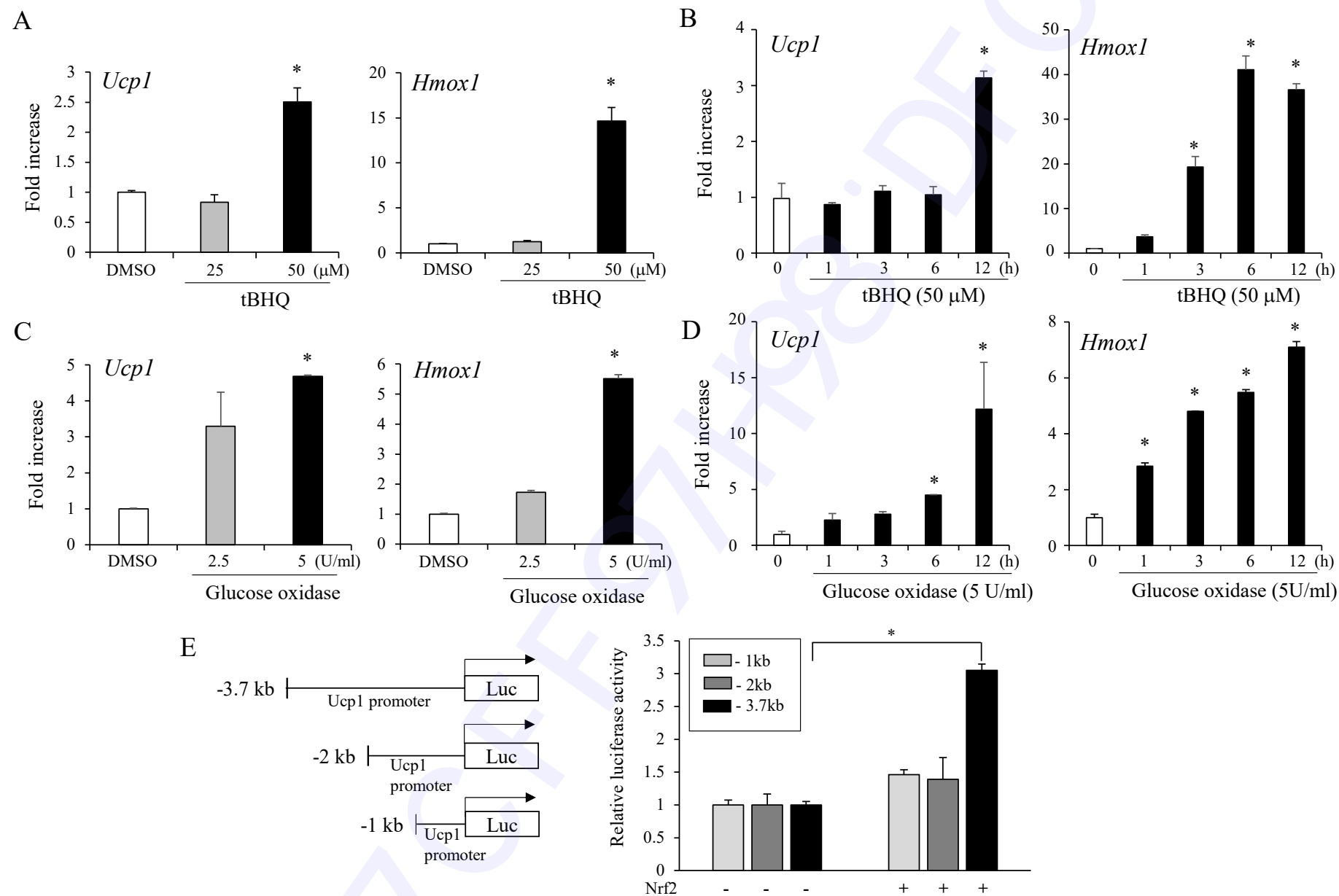


Fig. 2

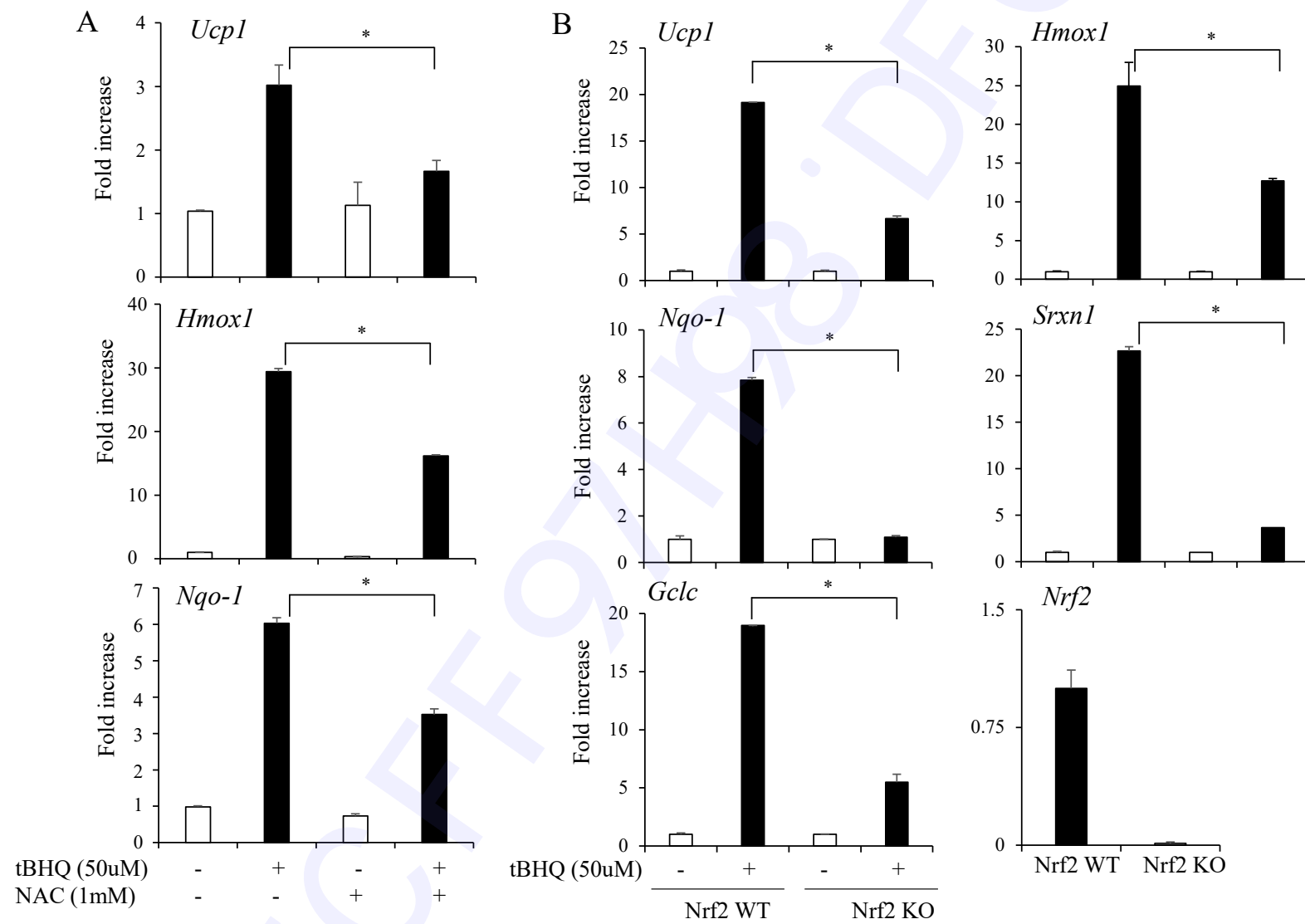


Fig. 3

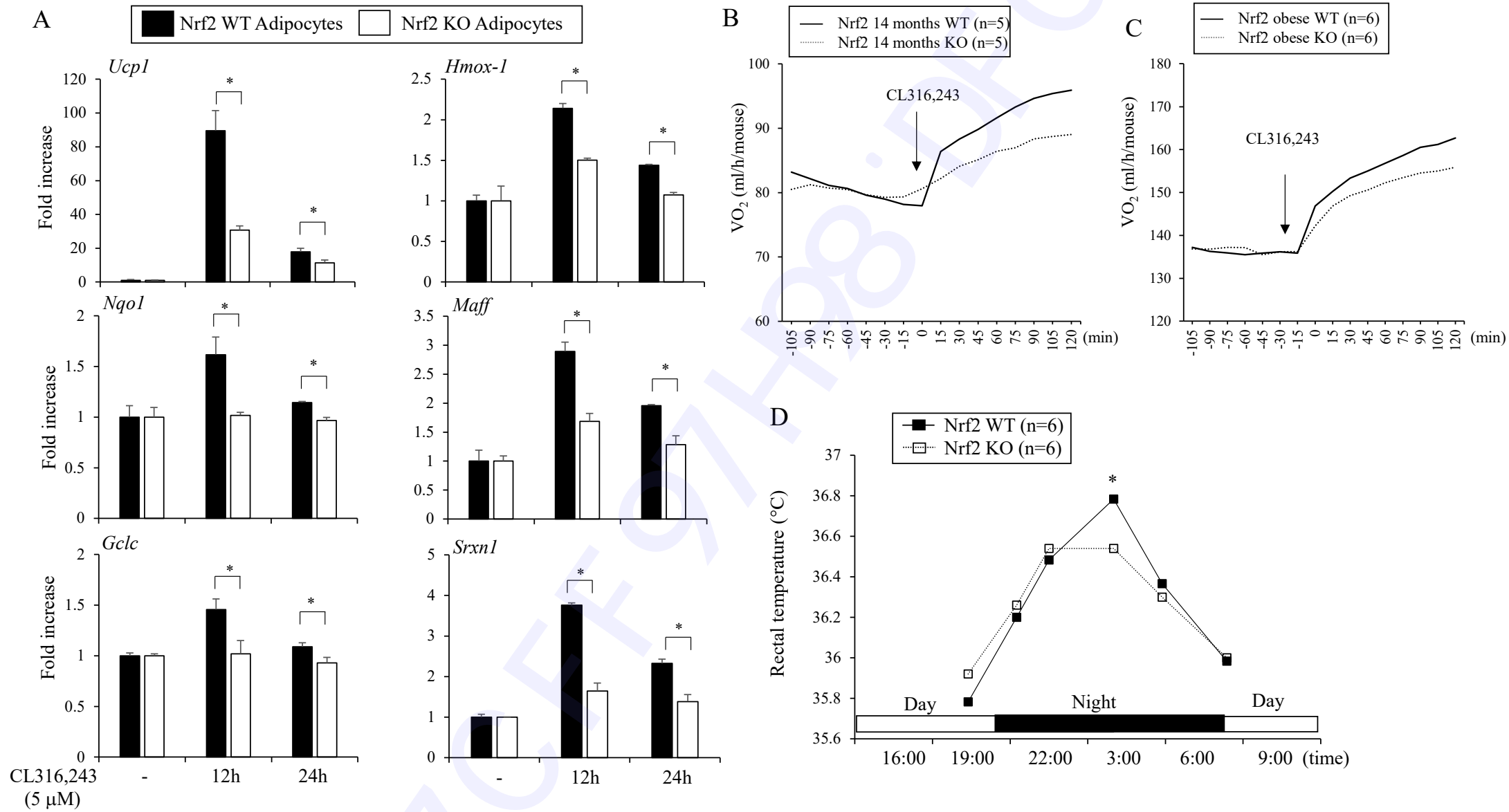


Fig. 4