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Nuclear factor-κB is a common upstream signal for growth differentiation factor-5 expression in brown adipocytes exposed to pro-inflammatory cytokines and palmitate

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ABSTRACT

We have previously demonstrated that genetic and acquired obesity similarly led to drastic upregulation in brown adipose tissue (BAT), rather than white adipose tissue, of expression of both mRNA and corresponding protein for the bone morphogenic protein/growth differentiation factor (GDF) member GDF5 capable of promoting brown adipogenesis. In this study, we evaluated expression profiles of *GDF5* in cultured murine brown pre-adipocytes exposed to pro-inflammatory cytokines and free fatty acids (FFAs), which are all shown to play a role in the pathogenesis of obesity. Both interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) were effective in up-regulating *GDF5* expression in a concentration-dependent manner, while similar upregulation was seen in cells exposed to the saturated FFA palmitate, but not to the unsaturated FFA oleate. *In silico* analysis revealed existence of the putative nuclear factor-κB (NF-κB) binding site in the 5′-flanking region of mouse *GDF5*, whereas introduction of NF-κB subunits drastically facilitated both promoter activity and expression of *GDF5* in brown pre-adipocytes. Chromatin immunoprecipitation analysis confirmed significant facilitation of the recruitment of NF-κB to the *GDF5* promoter in lysed extracts of BAT from leptin-deficient *ob*/*ob* obese mice. Upregulation o *GDF5* expression was invariably inhibited by an NF-κB inhibitor in cultured brown pre-adipocytes exposed to IL-1β, TNF-α and palmitate. These results suggest that obesity leads to upregulation of *GDF5* expression responsible for the promotion of brown adipogenesis through a mechanism relevant to activation of the NF-κB pathway in response to particular pro-inflammatory cytokines and/or saturated FFAs in BAT.

1. Introduction

Obesity is defined as abnormal and excessive fat accumulation with a high risk to induce health impairment, which is usually brought about by a combination of over-nutrition with low physical activity, in addition to a variety of genetic determinants of susceptibility [1]. White adipose tissue (WAT) is composed of adipocytes with large unilocular lipid droplets to serve as a storage depot for excess energy in maintenance and regulation of different physiologic functions through releasing a number of adipokines and free fatty acids (FFAs) into circulation as an endocrine organ [2,3]. In contrast, brown adipose tissue (BAT) contains multilocular lipid droplets to generate heat through mitochondrial uncoupling of lipid oxidation [4]. Although WAT is thought to be the main type of adipose tissues found in adult humans throughout the body in subcutaneous and visceral regions, recent studies have demonstrated that adult humans have substantial amounts of functioning BAT [5–7].

Growth differentiation factor-5 (GDF5) is a member of bone morphogenic protein (BMP)/GDF family belonging to the transforming growth factor-β superfamily, which generates diverse intracellular signals through a mechanism related to type I and

Abbreviations: BAT, brown adipose tissue; BMP, bone morphogenic protein; BSA, bovine serum albumin; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle medium; FFA, free fatty acid; GDF, growth differentiation factor; IL-1β, interleukin-1β; NF-κB, nuclear factor-κB; PCR, polymerase chain reaction; Smad, mothers against decapentaplegic homolog; TLR4, toll-like receptor-4; TNF-α, tumor necrosis factor-α; WAT, white adipose tissue; WT, wild-type.

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[2] Contributed to drafting the article or revising it critically for important intellectual content.

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type II serine/threonine kinase receptors [8]. In particular, GDF5 is well characterized as a gene implicated in joint integrity and homeostasis [9]. For instance, GDF5 mutations lead to congenital skeletal disorders and osteoarthritis in humans and mice [10]. We have previously demonstrated that GDF5 is selectively up-regulated in BAT from obesity model mice for promotion of brown adipogenesis in systemic energy expenditure *in vivo* [11]. Brown adipogenesis is markedly facilitated in association with activation of the BMP receptor/mothers against decapentaplegic homolog (Smad)/peroxisome proliferator-activated receptor gamma co-activator 1α pathway in cultured brown pre-adipocytes exposed to GDF5 *in vitro* [11], furthermore, while phosphatidylinositol 3-kinase/Akt signaling is involved in GDF5-induced promotion of brown adipogenesis through a mechanism relevant to the phosphorylation of Smad5 [12].

In order to investigate how obesity induces GDF5 expression in BAT, therefore, we have attempted to demonstrate upstream regulatory mechanisms for GDF5 upregulation under obesogenic conditions using cultured brown pre-adipocytes and BAT from leptin deficient *ob*/*ob* obese mice in this study.

2. Materials and methods

2.1. Materials

Brown pre-adipocyte cell lines derived from newborn wild-type (WT) mice were kindly provided by Dr. C.R. Kahn (Joslin Diabetes Center, Boston, MA, USA) [13]. Pre-adipocytic 3T3-L1 cells were obtained from ATCC (Manassas, VA, USA). The GDF5 promoter (−1101 to +367) construct was a generous gift from Dr. Ikegawa (RIKEN, Yokohama, Japan). RelA cFlag pcDNA3 (#20012), p50 cFlag pcDNA3 (#20018) and p52 cFlag pcDNA3 (#20019) were obtained from Addgene (Cambridge, MA, USA). Recombinant mouse interleukin-1β (IL-1β), recombinant tumor necrosis factor-α (TNF-α) and antibody against phosphorylated-p65 (p-p65) were purchased from Cell Signaling Technology (Danvers, MA, USA). An antibody against GDF5 was from R&D Systems International (Minneapolis, MN, USA). Sodium palmitate, sodium oleate and fatty acid-free bovine serum albumin (BSA) were from Sigma (St. Louis, MO, USA). BAY117082 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). THUNDERBIRD SYBR qPCR Mix was supplied by TOYOBO (Osaka, Japan). Other chemicals used were all of the highest purity commercially available.

2.2. Mice

WT mice and *ob*/*ob* mice on the C57BL/6 background were obtained from Japan SLC (Shizuoka, Japan). Mice were maintained at room temperature on a 12 h light/dark cycle with free access to food and water. Male mice were used throughout experiments. The protocol employed here meets the guideline of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Kanazawa University (approval number: AP-142976).

2.3. Cell culture, luciferase assay and FFA stimulation

Brown pre-adipocyte cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum except for FFA exposure experiments. For luciferase assay, cells were transiently transfected with reporter vectors by the lipofection method as previously described [14], followed by preparation of cell lysates and subsequent determination of luciferase activity. Transfection efficiency was normalized by determining the activity of *Renilla* luciferase. For FFA exposure experiments, FFAs were dissolved in ethanol and then incubated with 2% fatty acid-free BSA in DMEM. Brown pre-adipocytes were incubated in DMEM containing 2% fatty acid-free BSA in either the presence or absence of FFAs.

2.4. Real-time based quantitative polymerase chain reaction (PCR)

Total RNA was extracted from cells or tissues, followed by synthesis of cDNA with reverse transcriptase and oligo-dT primer. The cDNA samples were then used as templates for real-time PCR analysis by using specific primers for *GDF5* [11]. Expression levels of the genes examined were normalized by using the 36b4 expression levels as an internal control for each sample.

2.5. Immunoblotting

Cultured cells were solubilized in lysis buffer containing 1% Nonidet P-40. Samples were then subjected to SDS–PAGE, followed by transfer to polyvinylidene fluoride membranes and subsequent immunoblotting.

2.6. Chromatin immunoprecipitation (ChIP) assay

ChIP experiments were performed following the protocol provided with the ChIP assay kit as described previously [15]. In brief, BAT was cut into small pieces, and then incubated with 1% formaldehyde at room temperature for 20 min. After centrifugation of the crosslinked samples, the pellet was homogenized with a Dounce homogenizer in phosphate-buffered saline. The homogenate was centrifuged at 300*g* for 5 min, and the pellet was subsequently subjected to sonication in lysis buffer. Immunoprecipitation was performed with the anti-p-p65 antibody, followed by extraction of DNA with phenol/chloroform and subsequent PCR with specific primers: (a) 5′-CTTCTCAACATCTCTGCTCA-3′, (b) 5′-TTCATTTAAGT GCGATATAT-3′, (c) 5′-TTTAGACAGCATGACATCAG-3′ (d) 5′-TTGA ATCCTTTCCAGTGAAA-3′, (e) 5′-AATTAGAGGGAAAAAAAACT-3′ and (f) 5′-GCTCCCGTGTCCAGACGTGC-3′.

2.7. Statistical analysis

Results are all expressed as the mean ± standard error of the mean (SEM) and the statistical significance was determined by the two-tailed and unpaired Students' *t*-test or the one-way analysis of variance with Bonferroni/Dunnett *post hoc* test.

3. Results

3.1. Responsiveness to pro-inflammatory cytokines and FFAs

We have previously demonstrated that expression of both mRNA and corresponding protein for GDF5 is drastically up-regulated in BAT rather than WAT in different obese model mice [11]. Since obesity is highly associated with promoted production of inflammatory cytokines along with elevated plasma FFA levels [2,3], we tested whether *GDF5* expression is responsive to different extracellular signals related to obesity, such as pro-inflammatory cytokines and FFAs, in cultured brown pre-adipocytes. Brown pre-adipocytes were exposed to one of these mediators at two different concentrations for 6 h, followed by determination of *GDF5* expression. *GDF5* expression was significantly up-regulated in brown pre-adipocytes exposed to IL-1β (Fig. 1A) and TNF-α (Fig. 1B) in a concentration-dependent manner. Similar upregulation of *GDF5* expression was seen in brown pre-adipocytes exposed to the saturated FFA palmitate (Fig. 1C), but not in those exposed to the unsaturated FFA oleate (Fig. 1D). Similarly marked upregulation was seen in GDF5 protein expression in brown pre-adipocytes exposed to IL-1β, TNF-α and palmitate for 6 h (Fig. 1E), whereas

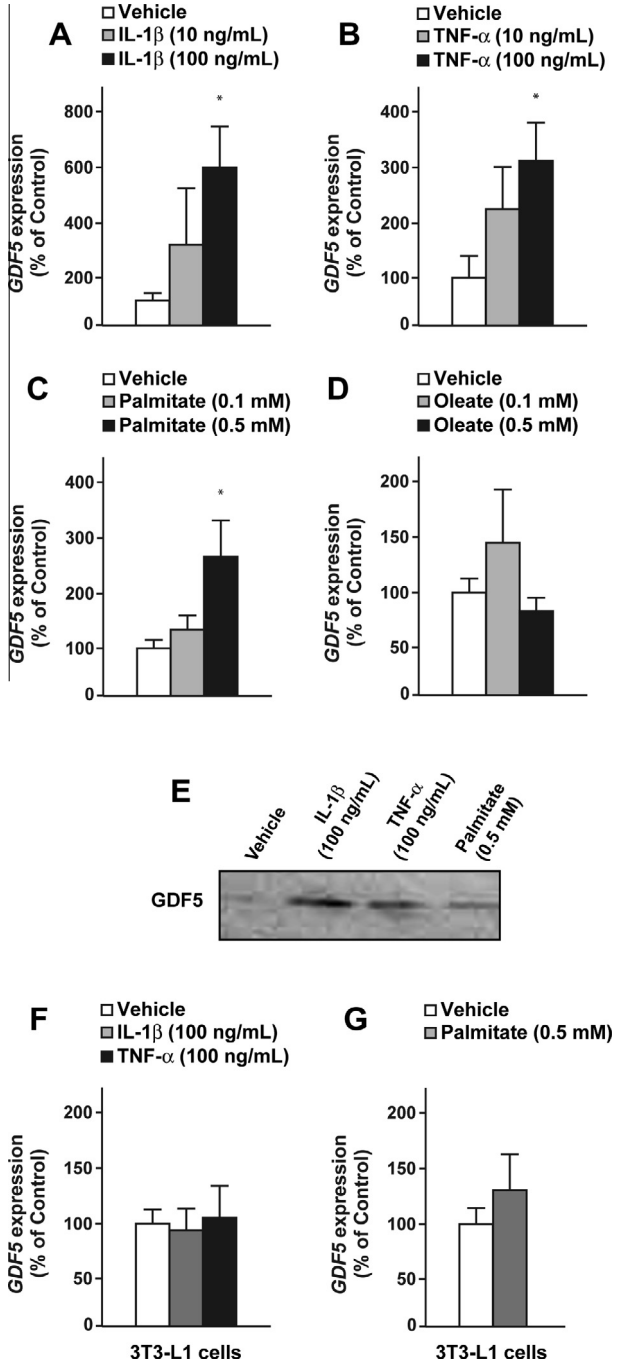

Fig. 1. *GDF5* expression is up-regulated by pro-inflammatory cytokines and palmitate in brown pre-adipocytes. Brown pre-adipocytes were exposed to (A) IL-1β, (B) TNF-α, (C) palmitate or (D) oleate at two different concentrations for 6 h, followed by determination of *GDF5* expression by qPCR. (E) Brown pre-adipocytes were exposed to IL-1β, TNF-α or palmitate for 6 h, followed by determination of GDF5 expression by immunoblotting. White pre-adipocytic 3T3-L1 cells were exposed to (F) IL-1β, TNF-α, or (G) palmitate for 6 h, followed by determination of *GDF5* expression by qPCR. $^{*}P < 0.05$, significantly different from each control value.

GDF5 expression was not significantly up-regulated in white pre-adipocytic 3T3-L1 cells after exposure to IL-1β (Fig. 1F), TNF-α (Fig. 1F) or palmitate (Fig. 1G) for 6 h.

3.2. Recruitment of NF-κB

We next tried to identify the transcription factor responsible for the upregulation of *GDF5* expression in BAT under obesogenic conditions. On computational analysis of the 5′-flanking region of mouse and human *GDF5* genes, we identified at least two putative NF-κB-binding elements in the 5′-flanking region of highly conserved mouse and human *GDF5* genes (Fig. 2A). Introduction of the NF-κB complex composed of RelA/p50 subunits, or of RelA/p52 subunits, induced a nearly 40-fold or 20-fold increase in *GDF5* promoter activity in brown pre-adipocytes, respectively (Fig. 2B). Indeed, introduction of the NF-κB complex composed of RelA/p50 subunits more than tripled *GDF5* expression in brown pre-adipocytes (Fig. 2C). To further verify the binding of NF-κB to putative binding sites (site 1 and site 2) located at the *GDF5* promoter, ChIP assay was conducted using lysed extracts of BAT from leptin-deficient *ob*/*ob* mice, in which *GDF5* expression was shown to be drastically up-regulated in our previous study [11]. Recruitment of p-p65 to the *GDF5* promoter regions encompassing site 1 and site 2 was markedly enhanced in lysed extracts of BAT from *ob*/*ob* mice compared with those from WT mice (Fig. 2D). Moreover, we confirmed the recruitment of p-p65 to the *GDF5* promoter regions in cultured brown pre-adipocytes exposed to IL-1β on ChIP assay (Fig. 2E).

3.3. Involvement of NF-κB signaling

We next investigated the effects of an inhibitor of the NF-κB signaling on upregulation of *GDF5* expression in brown pre-adipocytes exposed to IL-1β, TNF-α and palmitate. Brown pre-adipocytes were exposed to IL-1β, TNF-α or palmitate in either the presence or absence of the NF-κB inhibitor, BAY117082, at 10 μM for subsequent determination of *GDF5* expression. The addition of BAY117082 invariably led to significant inhibition of upregulation of *GDF5* expression in brown pre-adipocytes exposed to IL-1β (Fig. 3A), TNF-α (Fig. 3B) and palmitate (Fig. 3C), without significantly affecting basal *GDF5* expression. However, no significant upregulation was seen in the expression of *uncoupling protein-1* (*Ucp1*) and *peroxisome proliferator-activated receptor gamma co-activator 1α* (*Ppargc1a*) in brown pre-adipocytes exposed to IL-1β, TNF-α (Fig. 3D) and palmitate (Fig. 3E) under the experimental conditions employed.

4. Discussion

The essential importance of the present findings is that obesity-related pro-inflammatory cytokines and palmitate induced *GDF5* expression through a mechanism associated with activation of the NF-κB pathway in brown pre-adipocytes as summarized in Fig. 3F. Although the transcription factor sex determining region Y-box 11 is shown to directly up-regulate *GDF5* expression *in vitro* [16], upstream mechanisms are not well elucidated for GDF5 expression on the contrary to well-characterized downstream signaling pathways after activation by GDF5 of membrane BMP/GDF receptors. From this point of view, it should be emphasized that we identified NF-κB as a pivotal transcription factor responsible for upregulation of *GDF5* expression in brown pre-adipocytes under obesogenic conditions in this study. The transcription factor NF-κB is known to play a central key role in mechanisms underlying inflammation, autoimmune response, cell proliferation, differentiation and apoptosis through transcriptional regulation of the expression of genes involved in these physiological and pathological processes in most cell types [17]. Moreover, NF-κB has been implicated in the pathology as well as etiology in obesity. An increased NF-κB activity is observed in the abdomen in mice fed high fat diet on luciferase reporter analysis [18], for example, while diet-induced obesity is protected in transgenic mice overexpressing p65 in adipose tissue under the control by adipocyte protein-2 promoter along with enhanced energy expenditure [19]. To our knowledge, this is the first direct demonstration of the upregulation of *GDF5* expression through a mechanism

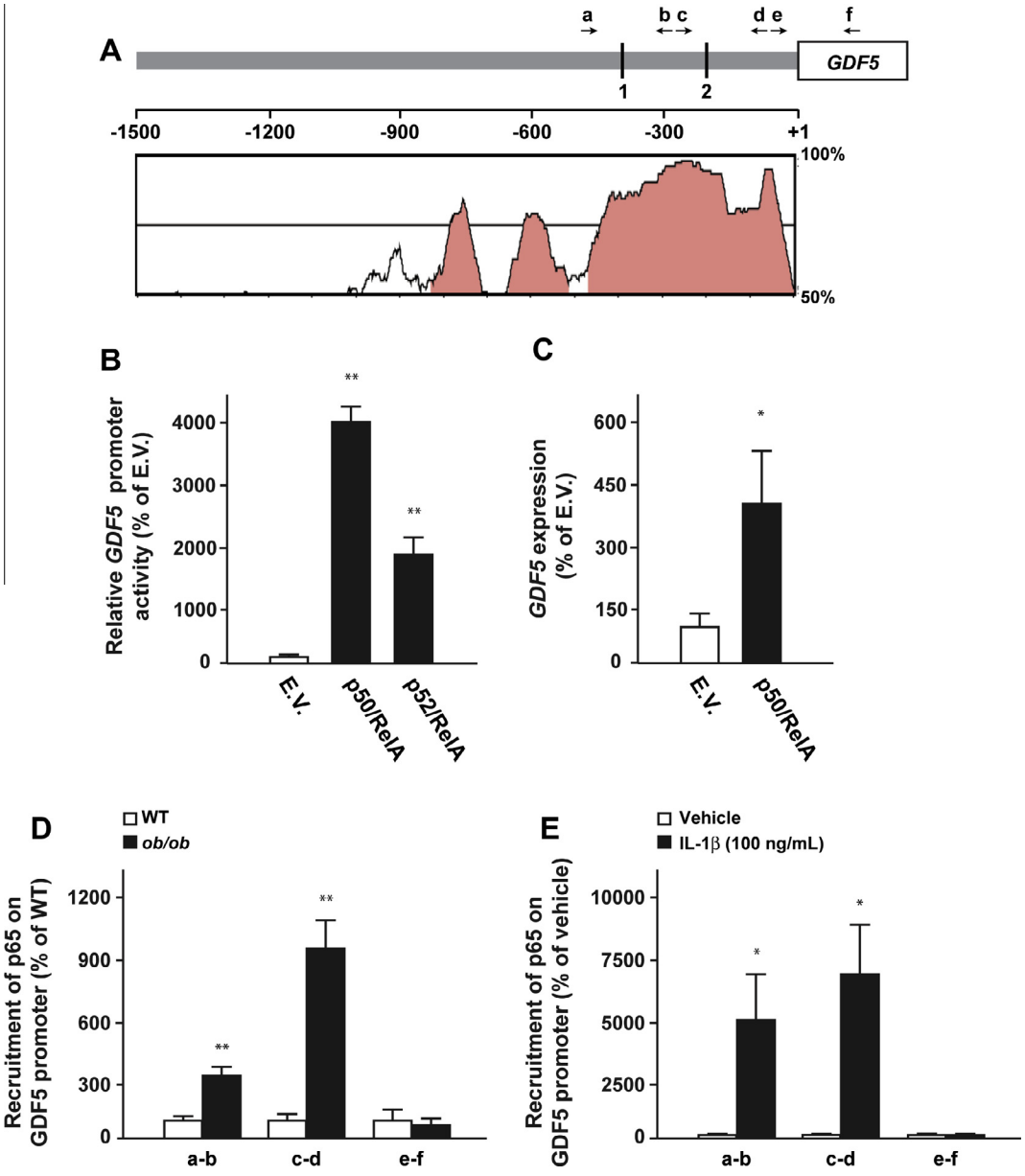

Fig. 2. NF-κB complex regulates *GDF5* expression in brown pre-adipocytes and BAT of *ob*/*ob* mice. (A) Schematic representation of the alignment of mouse and human *GDF5* promoter regions with putative NF-κB binding sites in addition to primers (a–f) used for ChIP assays. (B) Brown pre-adipocytes were co-transfected with GDF5-luc and RelA/p50 subunits or RelA/p52 subunits, followed by determination of luciferase activity. (C) Brown pre-adipocytes were transfected with RelA and p50 subunits, followed by determination of *GDF5* expression by qPCR. Lysed extracts of either (D) BAT from *ob*/*ob* mice or (E) brown pre-adipocytes exposed to IL-1β were subjected to ChIP assay using the anti-p-p65 antibody along with specific primers (a–b, c–d and e–f) to recognize *GDF5* promoter containing NF-κB binding site shown in the panel A. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from each control value obtained in (B, C) cells transfected with empty vector, (D) WT mice or (E) cells treated with vehicle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

relevant to the NF-κB signaling pathway in brown pre-adipocytes in response to extracellular obesogenic stimuli.

The reason why palmitate rather than oleate up-regulated *GDF5* expression in cultured brown pre-adipocytes is not clarified. Palmitate is a representative saturated FFA in terms of the abundance in plasma at concentrations close to 500 μM in patients with obesity and diabetes mellitus [20]. Emerging evidence is available for the involvement of intracellular FFAs and their metabolites with a property to stimulate inflammatory pathways in a variety of deleterious effects of FFAs in the literature [21]. In contrast, saturated FFAs such as palmitate are shown to stimulate transmembrane toll-like receptor-4 (TLR4) responsible for activation of the NF-κB pathway toward promotion of gene expression of different inflammatory cytokines with unsaturated FFAs being ineffective [22]. Taken together, one possible but hitherto unproven speculation is that extracellular saturated FFAs would activate membrane TLR4 endowed to promote intracellular NF-κB signaling to nuclear transactivation of the gene for GDF5 required for elevated energy expenditure in brown adipocytes in obesity. However, long-term exposure to saturated FFAs is shown to worsen and/or exacerbate insulin resistance and inflammation in cultured skeletal muscular cells [23]. The transcription factor NF-κB could be a common upstream signal leading to upregulation of *GDF5* expression by IL-1β, TNF-α and palmitate in brown adipocytes for promoted energy expenditure during obesity.

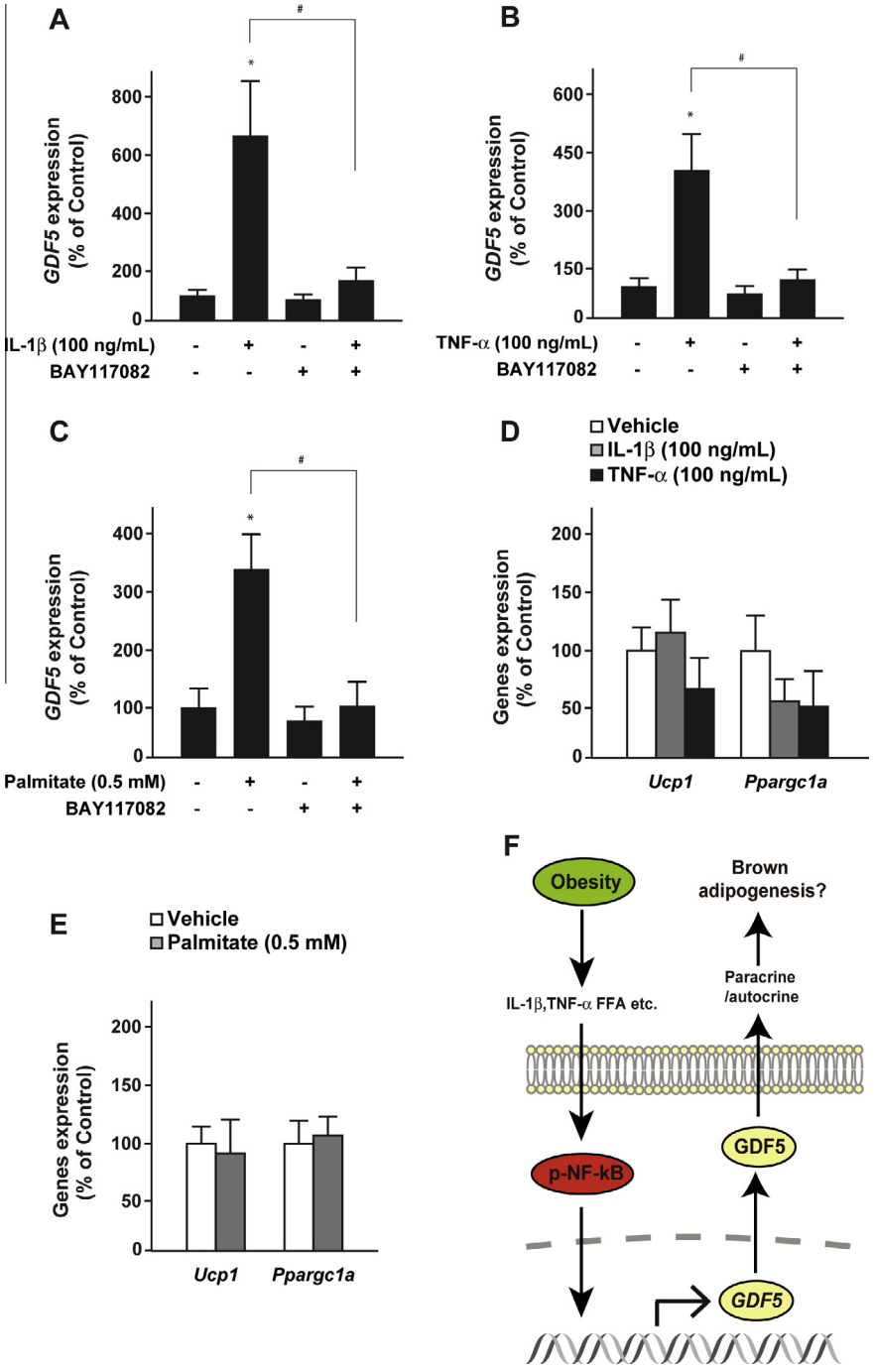

Fig. 3. An NF-B inhibitor prevents *GDF5* upregulation in brown pre-adipocytes. Brown pre-adipocytes were cultured with (A) 100 ng/mL IL-1β, (B) 100 ng/mL TNF-α or (C) 0.5 mM palmitate in either the presence or absence of BAY117082 at 10 μM for 6 h, followed by determination of *GDF5* expression by qPCR. Brown pre-adipocytes were exposed to (D) IL-1β, TNF-α, or (E) palmitate for 2 days, followed by determination of *Ucp1* and *Ppargc1a* expression by qPCR. (F) Proposed signaling pathway from obesity to brown adipogenesis. $^{*}P < 0.05$, significantly different from each control value obtained in cells not treated with (A, B) cytokine or (C) FFA. $^{\#}P < 0.05$, significantly different from the value obtained in cells treated with (A, B) cytokine or (C) FFA alone. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Nevertheless, the current results on the expression of both *Ucp1* and *Ppargc1a* in brown pre-adipocytes exposed to pro-inflammatory cytokines and palmitate are apparently in disagreement with the previous findings that energy expenditure is highly promoted through activation of the NF-κB signaling in transgenic mice with overexpression of p65 in adipose tissues [19]. The paradox could be accounted for by taking into consideration a variety of possible compensatory reactions often found in genetically modified animals *in vivo* as well as possible inadequateness of detailed experimental conditions *in vitro*. The possibility that saturated FFAs such as palmitate are secreted from white adipocytes into circulation to play a dual role as an extracellular agonist for TLR4 and an endogenous substrate for fatty acid transporters to promote energy expenditure and efficient lipid metabolism in brown adipocytes during obesity as a compensatory mechanism is not ruled out. The final conclusion should await comprehensive analysis on molecular biological and pharmacological profiling of a variety of cytokine receptors, TLR4, BMP/GDF receptors and fatty acid

transporters expressed at cell surface, in addition to intracellular to nuclear NF-κB signaling pathway, in both white and brown adipocytes during obesity.

Adipocytes are shown to produce different chemokines and cytokines, including monocyte chemotactic protein-1, IL-6, IL-1β and TNF-α, in obese mice [2,3]. In contrast to these adipokines with a pro-inflammatory property, both adiponectin and secreted frizzled-related protein-5 are believed to counteract different signal transduction processes mediated by those pro-inflammatory adipokines in obesity [24,25]. These previous findings thus give rise to a speculative idea that GDF5 could be among cytokines beneficial for the combat against obesity in brown adipocytes. The significance as well as the exact mechanism for predominant upregulation of *GDF5* expression in BAT rather than WAT under obesogenic conditions *in vivo*, however, remains to be elucidated. Future search for molecules responsible for GDF5 upregulation would give a clue for the discovery and development of novel strategies useful for the prophylaxis and treatment of obesity and related metabolic diseases in human beings, in addition to promoting further understanding of brown adipogenesis in systemic energy expenditure.

Conflict of interest

All authors have no conflict of interest.

Acknowledgments

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