Postprandial activation of protein kinase Cμ regulates the expression of adipocytokines via the transcription factor AP-2β

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Abstract. Abnormal secretion of adipocytokines promotes atherosclerosis, diabetes and insulin resistance, and is mainly induced by adipocyte hypertrophy. Recently, the circulating adipocytokine concentrations were reported to change in the postprandial period, as the levels of TNFα, IL-6 IL-8 and MCP-1 increased after a meal, whereas that of adiponectin decreased. These data suggest that prandial modulation of cytokines may be involved in the pathogenesis of atherosclerosis in type 2 diabetes. However, the regulatory mechanism of such change is still unclear. In the present study, we identified this mechanism with a special focus on the functions of protein kinase C (PKC) and of the transcription factor AP-2β, both of which are associated with the pathophysiology of adipocytokine regulation. PKCμ was highly phosphorylated in the re-feeding condition compared to the fasting condition in mouse adipose tissue, while other PKC isoforms remained unchanged. Furthermore, overexpression of PKCμ in 3T3-L1 adipocytes, but not other PKC isoforms, positively regulated the mRNA expression and promoter activity of MCP-1 and IL-6, and negatively regulated those of adiponectin. AP-2β had similar effects on the expression and promoter activity of these adipocytokines. Interestingly, overexpression of PKCμ enhanced the stimulatory and inhibitory effects of AP-2β on the expression of these adipocytokines. Finally, PKCμ could not activate a mutant MCP-1 promoter lacking the AP-2β binding domain. Our results suggest that postprandial activation of PKCμ plays a role in disordered postprandial adipocytokine expression through AP-2β.

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Introduction

The functions of adipocytes have been intensively investigated in recent years and adipocytes have been shown to act as endocrine cells that secrete various bioactive substances known as adipocytokines. It is well known that the circulating concentrations of inflammatory cytokines are elevated and anti-inflammatory cytokines are decreased among people with type 2 diabetes (T2DM) and among obese individuals (1-3). Abnormal secretion of adipocytokines can cause insulin resistance (4) and promote atherosclerosis (5). Inappropriate secretion of adipocytokines seems to be mainly due to adipocyte hypertrophy (4). It has also been shown that the plasma concentrations of several cytokines are affected by a single meal, as the levels of TNFα, IL-6 IL-8 and MCP-1 were found to increase after a meal (6), whereas those of adiponectin to decrease (7). Interestingly, these responses were more pronounced in people with T2DM than in healthy subjects (8). These data suggest that prandial modulation of cytokines may be involved in the pathogenesis of atherosclerosis in T2DM. However, the mechanisms involved in the postprandial changes in plasma concentrations of adipocytokines are unknown.

The protein kinase C (PKC) family plays many important roles in intracellular signaling events, such as cell growth and differentiation (9-12). This family comprises a number of individual isoforms, and each isoform has distinct roles and is regulated by specific pathways. PKC is also involved in insulin resistance, diabetes and atherosclerosis. Chronic and acute changes in the intracellular concentrations of glucose, insulin, and free fatty acid can regulate the activity of PKC (13) and the expression and activity of some PKC isoforms are regulated by feeding (14-16). Activation of PKCθ and PKCε are associated with insulin resistance (17,18), and PKCβ is implicated in the development of diabetic complications (19). PKCs also play important roles in adipocytes, including the modulation of insulin resistance (20) and adipocyte differentiation (21). Furthermore, they regulate the expression of adipocytokines, such as adiponectin and TNFα (22,23). However, the precise roles of PKCs in the regulation of adipocytokine expression are unknown.

We previously identified the transcription factor, activating enhancer-binding protein-2β (AP-2β), as a novel candidate gene that confers susceptibility to obesity and T2DM in a genome-wide association study (24). AP-2β is associated with insulin resistance and adiposity in healthy adolescents (25,26). We previously showed that AP-2β directly inhibits the expression of adiponectin (27), and enhances the expression of inflammatory adipocytokines, such as IL-6 and MCP-1 (27,28). Furthermore, we reported that the mRNA expression of TFAP2B was negatively correlated with that of adiponectin and positively correlated with that of IL-6 in human adipose tissue (29), supporting the role of AP-2β in the regulation of the expression of adipocytokines. Thus, AP-2β seems to be an important regulatory factor of the expression of adipocytokines. However, the transcriptional regulation of AP-2β is still unknown. It was recently reported that the transcriptional activity of AP-2α (another member of the AP2 family) is regulated by PKCμ, and that PKCα enhances the activation of AP-2β (30,31). These data led to us to hypothesize that PKCμ may regulate the activity of AP-2β in adipocytes.

In this study, we evaluated this possibility and demonstrated that PKCμ activity was stimulated by feeding in mouse adipose tissue and the transcriptional activity of AP-2β was activated by PKCμ in 3T3-L1 adipocytes. Our results suggest that postprandial activation of PKCμ may play a role in the postprandial changes in the expression of adipocytokines through an AP-2β-mediated pathway.

Materials and methods

Materials. Anti-phospho-PKCα/βII (Thr638/641), anti-phospho-PKCβII (Ser660), anti-phospho-PKCδ (Thr505), anti-phospho-PKCμ (Ser744/748 and Ser916), anti-PKCμ and anti-phospho-PKCθ (Thr538) antibodies, were purchased from Cell Signaling Technology (Beverly, MA). Anti-PKCα, anti-PKCβII anti-PKCδ and anti-PKCθ antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Gö6976 and Gö6983 were purchased from Calbiochem (San Diego, CA). The pGL3-basic luciferase and phRL-null vectors were purchased from Promega (Madison, WI), Dulbecco's modified Eagle's medium and fetal calf serum were obtained from Life Technologies (Grand Island, NY). All other reagents and chemicals were from standard suppliers.

Cell culture. 3T3-L1 cells were provided by Dr J.M. Olefsky (University of California, San Diego, CA) and were cultured and differentiated into adipocytes as previously described (32).

Animal experiments. All procedures were performed in accordance with the guidelines of the Research Center for Animal Life Science of the Shiga University of Medical Science. Twelve-week-old male C57BL/J6 mice were purchased from Charles River Japan (Kanagawa, Japan). The mice were housed in an environmentally controlled room with a 12-h light/dark cycle and were given free access to a normal laboratory diet and water. The diet (Oriental Yeast, Tokyo, Japan) consisted of 58% carbohydrate, 12% fat, and 30% protein (energy percent of diet). After intraperitoneal injection of pentobarbital (10 mg/kg) and under deep anesthesia, the epididymal fat depot was excised, immediately frozen in liquid nitrogen and stored until further experiments.

Preparation of expression plasmid vectors. Plasmid vectors encoding the mouse AP-2β gene (pcDNA3.1/AP-2β) were generated as previously described (27). Plasmid vectors encoding mouse PKCα, PKCβII, PKCδ and PKCμ were kindly provided by Dr A. Reifel Miller (Lilly Research Laboratories, Indianapolis, IN) (33). Plasmid vectors encoding mouse PKCθ were kindly provided by Dr Gottfried Baier (Innsbruck Medical University, Innsbruck, Austria) (34). The luciferase reporter plasmids for the human adiponectin promoter (pGL3/adiponectin promoter luc) and the human IL-6 promoter (pGL3/IL-6 promoter luc) were kindly provided by Dr Iichiro Shimomura (35) and Dr Kiyoshi Takeda (Osaka University, Osaka, Japan) (36), respectively. The luciferase reporter plasmids for the mouse MCP-1 promoter (pGL3/MCP-1 promoter luc) and pGL3/MCP-1 mutated promoter luc (putative AP-2 binding site mutant) were generated as previously described (28).

Cell transfection. Cell transfection was performed using the Amaxa Nucleofector method (Amaxa, Cologne, Germany) as previously described (28).

Isolation of cell fractions and immunoblot analysis. Nuclear and cytosolic protein fractionation and immunoblot analyses were conducted as previously described (28). The primary antibodies were diluted to 1:1000.

RNA preparation from adipocytes and quantitative RT-PCR. Real-time PCR was performed on a 7500 real-time PCR system (ABI Applied Biosystems) using Power SYBR-Green PCR Master mix. The primer sets were as follows: mouse MCP-1, 5'-GCCCCACTCACCTGCTGCTACT-3' and 5'-CCTGCTGCTGGTGATCCTCTTGT-3'; mouse adiponectin, 5'-GAAGATGACGTTACTACAAC-3' and 5'-TCAGTTGGTATCATGGAAGA-3'; mouse IL-6, 5'-ACAACCACGGCCTTCCCTACTT-3' and 5'-CACGATTTCCCAGAGAACATGTG-3'; and mouse β-actin, 5'-CGTGCGTGACATCAAAGAGAA-3' and 5'-TGGATGCCACAGGATTCCAT-3'.

Measurement of luciferase reporter gene activity. Luciferase activities were measured using a dual-luciferase reporter assay system (Promega) in accordance with the manufacturer's instructions and were normalized for phRL-null luciferase activity.

Statistical analysis. All values are expressed as means ± SEM unless otherwise stated. The Scheffe's multiple comparison test was used to determine the significance of any differences between more than three groups. Values of $p<0.05$ were considered statistically significant.

Results

PKCμ phosphorylation is increased in postprandial adipose tissue. We first analyzed the phosphorylation status of different PKC isoforms in postprandial adipose tissue. Since the catalytic activity of PKCs is regulated by the phosphorylation of serine/

threonine residues in the activation and autophosphorylation domains (37-39). As shown in Fig. 1, the phosphorylation of PKCμ was increased in the postprandial state at both Ser916 and Ser744/748, both of which are involved in the activation of PKCμ (40,41). In contrast, the expression of other PKC isoforms was unchanged (Fig. 1).

PKCμ regulates the mRNA expression and promoter activity of several adipocytokines in 3T3-L1 adipocytes. To examine whether PKCμ regulates the expression of adipocytokines, we overexpressed PKCμ in 3T3-L1 adipocytes. The mRNA expression of MCP-1 and IL-6 were increased by 1.6- and 1.9-fold respectively, whereas that of adiponectin was decreased by ~30% compared to control cells (Fig. 2A, upper panel). Furthermore, the promoter activities of MCP-1 and IL-6 were increased by 1.7 and 2.0-fold respectively, and that of adiponectin was decreased by ~20% compared to the control (Fig. 2A, lower panel). These findings suggest that PKCμ regulates the expression of several adipocytokines by modulating their transcriptional activities.

Other isoforms of PKCs do not stimulate MCP-1 promoter activity. To explore whether other PKCs also have an effect on adipocytokine expression, we overexpressed various PKCs and measured the transcriptional activity of MCP-1. The promoter activity of MCP-1 was increased by 1.8-fold only when PKCμ was overexpressed, while overexpression of PKCα, PKCβII, PKCδ and PKCθ did not affect MCP-1 promoter activity (Fig. 2B).

Overexpression of PKCμ augments the stimulatory effect of AP-2β on MCP-1 promoter activity. We previously reported that AP-2β regulates the expression of several adipocytokines. Overexpression of AP-2β directly inhibited adiponectin expression (27) and enhanced the expression of inflammatory adipocytokines, such as IL-6 and MCP-1 (27,28). Such disordered expression is also observed in obese subjects. However, the mechanisms involved in the regulation of AP-2β activity are unknown. It was reported that the transcriptional activity of AP-2α is regulated by its phosphorylation by PKCμ (30). Thus, it is possible that AP-2β may be regulated by PKCs. To test this possibility, we examined whether the stimulatory effects of AP-2β on MCP-1 promoter activity are affected by phorbol 12-myristate 13-acetate (TPA), which stimulates the activity of novel and conventional PKCs. TPA stimulation further augmented the stimulatory effects of AP-2β on MCP-1 promoter activity (Fig. 3A), which suggests that PKCs may be involved in the regulatory effects of AP-2β on adipocytokine expression. We next examined whether PKCμ enhances these effects of AP-2β. As shown in Fig. 3B, overexpression of PKCμ significantly increased the stimulatory effects of AP-2β on the mRNA expression and promoter activity of MCP-1 and IL-6 as well as the inhibitory effects of AP-2β on adiponectin. Furthermore, the stimulatory effects of AP-2β on MCP-1 promoter activity were specific to PKCμ overexpression (Fig. 3C).

The transcriptional activity of AP-2β is necessary for the stimulatory effects of PKCμ on MCP-1 promoter activity. We next examined whether PKCμ stimulates the expression of adipocytokines through AP-2β. As shown in Fig. 4, while the wild-type promoter activity of MCP-1 was significantly stimulated by the overexpression of AP-2β and PKCμ, the activity of a mutant MCP-1 promoter carrying a mutation in the AP-2β binding sites was not affected by either AP-2β or PKCμ. These results clearly suggest that the regulatory effects of PKCμ on MCP-1 expression require AP-2β transcriptional activity.

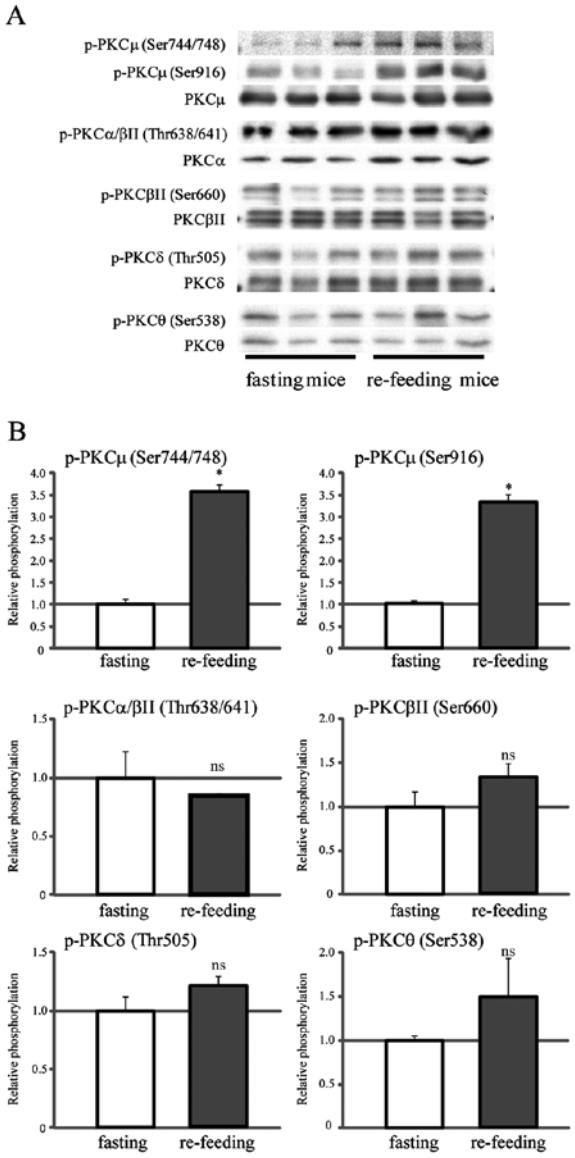

Figure 1. Short-term postprandial effects on PKC phosphorylation in mouse adipose tissue. (A) The day before the experiment, food was withdrawn from all animals at 4 pm. The next day, half of the animals were fed the normal diet in the dark from 8-10 am before the experiment, while the other half were kept in the fasting state. At 10 am, epididymal fat depots were excised. PKC phosphorylation was determined by immunoblot analysis. The epididymal fats were resuspended in lysis buffer. Homogenates were sonicated and the supernatants were retained. Each sample was suspended in loading buffer and electrophoresed on 7.5% polyacrylamide gels and electrophoretically transferred to a nitrocellulose membrane. Membranes were then probed with the primary antibodies at a dilution of 1:1000. (B) Quantitative analysis for the phosphorylation of PKCα, PKCβII, PKCδ PKCθ and PKCμ (Ser916 and Ser 744/748) was performed by measuring the relative density using the Scion Image software. All data are presented as the fold-change compared with the fasting group and are means ± SEM of more than three independent individuals. *p<0.05 vs. the fasting group; ns, not significant.

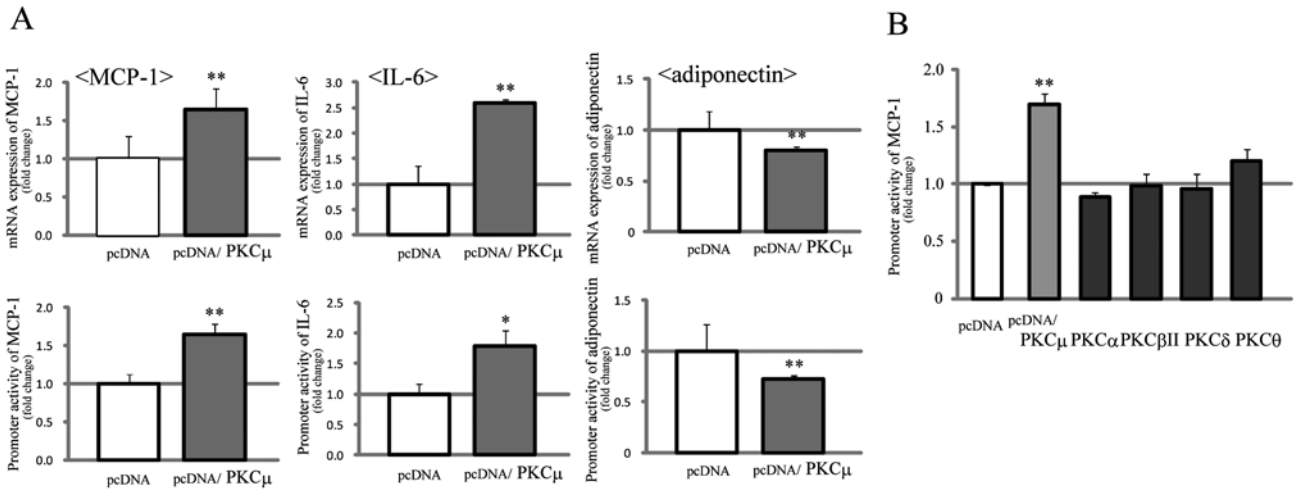

Figure 2. PKC*μ* regulates the mRNA expression and promoter activity of multiple adipocytokines (A) 3T3-L1 adipocytes were nucleofected with a PKC*μ* expression vector (pcDNA3.1/PKC*μ*). The mRNA expression of MCP-1, IL-6 and adiponectin was measured 24 h after transfection. Luciferase reporter constructs containing the 5'-flanking region of the mouse MCP-1 gene (pGL3/MCP-1 promoter luc), human IL-6 gene (pGL3/IL-6 promoter luc) and human adiponectin gene (pGL3/adiponectin promoter luc) were transfected into 3T3-L1 adipocytes with or without pcDNA3.1/PKC*μ* and the promoter activities of MCP-1, IL-6 and adiponectin were measured 24 h after transfection. (B) pGL3/MCP-1 promoter luc and a PKC expression vectors (PKCα, PKCβII, PKCδ, PKCθ or PKC*μ*) were nucleofected into 3T3-L1 adipocytes, and the promoter activity of MCP-1 was measured 24 h after transfection. All data are presented as the fold-change compared with cells transfected with the control vector and are the means ± SEM of three independent experiments. $^{*}p<0.05$ and $^{**}p<0.01$ vs. pcDNA control.

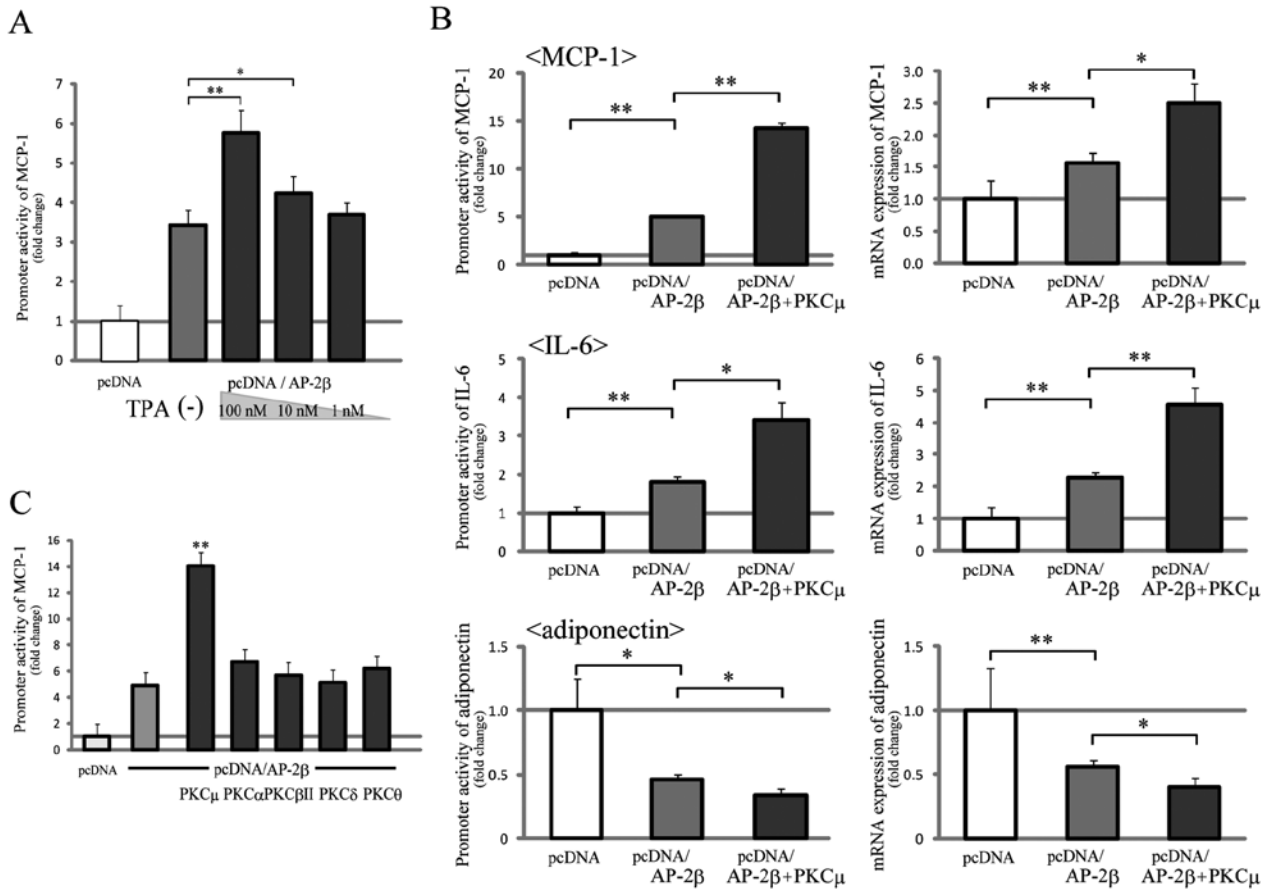

Figure 3. PKC*μ* augments the function of AP-2β in 3T3-L1 adipocytes. (A) 3T3-L1 adipocytes were nucleofected with the AP-2β expression vector (pcDNA3.1/AP-2β) and luciferase reporter constructs of the mouse MCP-1 gene (pGL3/MCP-1 promoter luc). Four hours after transfection, the medium was replaced with fresh medium with or without phorbol 12-myristate 13-acetate (TPA; 1, 10 or 100 nM) and the promoter activity of MCP-1 was measured 24 h later. (B) 3T3-L1 adipocytes were nucleofected with pcDNA3.1, pcDNA3.1/AP-2β, or pcDNA3.1/AP-2β plus pcDNA3.1/PKC*μ*, The mRNA expression and promoter activity of MCP-1, IL-6, adiponectin were measured 24 h after transfection. (C) pGL3/MCP-1 promoter luc, pcDNA3.1/AP-2β and one of the PKC expression vectors (PKCα, PKCβII, PKCδ, PKCθ or PKC*μ*) were nucleofected into 3T3-L1 adipocytes, and the promoter activity of MCP-1 was measured 24 h after transfection. All data are presented as the fold-increase compared with cells transfected with the control vector and are the means ± SEM of three independent experiments. $^{*}p<0.05$; $^{**}p<0.01$.

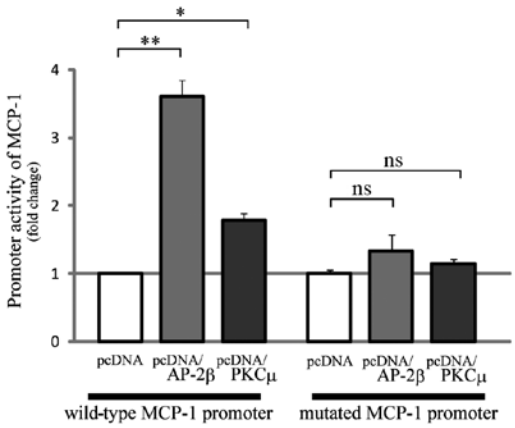

Figure 4. PKCμ increases the MCP-1 promoter activity through binding of AP-2β to the promoter region. 3T3-L1 adipocytes were transfected with either AP-2β (pcDNA3.1/AP-2β) or PKCμ (pcDNA3.1/PKCμ) expression vectors together with either pGL3/MCP-1 promoter Luc (wild-type) or the pGL3/MCP-1 mutated promoter Luc (putative AP-2 binding site mutant). The promoter activity of MCP-1 was measured 24 h after transfection. All data are presented as the fold-increase compared with cells transfected with the control vector and are the means ± SEM of three independent experiments. *p<0.05; **p<0.01; ns, not significant.

Discussion

In the current study, we show for the first time that PKCμ in adipose tissue is physiologically activated by feeding. We also observed postprandial activation of several PKC isoforms. Short-term hyperglycemia has been shown to activate PKCα, PKCβI and PKCβII in human platelets (15), and postprandial hyperinsulinemia to regulate PKCδ expression (16). However, in our preliminary studies, the activity of PKCμ in 3T3-L1 adipocytes was unchanged in cells exposed to high glucose or high insulin levels (data not shown). It is well known that hyperglycemia leads to the intracellular generation of diacylglycerol, which directly activates novel and conventional PKCs (14) and this activation may be involved in free fatty acid (FFA)-induced insulin resistance in the skeletal muscle (17,18). Furthermore, it was also reported that the phosphorylation of PKCμ is stimulated by FFAs in 3T3-L1 adipocytes (13). Based on these findings, we hypothesized that elevated intracellular concentrations of FFA in adipose tissue induced by dietary intake may activate PKCμ.

The importance of PKCμ in the cardiovascular system, particularly in the regulation of myocardial contraction, hypertrophy and remodeling, is well established (42). PKCμ integrates the regulatory pathways of insulin secretory capacity and pancreatic β-cell survival (43). Here, we have demonstrated a novel mechanism involving PKCμ in adipocytes as PKCμ regulates the expression of several adipocytokines. PKCμ stimulated the expression of MCP-1 and IL-6 and decreased the expression of adiponectin. These findings were similar to those of our previous results in which AP-2β induced changes in the expression of several adipocytokines (27,28,44-46). We have already proposed that AP-2β is a candidate gene that causes various dysfunctions that lead to the metabolic syndrome and T2DM. However, the pathways regulating its expression and activity have remained unknown. The expression of AP-2β was not affected by adiposity because it was unchanged in adipose tissue obtained from obese mice (ob/ob and KKAy mice) and high-fat-fed mice (unpublished data). Thus, it is likely that several pathways regulate the activity of AP-2β. It was recently reported that the DNA binding activity of AP-2α, another AP-2 isoform, is regulated by its phosphorylation through PKCμ (30). Thus, we hypothesized that PKCμ regulates the expression of adipocytokines by activating AP-2β. We clearly demonstrated that PKCμ enhanced the stimulatory and inhibitory effects of AP-2β on the regulation of adipocytokines and that AP-2β transcriptional activity was required for these effects.

In conclusion, PKCμ undergoes postprandial phosphorylation in adipose tissue. PKCμ activation can dysregulate the expression of adipocytokines, such as MCP-1, IL-6 and adiponectin via AP-2β in 3T3-L1 adipocytes. Our results suggest that postprandial activation of PKCμ may play a role in the postprandial changes in the expression of adipocytokines through an AP-2β-mediated pathway, which may lead to insulin resistance and atherosclerosis.

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