EXPERIMENTAL STUDY

Adipocyte-specific reduction of phosphodiesterase 3B gene expression and its restoration by JTT-501 in the obese, diabetic KK_{AY} mouse

Yan Tang, Haruhiko Osawa, Hiroshi Onuma, Masaaki Hasegawa, Tatsuya Nishimiya, Masaaki Ochi and Hideichi Makino

Department of Laboratory Medicine, Ehime University School of Medicine, Shigenobu, Ehime 791-0295, Japan

(Correspondence should be addressed to H Osawa; Email: harosawa@m.ehime-u.ac.jp)

Abstract

Objective: Phosphodiesterase (PDE) 3B is a key enzyme involved in the anti-lipolytic action of insulin in adipocytes. PDE3B activation results in a reduced output of free fatty acids (FFA), whereas elevated serum FFA is known to cause insulin resistance. We have recently reported that reduced PDE3B gene expression is restored by treatment with pioglitazone, in the adipose tissues of obese, insulin-resistant diabetic KK_{AY} mice. To determine whether the altered PDE3B gene expression is specific for adipocytes, the expression of this gene in liver and epididymal fat tissues of KK_{AY} mice was examined. The effect of JTT-501, another peroxisome proliferator-activated receptor (PPAR) γ ligand, which is different from thiazolidinedione, was also examined.

Methods: PDE3B mRNA and protein were quantified by an RNase protection assay and Western blotting respectively. Membrane-bound PDE activities were also measured.

Results: In adipose tissues of KK_{AY} mice, PDE3B mRNA, protein and membrane-bound PDE activity were reduced to 47%, 57% and 51% respectively relative to those in C57BL/6J control mice. JTT-501 increased PDE3B mRNA, protein and membrane-bound PDE activity by 2.2-, 1.6- and 1.7-fold respectively over those of untreated KK_{AY} mice. In the liver, PDE3B gene expression remained unchanged in KK_{AY} mice, and was not affected by JTT-501. JTT-501 reduced the elevated levels of serum insulin, glucose, FFA and triglyceride in KK_{AY} mice.

Conclusions: PDE3B gene expression was specifically reduced in the adipose tissues of KK_{AY} mice. JTT-501 restored this reduced gene expression with an accompanying improvement in elevated serum FFA and insulin resistance.

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Introduction

Eleven different gene families which involve mammalian cyclic nucleotide phosphodiesterase (PDE1 to 11) have been reported to date (1). PDE3B represents a major isoform of PDE in adipocytes. Insulin exerts its antilipolytic action via the phosphorylation and subsequent activation of PDE3B. The activation of this enzyme is accompanied by a decrease in intracellular cAMP which, in turn, leads to a reduction in cAMP-dependent protein kinase A (PKA) activity. This subsequently leads to the dephosphorylation and inactivation of hormone-sensitive lipase (HSL), leading to a decreased rate of hydrolysis of stored triglycerides and, as a result, to a reduction in the release of free fatty acids (FFA) from adipocytes (2, 3).

PDE3B is also thought to be the major, insulin-activated isoform of PDE in the liver (4). Glucagon and insulin, two antagonistic hormones, are the most important glucoregulatory factors in the liver. Glucagon

exerts its effect by enhancing the cAMP–PKA pathway, while insulin inhibits the effect of glucagon (5). The effect of insulin on glycogen synthesis and glycogenolysis is mediated, at least in part, by the enhancement of PDE, which inhibits the cAMP–PKA pathway (6–8). A reduction in the intracellular concentration of cAMP by insulin results in an increase in the rate of synthesis of fatty acids and triglycerides, as well as glycogen in this tissue (9, 10).

Type 2 diabetes mellitus is clinically characterized by insulin resistance in target tissues, which include adipose tissue, skeletal muscle and liver (11). It is possible that a reduced insulin sensitivity in adipocytes represents an event which occurs prior to overt insulin resistance in such tissues (12). When the antilipolytic action of insulin is inhibited, the rate of release of FFA from triglycerides is increased. It is well known that an elevation in serum FFA leads directly to insulin resistance in skeletal muscle and liver, as well as adipose tissues (13).

In prior studies, we were able to demonstrate that basal and insulin-induced membrane-bound PDE (mainly PDE3B) activities are impaired in the epididymal adipocytes of insulin-resistant diabetic KK mice, spontaneously obese rats and dexamethasone-treated rats (14–16). Other studies have also concluded that PDE activity is reduced in the adipocytes of diabetic patients or rats (17, 18). Nagaoka *et al.* have also shown that PDE3B mRNA is reduced in obese diabetic *cp/cp* rats (19). More recently, we demonstrated that PDE3B gene expression is reduced in the adipocytes of obese, insulin-resistant, diabetic KK^{ay} mice. Treatment with pioglitazone, an insulin-sensitizing thiazolidinedione, restores this altered PDE3B gene expression with a parallel improvement in insulin resistance (20). However, the issues of whether this alteration of PDE3B gene expression is specific for adipocytes and whether insulin-sensitizing compounds generally affect PDE3B gene expression have not yet been examined.

In view of this, we initiated an analysis of PDE3B gene expression in liver as well as the epididymal fat tissues of KK^{ay} mice. Our findings show that steady-state membrane-bound PDE activity, PDE3B protein or the corresponding mRNA levels are reduced only in the adipose tissues of these mice. Treatment with an isoxazolidinedione, JTT-501 (21, 22), a peroxisome proliferator-activated receptor (PPAR) γ ligand that is different from thiazolidinedione, restores all these parameters in parallel with an improvement in hyperglycemia, elevated FFA and insulin resistance.

Materials and methods

Animals and tissue preparation

Male non-diabetic C57BL/6J mice and insulin-resistant KK^{ay} mice were purchased from Clea Japan (Osaka, Japan) and were used experimentally when they reached an age of 10–11 weeks. KK^{ay} mice are the result of a cross between glucose-intolerant black KK female mice and yellow male obese Ay mice, and are known to serve as excellent models of obese insulin-resistant type 2 diabetes (23). C57BL/6J mice were generally employed as controls for KK^{ay} mice (20). The mice were housed individually, and were provided with food and water and allowed to feed *ad libitum*. The treatment group of KK^{ay} mice received JTT-501 in their food at a level of 0.18% (from Japan Tobacco Inc., Osaka, Japan), which resulted in the overall ingestion of 300 mg/kg per day over a 4-day period. The mice were killed by rapid decapitation under light diethyl ether anesthesia and the epididymal fat tissues and liver were removed rapidly. For the RNase protection assay, the tissues were homogenized with TRI REAGENT LS (Molecular Research Center Inc., Cincinnati, OH, USA), frozen in liquid nitrogen, and then stored at -80°C . For Western blotting, fat and liver tissues were homogenized with STS buffer (250 mM sucrose,

10 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, 15 $\mu\text{g/ml}$ pepstatine, 15 $\mu\text{g/ml}$ leupeptine, 3 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.0). For the assay of membrane-bound PDE activity, the liver was homogenized as described above, and free adipocytes were prepared from fresh fat tissues by the collagenase method (24). Blood samples were also collected when killed. Serum glucose, FFA, triglycerides and insulin were determined using previously described methods (20). All experimental procedures were approved by the Animal Experimentation Committee of Ehime University, School of Medicine.

RNase protection assay

Total RNA was isolated from liver and adipose tissues that had been homogenized in TRI REAGENT LS according to the manufacturer's instructions (Molecular Research Center Inc.). The integrity of each RNA sample was confirmed by intact 18S and 28S rRNA, which was stained with ethidium bromide on an agarose gel. The concentration and purity was assessed by absorbance at 260 nm and by the 260/280 nm ratio respectively.

RNase protection assay (RPA) was carried out as previously described (20). A 449 nt biotinylated cRNA probe which contained a 395 nt antisense mouse PDE3B RNA was transcribed using a MAXIscript *In Vitro* Transcription T7 Kit (Ambion, Austin, TX, USA) and Biotin-16-UTP (Boehringer Mannheim Corporation, Indianapolis, IN, USA). A 300 nt biotinylated RNA probe, which contained a 250 nt mouse β -actin antisense RNA, and RNA molecular markers were also produced using the pTRI- β -actin-mouse control template DNA and the RNA Century Marker Template Set respectively (Ambion). HybSpeed RPA (Ambion) was used to hybridize the purified cRNA probes of PDE3B and β -actin with 10 μg of each total RNA which had been extracted from liver and adipose tissues. The protected RNAs were separated by 5% acrylamide–8 mol/l urea gel electrophoresis, transferred to a Brightstar-Plus positively charged nylon membrane and were then visualized by the Brightstar BioDetect Kit (Ambion). The cRNAs, which were detected by chemiluminescence, were exposed and quantitated using 1D Image Analysis Software (Kodak Digital Science, Kodak, Rochester, NY, USA). Since the protected PDE3B and β -actin RNAs are of different sizes (395 and 250 nt respectively), both RNA species could be quantitated in a single RNA sample. The PDE3B/ β -actin mRNA ratio was calculated and the mean value in C57BL/6J mice defined as 1-fold. We were able to confirm that β -actin mRNA could be used to correct the PDE3B mRNA in each sample from liver as well (C57BL, 1.00 ± 0.06 ; KK^{ay}, 1.03 ± 0.07 ; JTT-KK^{ay}, 1.01 ± 0.03 ; $n = 6$), as shown in the case of adipose tissue (20).

Western blotting

Adipose and liver tissues were homogenized directly with STS buffer. In order to examine the difference between KKAY and C57BL mice, adipocytes were isolated from fresh epididymal fat tissues and were then homogenized in STS buffer, and the membrane-bound fraction was obtained as described previously (25, 26). Aliquots of protein samples (20 µg each) were electrophoresed on 7.5% polyacrylamide gels and the resulting size-fractionated proteins were electrophoretically transferred to Immobilon membranes (Millipore, Bedford, MA, USA). These membranes were incubated with an anti-rat PDE3B polyclonal antibody raised in rabbits against the NH₂-terminal peptide (amino acid residues 1–17) (20). Visualization of the PDE3B protein was carried out with an enhanced chemiluminescence detection system as described previously (20). The autographs were quantitatively analyzed by 1D Image Analysis Software (Kodak Digital Science). The specificity of our anti-rat PDE3B polyclonal antibody was confirmed by pre-incubating the antibody with the NH₂-terminus peptide (amino acids 1 to 17) of rat PDE3B as a competitor, as described previously (20).

Membrane-bound PDE (mainly PDE3B) catalytic activity

Membrane-bound PDE activity was assayed exactly as described previously (25, 26). Briefly, isolated adipocytes, epididymal fat pads, or liver were homogenized with STS buffer, the membrane-bound fraction was then suspended in pH 7.5 STS buffer, and the PDE activity was subsequently determined.

Statistical analysis

An unpaired Student's *t*-test was used for all the experiments, and results are expressed as means ± S.E.S. *P* < 0.05 was taken as significant.

Results

Effect of JTT-501 on serum insulin, glucose, triglyceride and FFA levels in KKAY mice

We first compared body weight, epididymal fat pad

weight, serum glucose, FFA, triglyceride and insulin concentrations of the KKAY mice to those of the control C57BL/6J mice (Table 1). The KKAY mice were obese, hyperinsulinemic, hyperglycemic and hyperlipidemic compared with the controls. A 4-day treatment with JTT-501 resulted in a reduction in serum glucose, FFA, triglycerides and insulin levels in the KKAY mice. The JTT-501-treated KKAY mice showed a slight increase (about 10%) in body weight and epididymal fat pad weight relative to the untreated KKAY mice.

Effect of JTT-501 on PDE3B mRNA in adipose tissues and liver of KKAY mice

To determine whether PDE3B mRNA is altered in KKAY mice, adipose tissue and liver PDE3B mRNA were compared with those of control C57BL mice using RPA (Fig. 1). The sizes of the protected probes were exactly the same, as expected, thus enabling us to determine PDE3B mRNA, as described previously (20). In the adipose tissue of the KKAY mice, the level of PDE3B mRNA was 47% that of the control mice (control 1.00 ± 0.08 vs KKAY 0.47 ± 0.03 fold induction, *n* = 6, *P* < 0.01). JTT-501 treatment restored this reduced level of PDE3B mRNA to the same as that in control C57BL mice (JTT-KKAY 1.01 ± 0.07 fold induction, *n* = 6, *P* < 0.01) (Fig. 1A). This increase was significant when compared with that of untreated KKAY mice. In the liver of the KKAY mice, the level of PDE3B mRNA was the same as that of the control mice (control 1.00 ± 0.07 vs KKAY 0.92 ± 0.05 fold induction, *n* = 6). JTT-501 had no effect on PDE3B mRNA levels in this tissue in KKAY mice (JTT-501-treated KKAY 0.99 ± 0.08 , *n* = 6) (Fig. 1B). Thus, steady-state PDE3B mRNA was decreased in the adipose tissues but was not affected in the liver of KKAY mice. Treatment with JTT-501 restored the levels of this reduced PDE3B mRNA in adipose tissues, but had no effect on the levels in the liver.

Effect of JTT-501 on PDE3B protein in adipose tissues and liver of KKAY mice

To examine whether the changes in PDE3B mRNA levels of the above-mentioned KKAY mice were accompanied by a corresponding change in the level

Table 1 The effect of JTT-501 on body and epididymal fat pad weight, serum glucose levels, FFA, triglycerides and insulin concentrations in KKAY mice.

Experimental groups	<i>n</i>	Body weight (g)	Epididymal fat pad weight (g)	Glucose (mg/dl)	FFA (mEq/l)	Triglycerides (mg/dl)	Insulin (ng/ml)
C57BL/6J mice	6	27.5±0.3	0.46±0.02	231±5	0.30±0.02	79±5	0.5±0.0
Untreated KKAY mice	8	41.8±0.6	1.72±0.07	700±46 ^a	1.68±0.07 ^a	592±77 ^a	51.7±5.7 ^a
JTT-KKAY mice	8	45.2±0.8 ^b	1.89±0.03	438±40 ^b	0.58±0.04 ^b	116±12 ^b	24.2±7.0 ^b

Data are the means ± S.E.S. ^aSignificant differences compared with C57 mice, *P* < 0.01. ^bSignificant differences compared with untreated KKAY mice, *P* < 0.01. JTT-KKAY: JTT-501-treated KKAY mice.

Figure 1 Effect of JTT-501 on PDE3B mRNA in (A) adipose tissues and (B) liver of KKAY mice. PDE3B mRNA was measured by RPA in epididymal fat tissues and liver from C57BL/6J control, KKAY and JTT-501-treated KKAY mice, as described in the Materials and methods section. The mice ($n = 6$ in each experimental group) were 10–11 weeks old. JTT-501 (300 mg/kg per day) was administered for a period of 4 days in the case of the treated KKAY mice. The 395 nt PDE3B protected probes and the 250 nt protected β -actin probes are shown in the upper panel. PDE3B mRNA is expressed as the intensity of the protected PDE3B band corrected by that of the protected β -actin band in each lane. The mean value in the untreated db/+ mice is then defined as 1.00 and the magnitude of the induction is shown as the mean $\pm$ S.E. (fold) in the lower panel. *Significant difference compared with C57BL/6J mice ($P < 0.01$). †Significant difference compared with untreated KKAY mice ($P < 0.01$). JTT-KKAY: JTT-501-treated KKAY mice.

of PDE3B protein, we next assessed the steady-state PDE3B protein by Western blotting using the specific PDE3B antibody as described previously (20) (Fig. 2). It has been confirmed that this antibody is specific to PDE3B in the liver as well as adipose tissue by incubation with the NH₂-terminus peptide (1 to 17 aa) of rat PDE3B as a competitor (data not shown). The level of PDE3B protein in adipose tissue of KKAY mice was 57% that of the control mice (control 1.00 ± 0.05 , vs KKAY 0.57 ± 0.03 fold, $n = 6$, $P < 0.01$). JTT-501 treatment restored this reduced level of PDE3B protein to a level which was nearly the same that in the control C57BL mice (JTT-KKAY 0.91 ± 0.08 fold, $n = 6$, $P < 0.01$) (Fig. 2A). This increase was also significant when compared with that of untreated KKAY mice. No significant difference in the level of PDE3B protein from the liver of KKAY and C57 mice (control 1.00 ± 0.07 vs KKAY 0.89 ± 0.04 fold, $n = 6$) were detected. JTT-501 had no effect on PDE3B protein levels in this tissue (JTT-KKAY 1.01 ± 0.08 fold, $n = 6$) (Fig. 2B). Thus, steady-state PDE3B protein was decreased in the adipose tissues but was not affected in the liver of KKAY mice. JTT-501 treatment restored this reduced PDE3B protein in adipose tissues but had no effect on that in the liver.

Effect of JTT-501 on membrane-bound PDE activities in adipose tissues and liver of KKAY mice

As a final output of PDE3B gene expression, the membrane-bound PDE (mainly PDE3B) activity was measured (Fig. 3). The activity in adipocytes was decreased in KKAY mice (control C57BL 105.6 ± 1.8 vs KKAY 53.6 ± 2.5 pmol/mg per min,

$n = 6$, $P < 0.01$) and was restored by treatment with JTT-501 (JTT-KKAY 89.2 ± 9.3 pmol/mg per min, $n = 6$, $P < 0.01$) (Fig. 3A). In the liver of untreated KKAY mice, the membrane-bound PDE activity was not affected compared with that of the control mice (control C57BL 20.5 ± 0.6 vs KKAY 19.9 ± 2.3 pmol/mg per min, $n = 8$). JTT-501 had no effect on this activity (JTT-KKAY 20.8 ± 1.5 pmol/mg per min, $n = 6$) (Fig. 3B). Thus, the membrane-bound PDE activity, and the levels of either steady-state protein or mRNA were decreased in adipose tissues but remained unchanged in the liver in KKAY mice. Treatment with JTT-501 restored all these parameters in adipose tissues but had no effect on those in the liver, in parallel with an improvement in hyperglycemia, elevated FFA and insulin resistance.

Discussion

The data herein show that PDE3B gene expression is reduced in adipocytes, but not in the liver of obese, insulin-resistant, diabetic KKAY mice. This reduced PDE3B gene expression in adipose tissue is restored by treatment with JTT-501, which is correlated with an improvement in elevated serum FFA and insulin resistance. The expression of this gene in the liver was not affected, suggesting that the altered gene expression in adipocytes is specific.

As well as thiazolidinediones, an isoxazolidinedione, JTT-501, is also a ligand for PPAR γ (21). In fat tissues, adipogenesis is stimulated via the activation of PPAR γ by ligands (27). Two types of thiazolidinediones, troglitazone and pioglitazone, cause an increase in the number of small adipocytes, which is probably the result of the induction of adipocyte differentiation

Figure 2 Effect of JTT-501 on PDE3B protein in (A) adipose tissues and (B) liver of KKAY mice. Western blotting was carried out using the anti-PDE3B antibody raised against the N-terminal peptide (1–17) of rat PDE3B as described in Materials and methods. All mice were 10–11 weeks old. JTT-501 (300 mg/kg per day) was administered for 4 days in the case of the treated KKAY mice. The specific 135 kDa PDE3B bands from the adipose tissue and liver are shown in the upper panel. The mean value of PDE3B protein intensity in the adipose tissues and liver of the C57BL/6J mice is defined as 1.00 and the fold induction is shown as mean $\pm$ s.e. (fold) in the lower panel. *Significant difference compared with C57BL/6J mice ($P < 0.01$, $n = 4$). †Significant difference compared with untreated KKAY mice ($P < 0.01$, $n = 4$). JTT-KKAY: JTT-501-treated KKAY mice.

(28, 29). PDE3B mRNA levels are also increased during differentiation from pre-adipocyte to adipocytes in 3T3-L1 cells (30). Therefore, it is possible that the effect of JTT-501 on PDE3B gene expression, as demonstrated in the present study, is related to the differentiation state of the adipocytes. PPAR γ is mainly expressed in adipose tissues, whereas PPAR α is mainly expressed in the liver. This could account for the difference in regulation in PDE3B gene expression by JTT-501 for the liver vis-à-vis adipose tissue (31, 32).

An enlargement of adipocytes and an increase in fat mass are some of the characteristics of obesity (33). The basal and catecholamine-stimulated rate of lipolysis as well as intracellular cAMP concentration and HSL activity is positively correlated with fat cell size (34–36). In fact, both the level of lipolysis and that of circulating FFA are increased in obese humans and animals (37, 38). It is possible that the inactivation of PDE3B results in an increase in HSL activity, which, in turn, would lead to an enhanced rate of release of FFA. Thus, a reduction in PDE3B gene expression in the

adipocytes of KKAY mice could contribute to an increase in the rate of FFA release.

The PDE3B gene expression was reduced in adipose tissues of polygenic obese insulin-resistant models, KKAY mice, and was restored by JTT-501. This result is consistent with that found for db/db mice, the monogenic obese insulin-resistant models, due to an abnormality in the leptin receptor (39). Thus, the reduced PDE3B gene expression in adipocytes could represent the common event in insulin resistance which is observed in these obese insulin-resistant diabetic mice regardless of their genetic backgrounds (20). The restoration of this reduced gene expression in adipose tissue by three types of PPAR γ ligands, namely JTT-501, pioglitazone and troglitazone (39), with a paralleled improvement in insulin resistance, further supports this hypothesis. Likewise, in human obese type 2 diabetes mellitus, the reduced PDE3B gene expression in adipocytes could account for the development of insulin resistance.

In summary, PDE3B gene expression is reduced in adipocytes but remains unchanged in the liver of KKAY

Figure 3 Effect of JTT-501 on membrane-bound PDE (mainly PDE3B) activity in the (A) adipose tissues and (B) liver of KKAY mice. All mice were 10–11 weeks old. JTT-501 (300 mg/kg per day) was administered for 4 days in the case of the treated KKAY mice. The membrane-bound PDE activity was determined in the epididymal adipocytes and liver of C57BL/6J control mice, KKAY mice and JTT-501-treated KKAY mice as described in the Materials and methods section. Columns represent means $\pm$ s.e.s of PDE catalytic activity (pmol/mg per min). *Significant difference compared with C57BL/6J control mice ($P < 0.01$, $n = 8$). †Significant difference compared with untreated KKAY mice ($P < 0.01$, $n = 6$). JTT-KKAY: JTT-501-treated KKAY mice.

Figure 4 A possible role of PDE3B gene expression in insulin resistance. PDE3B mediates antilipolytic action of insulin and the activation of PDE3B results in reduced FFA output from adipocytes. An elevation in serum FFA is known to cause insulin resistance in the skeletal muscle and liver and, of these insulin target tissues, adipocytes are the most sensitive to insulin. PDE3B gene expression is reduced specifically in adipose tissue of obese, insulin-resistant rodents. This reduced gene expression could cause insulin resistance through elevated serum FFA. PPAR γ ligands such as JTT-501 increase PDE3B gene expression in adipocytes, which could improve insulin resistance through the reduction of serum FFA.

mice (Fig. 4). JTT-501 restores this altered gene expression only in adipocytes with an accompanying improvement in insulin resistance. The issue of how PDE3B gene expression is regulated under these conditions, and whether the altered PDE3B gene expression is the primary cause of insulin resistance observed with human obese type 2 diabetes is not presently clear. These topics merit further investigation.

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