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Activation of mouse phosphodiesterase 3B gene promoter by adipocyte differentiation in 3T3-L1 cells

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Abstract Activation of phosphodiesterase (PDE) 3B reduces free fatty acid output from adipocytes. Induction of PDE3B gene expression by adipocyte differentiation could improve insulin resistance. To examine whether the PDE3B promoter is activated by this differentiation, the 5′ flanking sequence of the mouse PDE3B gene was isolated. The transcription initiation site was determined to be located 195 bp upstream of the translation start site. No putative binding site for peroxisome proliferator-activated receptor γ was found within 2 kb upstream of the transcription initiation site. This region had promoter activity, which was further activated on adipocyte differentiation in 3T3-L1 cells.

Key words: Phosphodiesterase 3B; Adipocyte; Differentiation; Transcription; Promoter; Insulin resistance

1. Introduction

Phosphodiesterase 3B (PDE3B) is a major isoform of the PDE families in adipocytes [1]. Insulin exerts an anti-lipolytic action via the phosphorylation and activation of PDE3B. This activation results in a decrease in intracellular cAMP levels, which leads to a reduction in protein kinase A activity. This subsequently leads to the inactivation of hormone-sensitive lipase, leading to a reduced output of free fatty acids (FFA) from adipocytes [2–5].

Type 2 diabetes mellitus is clinically characterized by insulin resistance in target tissues such as adipose tissue, skeletal muscle, and liver. Reduced insulin sensitivity in adipocytes could represent an event which occurs prior to overt insulin resistance [6–8]. When the anti-lipolytic action of insulin is impaired, the FFA output from adipocytes is increased. The elevation of serum FFA causes insulin resistance in skeletal muscle and liver as well as adipose tissues [9–11].

In prior studies, we demonstrated that basal and insulin-induced membrane-bound PDE (mainly PDE3B) activities are impaired in the epididymal adipocytes of insulin-resistant diabetic KK mice and spontaneously obese rats [12,13]. PDE3B gene expression is also reduced in the adipocytes of obese insulin-resistant KKAy and db/db mice and cp/cp rats [14,15]. These findings suggest that the reduced PDE3B gene expression in adipocytes is a general phenomenon, which is associated with the obese insulin-resistant state.

In earlier studies, we reported that an insulin-sensitizing drug, a derivative of thiazolidinediones, increases PDE3B gene expression in adipose tissues of KKAy or db/db mice, and thus improves insulin resistance [14,16]. Thiazolidinediones are ligands which bind to the peroxisome proliferator-activated receptor (PPAR) γ. Adipogenesis is stimulated on PPARγ activation by ligands [17]. Thiazolidinediones increase the number of small adipocytes, probably by inducing adipocyte differentiation [18,19]. PDE3B mRNA levels are also increased in differentiated 3T3-L1 adipocytes [20]. Therefore, thiazolidinediones might induce PDE3B gene expression via the induction of adipocyte differentiation.

In view of this, we initiated an analysis of the promoter of the mouse PDE3B gene. The 5′ flanking sequence of the mouse PDE3B gene was isolated, and an analysis of a 2-kb portion of the 5′ flanking sequence revealed the presence of putative binding sites for some important transcription factors, but not PPARγ. This isolated region had promoter activity and was activated on adipocyte differentiation.

2. Materials and methods

2.1. Cell culture

3T3-L1 cells, purchased from Human Science Research Resources Bank (Osaka, Japan), were grown in 10 cm dishes with low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Confluent 3T3-L1 cells were treated with differentiation medium, namely, DMEM containing 10% FCS, insulin (1.6 μmol/l), dexamethasone (1.0 μmol/l), and isobutylmethylxanthine (0.5 mmol/l) for 3 days, and were then incubated with DMEM with 10% FCS for 4–7 days until fully differentiated.

2.2. RNA isolation and real-time RT-PCR

Total RNA was isolated from cell homogenates as described previously [14]. Mouse PDE3B mRNA and internal control mouse β-actin were quantitated using the TaqMan RT-PCR method using ABI PRISM 7700 (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). All primers and probes were designed using the Primer Express software (Perkin-Elmer Applied Biosystems) (Table 1). The reporter dye FAM (6-carboxyfluorescein) and VIC were used to label the 5′ terminus of PDE3B and β-actin probes, respectively. TAMRA (6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine), which was attached at the 3′ terminus of each probe, was used as the universal quencher.

100 ng of total RNA with 1.5 μl of 5 μM primers and 0.3 μl of 6.8 μM PDE3B probe or 6.4 μM β-actin probe were incubated with 12.5 μl of 2× master mix without uracil-*N*-glycosylase (Perkin-Elmer Biosystems, Foster City, CA, USA), and 0.625 μl 40× Multi Scribe

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Table 1
Primers and probes of real-time RT-PCR

	Forward primer 5′ to 3′	Probe 5′ to 3′	Reverse primer 5′ to 3′
mPDE3B	TGAGTGGCAGAACCAGTTTCC	[a]CTCTGGAGCCTCATCGTTGCTGACTAAT[c]	TGCGATCCCACCTTGAACA
mβ-Actin	AGGTCATCACTATTGGCAACGA	[b]ATGCCCTGAGGCTCTTTTCCAGCCTT[c]	CACAGGATTCCATACCCAAGAAG

[a]The reporter dye FAM.
[b]The reporter dye VIC.
[c]The universal quencher TAMRA.

and RNase inhibitor mix. The thermal cycle parameters described in the protocol for the TaqMan One-Step RT-PCR Master Mix Reagent Kit (Perkin-Elmer) were used. The comparative cycle of the threshold method was used for quantitating mPDE mRNA following the manufacturer's protocol (Perkin-Elmer).

2.3. Primer extension

Primer extension was done as described previously [21]. Total RNA was isolated from undifferentiated or differentiated 3T3-L1 cells and epididymal adipose tissue of a 10-week-old C57BL/6J mouse as described [14]. 20 μg of tRNA or each total RNA was used as a template for a reverse transcriptase reaction using the mPDE0R primer 5′-end-labeled with [^{32}P]ATP (Table 2). The M13mp18 sequence ladder was electrophoresed to determine the size of each cDNA product.

2.4. Isolation of the 5′ flanking sequence of the mouse PDE3B gene and DNA sequencing

A mouse genomic library constructed by EMBL-3 with a genomic DNA isolated from the liver of an adult DBA/2J mouse (Clontech Laboratories, Inc., Palo Alto, CA, USA) was screened with rat PDE3B cDNA as a probe using standard hybridization methods [22]. 15 positive clones were obtained, and one clone, which contained exon 1, was selected by DNA sequencing. This vector was digested with *Sal*I, and an approximately 15-kb DNA fragment, including exon 1, was isolated. This *Sal*I fragment was then ligated into the *Sal*I site of the pBluescript KS (+) and is hereafter referred to as pSalI PDE. The pSalI PDE contains about 5 kb of 5′ flanking sequence of the PDE3B gene. The pSalI PDE was digested with *Eco*RI and *Xba*I, and a 5-kb fragment was isolated. This fragment was ligated into the *Xba*I/*Eco*RI sites of pGEM7, which was named pGEM7-mPDE3B RX. This construct was then digested with *Xba*I and *Nhe*I, and self-ligated. This pGEM7-mPDE3B RN construct contained about 2.3 kb of the mouse PDE3B 5′ flanking region and the 712-bp 5′ region of exon 1.

The 5′ flanking sequence was determined using the pGEM7-mPDE3B RN as a template. The sequence was initially determined using a reverse primer designed in exon 1 (mPDE5-R), and forward and reverse primers were then subsequently designed based on the determined sequence (Table 2). The DNA sequencing reaction was performed using previously described methods [23,24].

2.5. Plasmid construction

We used the pGEM7-mPDE3B RN to prepare vectors for a luciferase assay of promoter activity. A 2.1-kb of the *Kpn*I/*Bam*HI and a 0.4-kb of the *Sac*I/*Bam*HI fragments of the pGEM7-mPDE3B RN were isolated and subcloned into the *Kpn*I/*Bgl*II and *Sac*I/*Bgl*II sites of the pGL3-Basic vector (Promega, Madison, WI, USA) to obtain pGLmPDE3B-1 and pGLmPDE3B-2, respectively. The pRL-CMV vector, which contained renilla luciferase (Promega), was used as an internal control for correcting transfection efficiency.

2.6. Transfection and luciferase assay

The day prior to transfection, 3T3-L1 cells were plated on a 24-well plate so as to reach a 50–90% confluent state on the day of the transfection. 1 μg of a reporter vector, such as the pGLmPDE3B-1 or pGLmPDE3B-2, and 5 ng of the pRL-CMV internal control vector were diluted with 20 μl of serum-free DMEM per well. 5 μl of PLUS Reagent® (Gibco BRL, Rockville, MD, USA) was then added and the resulting solution was incubated at room temperature (RT) for 15 min to prepare pre-complexed DNA. In a second tube, 2 μl of Lipofect AMINE Reagent® (Gibco BRL) was mixed with 23 μl of serum-free DMEM per well. These pre-complexed DNAs and the diluted Lipofect AMINE Reagent were combined and then incubated for 15 min at RT. This DNA/Lipofect AMINE Reagent mixture was then transferred to each well, which contained 200 μl of fresh serum-free DMEM placed on 3T3-L1 cells, and then incubated at 37°C at 5% CO_2 for 3 h. The medium was exchanged to DMEM containing 10% FCS, and the cells were incubated at 37°C at 5% CO_2 for 24 h. The medium was exchanged to a differentiation medium when differentiation was the objective, whereas it was changed to new DMEM containing 10% FCS for undifferentiated controls. After 48 h of incubation at 37°C at 5% CO_2, the medium was removed, washed with phosphate-buffered saline, and the cells were then lysed with 50 μl of passive lysis buffer for 15 min. The firefly luciferase activity and renilla luciferase activity of these lysates were measured using the Dual-Luciferase™ Reporter Assay System following the manufacturer's protocol (Promega, Madison, WI, USA) and a Lumicounter 700 (MicroTech NITI-ON, Chiba, Japan).

2.7. Statistics

The Student's *t*-test was used for statistical analysis unless otherwise indicated.

3. Results

3.1. Increase in PDE3B mRNA upon adipocyte differentiation in 3T3-L1 cells

To examine whether the level of PDE3B mRNA begins to increase during early adipocyte differentiation, we employed the TaqMan RT-PCR method (Fig. 1). The level of mPDE3B mRNA was increased on differentiation, to about two-fold at 12 h and to 3.5-fold at 24–48 h. Actinomycin D (5 μg/ml), a general transcription inhibitor, blocked this increase in

Table 2
Oligonucleotide for sequencing of mPDE promoter region

Primer name	Forward primer 5′ to 3′	Reverse primer 5′ to 3′	Location
mPDE11-F&R	TCTCAGATGA CAAGCTCGA	TCGAGCTTGT CATCTGAGA	−1851
mPDE12-F&R	CACACAGTAA GGTTTCTCA	TGAGAAACCT TACTGTGTG	−1296
mPDE10-F&R	CTATGTGAAA CAGTGCATG	CATGCACTGT TTCACATAG	−878
mPDE9-F&R	CCATCTGCCA AAGAGCTCG	CGAGCTCTTT GGCAGATGG	−444
mPDE8-F&R	CGGCCGTGAG GTAACTGTT	AACAGTTACC TCACGGCCG	−114
mPDE4-F&R	ACACCAAGAA GCCCTTCTC	AGAAGGGCTT CTTGGTGTC	−3
mPDE1-F&R	GGAGGGCGGC GCGGGGTCGG	CCGACCCCGC GCCGCCCTCC	143
mPDE0-R		CTCGCGCTCG TCCTTCCTC	197
mPDE5-F&R	AACGGCTACG TGAAGAGCT	AGCTCTTCAC GTAGCCGTT	288

Annealing temperature is 55°C. The standard buffer (TAKARA SHUZO Co., Ltd. Biomedical Group, Shiga, Japan) containing DMSO was used. The numbers of location indicate the 5′ start site (base) of each forward primer by defining the transcription initiation site as +1.

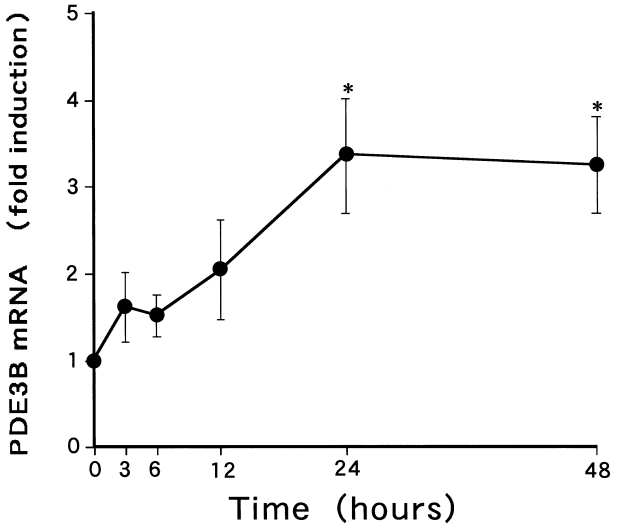

Fig. 1. Increase in PDE3B mRNA on adipocyte differentiation in 3T3-L1 cells. Total RNA was isolated from the cells at the indicated times after the addition of the differentiation medium. The TaqMan RT-PCR method was employed to quantitate PDE3B mRNA as described in Section 2. The level of mouse PDE3B mRNA was normalized by the level of mouse β-actin mRNA in each sample for meaningful comparisons, and the relative amounts of PDE3B mRNA were determined by calculating from the threshold cycles. *$P < 0.01$ compared to 0 h.

PDE3B mRNA at 24 h (data not shown). This suggests that the increase in PDE3B mRNA by adipocyte differentiation could result, at least in part, from increased gene transcription.

3.2. Determination of the transcription initiation site of the mouse PDE3B gene

To determine the transcription initiation site of the mouse PDE3B gene, primer extension was employed (Fig. 2A). When tRNA was used as a template, no product was detected (Fig. 2A, lane 1). In contrast, only one specific band was detected with the total RNA from differentiated 3T3-L1 cells or mouse adipose tissue, but not with that from undifferentiated 3T3-L1 cells (Fig. 2A, lanes 2–4). Thus, the transcription initiation site is located 195 bp upstream of the translation start site. The sequence analysis around the transcription initiation site revealed that a putative TATA or GATA box was located between −38 and −33 bp upstream of this site (Fig. 2B).

3.3. Analysis of the 5′ flanking sequence of the mouse PDE3B gene

To examine the function of the PDE3B promoter, we first isolated the 5 kb of the 5′ flanking sequence of the mouse PDE3B gene. The sequence of about 2 kb of the promoter region is available in EMBL (#AJ344077). Analysis by the TFSEARCH program (http://pdap1.trc.rwcp.or.jp/research/db/TFSEARCH.html) revealed that this region contains several putative binding sites for some important transcription factors, except PPARγ (Fig. 3). There are two highly conserved regions between this mouse PDE3B promoter and human PDE3B promoter in the draft sequence. The putative binding sites for factors such as the CCAAT/enhancer binding protein (C/EBP) and p300 were located within the distal conserved region. The proximal conserved region includes the binding sites for the cAMP response element binding protein.

3.4. Basal and adipocyte differentiation-induced promoter activity of the mouse PDE3B gene in 3T3-L1 cells

To examine whether the isolated PDE3B promoter has basal activity, we checked the two luciferase reporter constructs in transfection experiments, namely pGLmPDE3B-1, which contains the region between −2075 bp and +74 bp, and pGLmPDE3B-2, the region between −373 and +74 bp (Fig. 4). The basal promoter activities of pGLmPDE3B-1 and pGLmPDE3B-2 were both increased compared to those of the pGL3-Basic vector, which has no promoter regions. This suggests that these two regions have basal promoter activity.

To examine whether these promoter regions were further activated on adipocyte differentiation, transfected cells were treated with the differentiation medium for 48 h. The promoter activities of pGLmPDE3B-1 and pGLmPDE3B-2 were induced to 1.8-fold ($n = 6$, $P < 0.05$) and 1.6-fold ($n = 8$, $P < 0.05$), respectively. Therefore, these PDE3B promoter regions appear to be further activated as the result of adipocyte differentiation.

4. Discussion

We isolated approximately 2 kb of the 5′ flanking sequence of the mouse PDE3B gene. When a search was made for binding sites related to adipocyte differentiation, C/EBP binding sites were found at −1104/−1092, −1142/−1136, and −1303/−1290 bp. Consensus binding sites for PPAR and sterol regulatory element binding protein (SREBP) were not detected, although a MyoD binding site similar to E box, another binding motif of SREBP, was found at −445/−436 and −686/−677 bp.

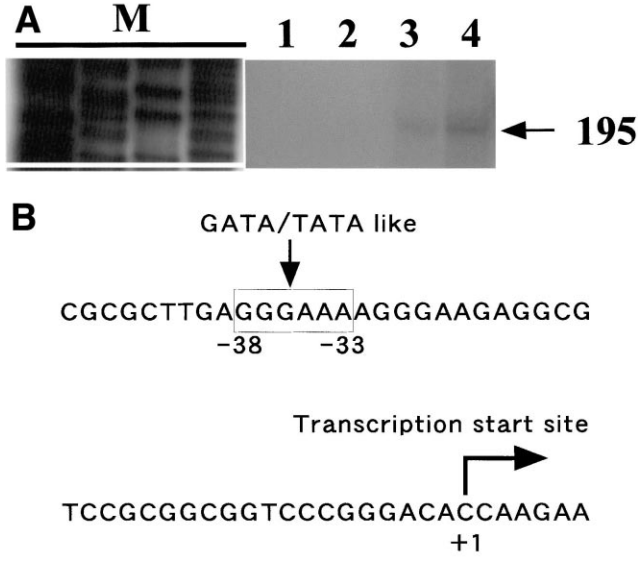

Fig. 2. Determination of the transcription initiation site of the mouse PDE3B gene. A: Primer extension was done as described in Section 2. 20 μg of tRNA (control) or total RNA isolated from the indicated materials was used as a template for a reverse transcriptase reaction using the mPDE0R primer 5′-end-labeled with [^{32}P]ATP. Transcription initiation site is located 195 bp upstream of the translation start site as indicated by an arrow. Lanes 1: tRNA; 2: undifferentiated 3T3-L1 cells; 3: differentiated 3T3-L1 adipocytes; 4: mouse epididymal adipose tissue; M: M13mp18 sequence ladder. B: The sequences in the vicinity of the transcription initiation site are shown. A putative TATA or GATA box and the transcription initiation site are indicated by arrows.

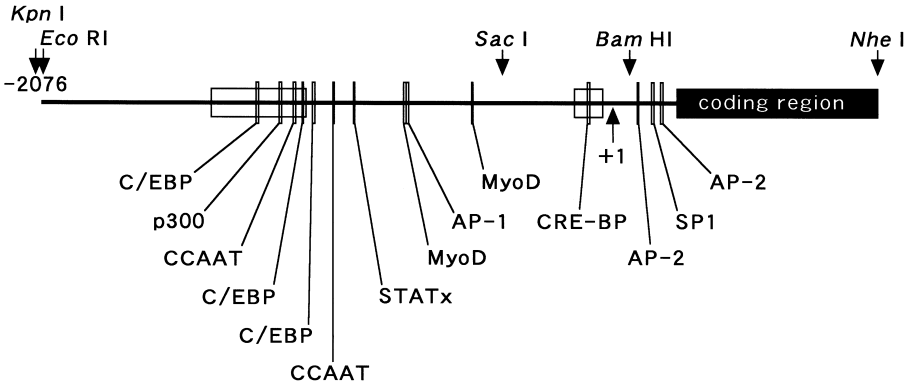

Fig. 3. Schematic representation of the mouse PDE3B gene promoter. The 5′ flanking sequence of the mouse PDE3B gene was sequenced with the primers shown in Table 2 as described in Section 2. The first base of the transcription initiation site is defined as +1. Restriction enzyme recognition sites are also shown, along with their names. The highly conserved region between this mouse sequence and human draft sequence are shown as boxes. The putative binding sites for important transcription factors, as determined by the TFSEARCH program, are also boxed, and include C/EBP, p300, CRE-BP, SP1, etc. It should be noted that these cites have not been proven to be functional.

The transcription initiation site is located 195 bp upstream of the translation start site of the mouse PDE3B gene. The proximal conserved region between the mouse and human PDE3B promoter is located between −172 bp and −30 bp, suggesting that the basic transcription machinery may function around this conserved region. In fact, a putative TATA or GATA box was also found between −38 and −33 bp in this promoter.

Our findings show that PDE3B mRNA is increased at 12 h after the induction of adipocyte differentiation using sensitive real-time RT-PCR. Previously, Taira et al. showed that fully differentiated 3T3-L1 adipocytes contain increased levels of PDE3B mRNA compared to 3T3-L1 fibroblasts [20]. Landström et al. showed that PDE3B mRNA levels are increased after 4 days of differentiation [25]. These differences may be due to the sensitivity of the assays employed.

The present data show that PDE3B promoter activity, as well as PDE3B mRNA levels, is increased on adipocyte differentiation in vitro. This enhanced PDE3B gene expression could improve insulin resistance through a reduced FFA output from adipocytes. Thiazolidinediones improve insulin resistance, probably via the induction of adipocyte differentiation in vivo [18,19]. Since the isolated PDE3B promoter lacks the putative PPAR binding sites, this would be of particular interest for a target for novel anti-diabetic drugs, which specifically induce the PDE3B gene expression to reduce FFA output.

In summary, we have isolated the promoter region of the mouse PDE3B gene. The 5′ flanking sequence, including the highly conserved regions between human and mouse had promoter activity, which was further activated on adipocyte differentiation. It is not clear which DNA elements are responsible for the basal and differentiation-induced promoter activity, and which transcription factors bind these elements. Further experiments will be needed to clarify these points.

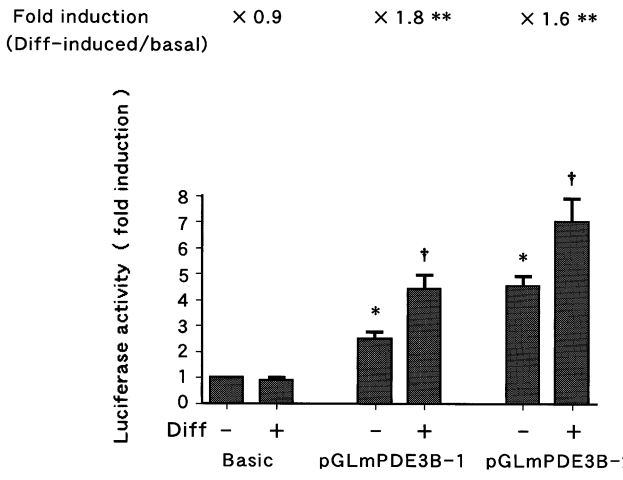

Fig. 4. Basal and adipocyte differentiation-induced promoter activity of the mouse PDE3B gene in 3T3-L1 cells. Undifferentiated 3T3-L1 cells were transfected as described in Section 2. The transfected 3T3-L1 cells were then treated with or without differentiation medium for 48 h. The pGLmPDE3B-1, containing the region between −2075 and +74 bp, and pGLmPDE3B-2, containing the region between −373 and +74 bp, were used as a firefly luciferase reporters. Renilla luciferase construct, the pRL-CMV, was used for an internal control to correct transfection efficiency. Promoter activities were determined as firefly luciferase activity from the pGLmPDE3B-1 or pGLmPDE3B-2, as corrected by renilla luciferase activity from the pRL-CMV. The relative promoter activities were determined by defining the pGL3-Basic vector activity as 1. Data represent mean ± S.E.M. $*P < 0.01$ compared to control; $^{\dagger}P < 0.05$ compared to undifferentiated cells (Diff-); $**P < 0.05$ compared to pGL3-Basic. Diff, differentiation was induced by the reagents described in Section 2.

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