Methylglyoxal attenuates isoproterenol-induced increase in uncoupling protein 1 expression through activation of JNK signaling pathway in beige adipocytes

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1. Introduction

Adipocytes, which play an important role in lipid metabolism, are generally classified into white and brown adipocytes according to their function [1]. White adipocytes store excess energy in the form of triglycerides (TG), while brown adipocytes produce heat associated with energy consumption through the degradation of TG, which plays a vital role in regulating systemic metabolism and thermogenesis [1]. Uncoupling protein 1 (UCP1), a proton carrier located in the inner membrane of the mitochondria, is involved in the thermogenic function of brown adipocytes [2,3]. UCP1 activation causes dissipation of the electrochemical proton gradient and a decrease in the proton motive force used to synthesize ATP. Proton leakage induced by UCP1 uncouples the proton gradient from ATP synthesis, releasing the free energy as heat [2,3]. Recently, some white adipocytes located in subcutaneous fat, such as the inguinal white adipose tissue (iWAT), have been shown to increase Ucp1 expression under cold exposure or adrenergic stimulation (i.e., brown-like “beige” adipocytes) [4,5]. Beige adipocytes also expend energy through UCP1-mediated thermogenesis [4,5]. Therefore, the elucidation of the molecular mechanisms underlying the regulation of thermogenesis in both brown and beige adipocytes is of interest because it can provide novel insights into approaches to control and treat obesity and obesity-related metabolic diseases such as type 2 diabetes and diabetic complications [4,5].

Although the development of diabetic complications is the result of a highly complex process, the accumulation of advanced glycation end...
products (AGEs), the synthesis of which is initiated by a non-enzymatic reaction between the aldehyde groups of glucose and amino groups of proteins, is recognized as one of the major factors linked to it [6]. Methylglyoxal (MG, CH$_2$COCHO) is a ubiquitous 2-oxoaldehyde derived from glycolysis [7–9]. Although MG is a natural metabolite, it is highly reactive because it contains two carbonyl groups and has a higher potential than glucose to produce AGEs. Plasma and tissue MG levels are higher in patients with diabetes than in healthy individuals, suggesting that MG plays a role in the development and progression of diabetic complications [10–12]. MG is mainly metabolized to β-lactate within cells through a ubiquitous glutathione-dependent glyoxalase system consisting of glyoxalase I and glyoxalase II [7,9]. Functional genome analyses suggest a relationship between a deficiency in this system, which implies increased MG and the development of diabetic complications [13,14]. However, it remains to be determined whether high MG levels are a cause or consequence of diabetes.

In this study, we investigated the effect of MG on Ucp1 expression induced by treatment with the β-adrenergic receptor agonist, isoproterenol, in differentiated immortalized iWAT-derived pre-adipocytes. Although the isoproterenol-induced expression of Ucp1 was inhibited by MG, downregulation of the protein kinase A (PKA) pathway, which contributes to Ucp1 expression by isoproterenol, was not responsible for this inhibitory effect. We found that MG enhanced the phosphorylation of c-Jun N-terminal kinase (JNK) mitogen-activated protein kinase (MAPK), and an inhibitor of JNK suppressed the inhibitory effect of MG on Ucp1 expression. These results indicate that activation of JNK MAPK is necessary for the MG-induced inhibition of Ucp1 expression.

2. Materials and methods

2.1. Materials

Dulbecco’s modified Eagle’s medium (DMEM)-high glucose, insulin, indomethacin, SB203580, and N-acetyl-l-cysteine (NAC) were purchased from Wako (Osaka, Japan). Fetal bovine serum was purchased from Gibco (FBS; Grand Island, NY, USA). Penicillin-Streptomycin mixed solution, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, and dihydroxyacetone (DHA) were purchased from Nacalai Tesque (Kyoto, Japan). Rosiglitazone was purchased from LKT Laboratories (Minneapolis, MN, USA). 3,3’,5-triiodo-l-thyronine (T$_3$), S-p-bromobenzylglutathione cyclooctenyl diester (BBGC), MG, and isoproterenol were purchased from Sigma-Aldrich (MO, USA). SP600125 was purchased from Enzo Life Sciences (NY, USA).

2.2. Cell culture

Immortalized primary pre-adipocytes from mouse iWAT were a kind gift from Dr. Shingo Kajimura (Harvard Medical School, MA, USA). C3H10T1/2 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). These cells were maintained in a humidified 5% CO$_2$ atmosphere at 37 °C in basic medium (DMEM-high glucose supplemented with 10% (v/v) FBS, 10,000 units/ml penicillin, and 10,000 μg/ml streptomycin). To differentiate the pre-adipocytes into mature adipocytes, cells were cultured to confluence before stimulation with 1 nM T$_3$, 5 μg/ml insulin, 0.5 μM rosiglitazone, 2 μg/ml dexamethasone, 0.5 mM IBMX, and 125 μM indomethacin in a basic medium for 48 h. The medium was then replaced with a growth medium (basic medium supplemented with 1 nM T$_3$, 5 μg/ml insulin, and 0.5 μM rosiglitazone). This step was repeated every 2 days until the adipocytes reached the 6th day after the induction of differentiation. After 6 days of differentiation, the matured adipocytes were incubated in a serum-free medium for 3–5 h before being subjected to 1 μM isoproterenol stimulation. The cells were pre-treated with either MG, BBGC or DHA in serum-free medium for 30 min before isoproterenol stimulation. The use of 10 μM SP600125 (JNK inhibitor), 10 μM SB203580 (p38 inhibitor), or 10 mM NAC (reactive oxygen species [ROS] inhibitor) precedes MG or BBGC treatment by 30 min.

2.3. RNA preparation and quantification of gene expression

Total RNA was isolated from cultured cells using Sepasol Super-I (Nacalai Tesque, Kyoto, Japan) following the manufacturer’s protocol. Total RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega, WI, USA) according to the manufacturer’s instructions using a thermal cycler (Takara PCR Thermal Cycler SP, Takara, Shiga, Japan). mRNA expression was quantified by real-time PCR using the SYBR® Green I assay system performed with a LightCycler (Roche Diagnostics, Mannheim, Germany). The protocol for amplification was as follows: denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72 °C for 45 s. The expression levels of these genes were normalized to those of 36B4. The primer sequences are listed in Supplementary Table 1.

2.4. Luciferase reporter assay

4.5 μg pUCP1-pro-Luc, a tk-LUC luciferase reporter plasmid containing the 3.8-kb portion of the 5′-flanking region of the mouse Ucp1 gene [15], was transfected into 90% confluent C3H10T1/2 cells growing on a 100-mm culture dish along with 500 ng pGL4.74 (hRluc/TK) vector as an internal control using Lipofectamine 2000 (Thermo Fisher Scientific, MA, USA). Four hours after transfection, the cells were seeded in 96-well-plates overnight. The next day, after 3–5 h of serum starvation, cells were treated with 1 mM MG for 20 min before 1 μM isoproterenol stimulation for 6 h. The cells were lysed and luciferase assay was performed using a Dual-Luciferase Reporter Gene Assay System (Promega, WI, USA) in accordance with the manufacturer’s protocol. Luminescence was measured using the Centro XS2 LB 960 Microplate Luminometer (Berthold Technologies, Bad Wildbad, Germany).

2.5. Western blotting

Western blotting was performed to evaluate protein phosphorylation, as described previously [16]. The antibodies used were anti-phospho-HSL Ser660, #4126; anti-HSL, #4107; anti-phospho-PKA substrate, #9624; anti-perilipin, #9349; anti-phospho-CREB Ser133, #9198; anti-CREB, #9197; anti-β-actin, #4967; anti-phospho-p38 Thr183/Tyr185, #9215; anti-p38, #9212; anti-phospho-JNK Thr183/Tyr185, #9251; anti-JNK, #9252; anti-phospho-ERK Thr202/Tyr204, #9101; and anti-ERK, #9102. All primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Immunoreactive bands were detected using anti-rabbit HRP secondary antibody, NBP1-75297 (Novus Biologicals, CO, USA) with Immobilon Western Chemiluminescent Horseradish Peroxidase Substrate (Millipore, Burlington, MA, USA) and an LAS-4000 mini-imaging system (Fujifilm, Tokyo, Japan).

2.6. Lipolysis assay

Mature adipocytes (day 6 post differentiation induction) were starved of serum for 3–5 h before adrenergic stimulation with 1 μM isoproterenol in serum-free medium containing 2% bovine serum albumin (Nacalai Tesque, Kyoto, Japan) for 3 h. Non-esterified fatty acids (NEFA) and glycerol levels within the cell culture medium were measured using NEFA C (Wako, Osaka, Japan) and triglyceride E (Wako, Osaka, Japan) assay kits, respectively. NEFA and glycerol levels were then normalized to the cellular protein levels.

2.7. Intracellular ROS assay

The intracellular levels of ROS were detected using the fluorescent probe H$_2$DCFDA (Thermo Fisher Scientific, MA, USA). The adipocytes were incubated with 2 μM of the dye in either the presence or absence of
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3.1. MG attenuates the isoproterenol-induced expression of Ucp1

To examine whether MG affects Ucp1 expression, we determined Ucp1 expression following stimulation by a non-selective β-adrenergic receptor agonist, isoproterenol, in differentiated immortalized iWAT-derived pre-adipocytes. As shown in Fig. 1A, pretreatment with MG decreased Ucp1 expression induced by 3 h of isoproterenol treatment.

The inhibitory effect of MG on the isoproterenol-induced expression of Ucp1 was dose-dependent (Fig. 1B). In addition, usage of a luciferase reporter system show that MG significantly decreased the luciferase activity of the mouse Ucp1 gene promoter reporter plasmid under isoproterenol stimulation, which suggests that MG attenuates the isoproterenol-induced expression of Ucp1 through decreasing its promoter activity (Fig. 1C).

Glyoxalase I is the main metabolic enzyme for MG; therefore, treatment with BBGC, an inhibitor of glyoxalase I, increases the concentration of intracellular MG [17]. Moreover, BBGC pretreatment negatively affected the isoproterenol-induced expression of Ucp1 (Fig. 1D). DHA is the smallest ketotriose that is utilized by many organisms as an energy source. It has been previously reported that DHA is non-enzymatically converted to MG [18], and intracellular MG levels in yeast are increased by cultivation under presence of DHA [19]. The isoproterenol-induced expression of Ucp1 was attenuated by treatment with DHA (Fig. 1E). These results suggest that intracellular MG negatively influences the expression of Ucp1 induced by adrenergic stimulation. Next, we set out to examine the mechanism by which intracellular MG affects Ucp1 expression.

Fig. 1. MG represses isoproterenol-stimulated Ucp1 expression in differentiated immortalized iWAT-derived pre-adipocytes.

Adipocytes were treated with 1 mM MG (A), or 0.5, 1, or 2 mM MG (B) for 30 min, followed by stimulation with 1 μM isoproterenol (Iso) for 3 h. mRNA expression levels of Ucp1 in the cells were then determined by real-time PCR. Data are presented as the mean ± SEM (error bars), with n = 3–4 per group. (C) C3H10T1/2 fibroblasts were transfected with the Ucp1 promoter reporter plasmid, pUCP1-pro-Luc. After that, the cells were treated with 1 mM MG for 30 min, followed by stimulation with 1 μM isoproterenol for 6 h. Cells were lysed and luciferase assay was then performed. Data are presented as the mean ± SEM (error bars), with n = 5 per group. 20 μM BBGC (D), or 12.5, 25 or 50 mM DHA (E) were pre-treated in adipocytes for 30 min, followed by stimulation with 1 μM isoproterenol for 3 h. Then, mRNA expression levels of Ucp1 in the cells were determined by real-time PCR. Data are presented as the mean ± SEM (error bars), with n = 3–4 per group. One-way ANOVA followed by Dunnett’s (A,D) or Sidak’s (B,C,E) post-hoc tests were done to determine statistical significance. *, P < 0.05; **, P < 0.01 between the non-stimulated vehicular control group and the respective groups. #, P < 0.05; ##, P < 0.01.

2.8. Statistical analysis

Statistical analyses were conducted using GraphPad Prism (version 9.2; San Diego, CA, USA). After confirming that datasets fulfill the Shapiro-Wilk normality test, statistical significance was determined using one-way ANOVA followed by Sidak’s or Dunnett’s multiple comparison test, as indicated in the figure legends. Differences were considered significant at P < 0.05.

3. Results and discussion

3.1. MG attenuates the isoproterenol-induced expression of Ucp1

10 mM NAC for 30 min at 37 °C in the dark, followed by treatment with either 500 μM H2O2 (Wako, Osaka, Japan) or 1 mM MG for 3 h. Fluorescence levels were then measured in the fluorescence reader, Tecan Infinite F-200 microplate reader (Tecan Inc., Mannefors, Switzerland) with excitation/emission at 485 nm/535 nm.
agonists increases the intracellular level of cAMP, which in turn activates the PKA signaling pathway, which contributes to the increased expression of Ucp1 and other thermogenic genes. Since MG did not activate PKA signaling, MG might have enhanced CREB phosphorylation through the activation of MSK1. Since MG did not activate PKA signaling, MG might have enhanced CREB phosphorylation through the activation of MSK1. 

3.3. MG attenuates Ucp1 expression through the activation of JNK signaling pathway

MG is involved in the activation of signaling pathways as a signaling molecule in diverse organisms from yeast to mammalian cells [7,9]. Moreover, MG induces the activation of JNK, p38, and extracellular signal-regulated kinases (ERK) [8]. These kinases are involved in the regulation of gene expression and cellular processes [9]. The molecular mechanisms by which MG regulates JNK signaling are not yet fully understood. Further studies are needed to elucidate the role of MG in JNK signaling and its potential therapeutic applications.
receptor kinase (ERK) MAPK signaling pathways in cultured cells [32–34]. As shown in Fig. 3A, MG enhanced the phosphorylation of JNK and p38 MAPKs in adipocytes. An increase in the phosphorylation levels of JNK and p38 was also observed following treatment with BBGC (Fig. 3B). In contrast, ERK phosphorylation levels were not significantly affected by MG or BBGC (Fig. 3A and B).

To determine whether the activation of JNK and p38 participates in the negative effect of MG on Ucp1 expression, we examined the effect of MAPK inhibitors on the MG-induced inhibition of Ucp1 expression. Although the p38 MAPK inhibitor, SB203580, did not affect the decrease in the isoproterenol-induced expression of Ucp1 by MG or BBGC treatment, the JNK MAPK inhibitor, SP600125, recovered this decrease (Fig. 3C and D). These results suggest that MG attenuates the isoproterenol-induced expression of Ucp1 through the activation of the JNK signaling pathway.

Peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear receptor family of ligand-activated transcription factors, is the master regulator of adipogenesis [35]. In addition, PPARγ controls the transcriptional regulation of Ucp1 [35]. ERK and JNK MAPK phosphorylates PPARγ at Ser112, which attenuates PPARγ activity [36], and this phosphorylation is also associated with Ucp1 expression [37]. Although these findings raise the possibility that MG inhibits Ucp1 expression via the negative regulation of PPARγ by increasing its phosphorylation, we found no increase in Ser112 phosphorylation of PPARγ following treatment with MG (data not shown).

The number of mitochondria and UCP1 protein in beige adipocytes strongly depends on the activity of mitophagy [38,39], which is repressed in response to thermogenic activation [40,41]. Meanwhile, MG was recently shown to increase mitophagy in brain endothelial cells [42], and the involvement of JNK as an upstream regulator of mitophagy has been reported [43,44]. Hence, one of the possible molecular mechanisms underlying the MG-induced inhibition of Ucp1 expression through the activation of the JNK signaling pathway may involve altered mitophagy activity. However, further investigations are necessary.

3.4. Effect of ROS generation on the MG-induced inhibition of Ucp1 expression

An increase in ROS levels can adversely affect cell function and homeostasis, leading to oxidative stress [45]. In some cell lines, such as human umbilical vascular endothelial cells and pancreatic β-cells, MG has been reported to activate the JNK signaling pathway through ROS generation [46,47]. Therefore, we examined whether the MG-induced phosphorylation of JNK is due to ROS generation in differentiated immortalized iWAT-derived pre-adipocytes using the ROS inhibitor, NAC. Similar to other cell lines, 3 h treatment with MG also generates ROS, which could be inhibited by NAC pretreatment, in the adipocytes (Fig. 4A). However, as shown in Fig. 4B and C, the increase in JNK phosphorylation following treatment with MG was not significantly affected by NAC. Meanwhile, the MG-induced inhibition of Ucp1 expression also did not significantly recover in the presence of NAC (Fig. 4D). These results suggest that ROS generation is not involved in the MG-induced phosphorylation of JNK. At this stage, the molecular machinery by which MG activates the JNK signaling pathway independent of ROS generation in differentiated immortalized iWAT-derived pre-adipocytes is unknown, but if this can be clarified, it will provide new insights into the regulation of Ucp1 expression.

Thermogenesis in adipose tissue is related to metabolic diseases such as obesity and type 2 diabetes mellitus [48]. Thermogenesis mediated by UCP1 is an important component of total energy expenditure and contributes to the overall energy balance [48]. Based on our findings, the analysis of MG on the thermogenic function in adipose tissues regarding the inhibitory effect of MG on Ucp1 expression may lead to further understanding of the relationship between MG and diabetes mellitus.
However, since this study especially focused on the regulatory mechanisms of Ucp1 mRNA expression, further investigations into MG’s effect on UCP1 protein levels as well as its thermogenic functionality are needed to understand the physiological functions on adipocytes.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.101127.

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