Evidence for the presence of several phosphodiesterase isoforms in brown adipose tissue of Zucker rats: modulation of PDE2 by the fa gene expression

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Received 2 July 1999

Abstract The present study was undertaken to characterise the phosphodiesterases (PDEs) present in brown adipose tissue (BAT) of Zucker rat pups and to determine whether the capacity for degradation of cyclic nucleotides was affected by the fatty genotype. Regardless of the genotype, PDE2–4 contributed to total PDE activity, the PDE3 activity equalling the sum of PDE2 and 4 activities. In fa/fa compared to Fa/fa rats, (a) PDE2 activity was significantly increased, (b) Western blot analysis of PDE2 revealed two signals at 71 and 105 kDa, with changes in protein being in good parallelism with changes in activity, (c) the PDE2 mRNA concentration was also significantly increased. In good agreement, the cGMP concentration was decreased in BAT from fa/fa pups.

Key words: Type 3 phosphodiesterase; Type 4 phosphodiesterase; Obesity; Cyclic GMP

1. Introduction

In the Zucker obese (fa/fa) rat, obesity is genetically transmitted as an autosomal recessive trait due to a mutation on a single gene that has been called 'fa', for fatty. A single nucleotide substitution in the fa allele of the leptin receptor is responsible for the obese phenotype [1]. Because leptin acts on the brain to inhibit feeding and to increase thermogenesis [2], this mutation produces profound obesity of early onset caused by defective non-shivering thermogenesis [3,4] and preferential deposition of energy into white adipose tissue [5].

Brown adipose tissue (BAT) is the main effector of non-shivering thermogenesis in a new-born rat and therefore plays an important role in the positive energy balance at the onset of obesity. In Zucker fa/fa rats, before obesity is clearly apparent, numerous metabolic abnormalities are present in the BAT [4,6,7] and can be corrected by administration of thermogenic β-adrenergic agonists [8,9], suggesting that impaired sympathetic activity is one of the first consequences of the gene's expression. Furthermore, cellular responsiveness to catecholamines is altered in BAT [10], thus demonstrating that the capacity for 3′,5′-cyclic monophosphate (cAMP) production is decreased in fa/fa neonates.

The intracellular concentration of cAMP results from an equilibrium between production and degradation. The 3′,5′-cyclic nucleotide phosphodiesterases (PDEs) are responsible for the degradation of the second messenger cyclic nucleotides cAMP and cGMP. They are important in regulating intracellular concentrations of cyclic nucleotides and, consequently, biological responses to these molecules. Mammalian PDEs include a large group of structurally related isoenzymes, which belong to at least seven distinct but related gene families [11]. Because they are differently expressed and regulated, PDEs are found in different amounts, proportions and subcellular locations in different cells and tissues. In white adipocytes, cGMP-inhibited cAMP phosphodiesterase plays a key role in the anti-lipolytic action of insulin [12] and accounts for 80% of the phosphodiesterase activity in the non-soluble fraction of these cells [13]. However, cGMP-stimulated PDE (PDE2) and cAMP-specific PDE (PDE4) have also been characterised in a soluble fraction from extract of rat epididymal fat cells [13].

In BAT, PDE3 is present and the hormonal regulation of this PDE form is thought to be similar to that in white adipose cells [14]. However, neither the presence of the other PDE forms nor their relative contributions to phosphodiesterase activity has been investigated in this tissue. The present study was undertaken to characterise the PDEs present in BAT of Zucker rats and to determine whether the capacity for cAMP degradation by these different phosphodiesterase isoforms was affected by the fatty genotype. Because the endocrine status of obese rats is strongly modified after weaning, we examined PDE(s) activities in young pre-obese (fa/fa) and lean (Fa/fa) Zucker rats at 14 days of age.

2. Materials and methods

2.1. Animals and protocols

Zucker rats were bred in our laboratory from obese (fa/fa) male and heterozygous lean (Fa/fa) female crosses. The pups and their mother were housed in a temperature-controlled room (22 ± 1°C) on a fixed 12:12 h light/dark cycle (lights on from 07:00 h to 19:00 h). The dams were fed on a stock diet (UAR, France) ad libitum. The litter size was standardised at 10 pups which were used at 14 days of age. The genotype of the pups was identified through study of the correlation between inguinal adipose weight and body weight [15].

2.2. Tissue sampling and processing

Rats of either sex were killed by decapitation and interscapular BAT was carefully dissected and immediately frozen in liquid nitrogen

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Abbreviations: Cilostamide, *N*-cyclohexyl-*N*-methyl-4-(1,2-dihydro-2-oxo-6-quinolyloxy) butiramide; TES, *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid

0014-5793/99/$20.00
PII: S0014-5793(99)00934-5

for further homogenisation or for isolation of RNA. Three tissues (300–400 mg) were homogenised with a glass-Teflon homogeniser in 3 ml of ice-cold 10 mM TES/250 mM sucrose buffer (pH 7.0) containing 0.5 μg/ml each of leupeptin, pepstatin, aprotinin and 100 μm 4-(2-aminoethyl)benzensulfonyl fluoride hydrochloride. The extract was defatted by centrifugation at low speed (1000×*g* for 10 min at 4°C). Soluble and particulate fractions were prepared from the resulting infranatant by high speed centrifugation (100 000×*g* for 40 min at 4°C). The resulting pellet was resuspended in 200 μl of homogenisation buffer. In defatted homogenates and in both the soluble and the particulate fractions, the proteins were measured according to the method of Bradford [16] and PDE activity was determined.

2.3. Preparation of BAT cells

10 Tissues were minced and digested at 37°C with the combination of collagenase and DNAse I as described by Omatsu-Kanbe et al. [14]. The floating fractions containing brown adipose cells were washed three times in Krebs Ringer bicarbonate HEPES buffer (120 mM NaCl, 4 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM $NaHCO_3$, 30 mM HEPES, pH 7.4) containing 2.5 mM glucose and 200 nM adenosine and then homogenised and processed as described above. Cells were kept at 37°C until homogenisation.

2.4. cAMP-PDE assay

cAMP-PDE activity was assayed according to Degerman et al. [17]. Radiolabelled [^{3}H]cAMP substrate was purified by thin layer chromatography on cellulose plates and by anion-exchange chromatography on DEAE-Sephadex. Samples were incubated at 30°C for 10 min, in a total volume of 0.3 ml containing 50 mM HEPES (pH 7.4), 0.1 mM EGTA, 8.3 mM $MgCl_2$ and 0.1 μM [^{3}H]cAMP (20 000 dpm). The reaction was stopped by addition of 0.1 ml HCl (1 N) containing 12 μM cAMP and 6 μM 5′-AMP. After neutralisation by 0.1 ml Tris (0.25 M, pH 8.0) and incubation of the reaction mixture at 30°C for 20 min with 2 μg snake venom nucleotidase (Crotalux atrox, Sigma, St. Louis, MO, USA) in 0.1 ml Tris (0.1 M, pH 8.0), [^{3}H]adenosine was separated from intact [^{3}H]cAMP by chromatography on DEAE-Sephadex and collected into vials for liquid scintillation counting. Hydrolysis of substrate did not exceed 20%. Under these conditions, cAMP-PDE activity was called 'basal activity' and was proportional to the enzyme concentration. All assays were done in duplicate. PDE activities are expressed as units (U), where 1 U catalyses the hydrolysis of 1 μmol of cyclic nucleotide in 1 min at 30°C.

Substrate-specificities and inhibitors were used for distinguishing PDE activities [11]. Inhibitors were routinely dissolved as stock solutions in dimethyl sulfoxide (DMSO) and subsequently diluted with a maximum concentration of 0.3% being employed in the assay. Such concentrations of DMSO did not affect the various PDE activities.

PDE4 activity was estimated by the difference between the activity measured in basal conditions and the activity measured in the presence of 5 μM 4-(3-cyclopentyloxyphenyl)-2-pyrrolidone (a highly specific PDE4 inhibitor).

PDE3 activity was estimated as the fraction of basal PDE activity inhibited by 0.5 μM *N*-cyclohexyl-*N*-methyl-4-(1,2-dihydro-2-oxo-6-quinolyloxy) butiramide (cilostamide).

PDE2 activity could be gauged by measuring the increase in cAMP hydrolysis caused by a low cGMP concentration. Addition of low concentrations of cGMP might also be expected to inhibit PDE3 activity leading to a reduction of cAMP. However, using both the highly selective PDE3 inhibitor, cilostamide [17], and a low concentration of cGMP (1 μM), PDE2 activity could be assessed by the difference between activity measured in the above conditions and the basal activity.

Table 1
PDE activity in BAT of 14 days old Zucker rats

PDE activity (U per whole tissue)				
Genotype	*n*	Homogenate	Particular fraction	Soluble fraction
Fa/fa	4	871 ± 47	227 ± 11	402 ± 40
fa/fa	4	827 ± 39	208 ± 9	339 ± 23

Homogenate, particulate and soluble fraction were prepared as described in Section 2. Data are means ± S.E.M., *n* is the number of animals.

2.5. Western blot analysis

5 μg protein of the soluble fraction was electrophoresed on a 10% SDS-polyacrylamide gel, electroblotted and incubated with a 1/1000 dilution of a biotinylated anti-PDE2 protein antibody from rabbit. The immune complex was incubated with streptavidin-horseradish peroxidase conjugate (RPN.1231, Amersham, France) and detected by an ECL Western blotting system (Amersham, France).

2.6. Northern blot analysis

Total RNA from frozen tissues was extracted by guanidinium thiocyanate according to Chirgwin et al. [18]. RNA was analysed by Northern blotting after electrophoresis on a 1% agarose and 0.66 M formaldehyde gel and was transferred by capillarity to nylon membranes (Nybon N$^+$, Amersham). Hybridisation was performed with a [^{32}P]cDNA probe encoding bovine brain PDE2 [19].

To verify the amount of total RNA in each lane, the blots were also hybridised with a synthetic oligonucleotide (ACGGTATCTGATCGTCTTCGAACC), specific for ribosomal 18S RNA. Quantification was performed by scanning densitometry. Assuming that 18S RNA did not vary with the genotype, values were normalised for the corresponding amount of 18S RNA.

2.7. cGMP assay

cGMP was extracted from tissue homogenates using columns containing anion-exchange silica sorbents (Amprep SAX, Amersam) and the level of cyclic nucleotide was determined by a radioimmunoassay (Amersham TRK 500).

2.8. Statistical analysis

Results are expressed as mean ± S.E.M. The level of significance in the difference between groups was calculated by Student's *t*-test.

3. Results

Activities of total cAMP-PDE in homogenates, cytosolic and particular fractions of BAT are presented in Table 1. Within each fraction, the total PDE activity was not affected by the genotype. Independently of the genotype, 40–45% of the PDE activity was recovered in the cytosolic fraction. PDE3 activity (Table 2) was found to account for half of the total PDE activity in BAT homogenates and was mainly located in the particulate fraction. Taken together, these data show that a large part of the PDE activity was recovered in the cytosolic fraction and could not be attributable to PDE3.

Fig. 1 shows activities of PDE3 in the particular fraction and those of PDE2 and PDE4 in the cytosolic fraction of (A) isolated brown adipose cells and (B) tissue homogenates. In fa/fa compared to Fa/fa rats, the specific activity of PDE2 in brown fat cells was increased by 50% (Fig. 1A).

When measurements were performed in fractions from tissue homogenates (Fig. 1B), PDE(s) activities displayed a similar pattern except for PDE4 which was slightly but significantly increased in fa/fa compared to Fa/fa rats. However, it could not be excluded that a part of the PDE4 activity in BAT homogenates was due to contamination by monocytes, lymphocytes and leukocytes in which several subtypes of

Table 2
Activity of PDE3 in BAT of 14 days old Zucker rats

PDE3 activity (percentage of total activity per whole tissue)				
Genotype	*n*	Homogenate	Particular fraction	Soluble fraction
Fa/fa	4	47 ± 1.1	73 ± 1.8	31 ± 2.8
fa/fa	4	43 ± 1.4	68 ± 1.8	31 ± 1.9

Homogenate, particular and soluble fraction were prepared as described in Section 2. PDE3 activity was estimated as the fraction of basal activity that could be inhibited by cilostamide. Data are means ± S.E.M., *n* is the number of animals.

PDE4 are highly expressed [20]. Thus, the very small difference between fa/fa and Fa/fa pups could result from blood contamination.

Immunoblotting of PDE2 in BAT is presented in Fig. 2. In BAT from Zucker rats, the antiserum detected a signal at 105 kDa close to purified PDE2 from bovine cardiac tissue, bovine adrenal tissue and rabbit brain [11] but also one signal at 71 kDa which corresponds probably to a proteolytic product of the larger form as it has been observed in rat liver [21]. Both the 105 and the 71 kDa signals were multiplied by two in fa/fa compared to Fa/fa rats. These variations of PDE2 concentrations in BAT paralleled those observed for enzyme activities (Fig. 1).

Northern blot analysis of PDE2 (Fig. 3) revealed that the concentration of mRNA was significantly increased in BAT of fa/fa compared to Fa/fa pups (Fig. 3).

cGMP concentrations were measured in BAT of Zucker rats. There was a significant 25% decrease in the level of cGMP in BAT of pre-obese compared to lean rats (22.1 ± 1.06 versus 29.3 ± 1.42, $P < 0.01$ for 12 fa/fa versus 13 Fa/fa).

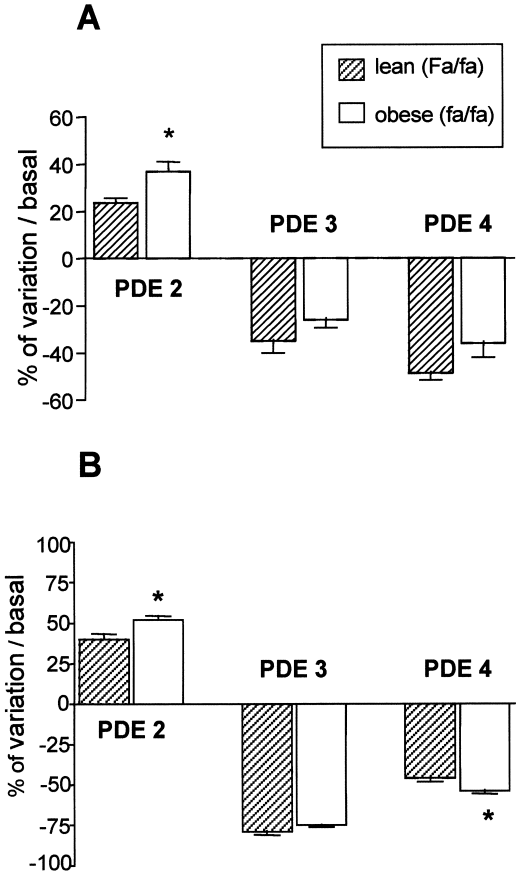

Fig. 1. Activities of PDE(s) in (A) isolated brown adipose cells and in (B) BAT of 14 days old fa/fa and Fa/fa Zucker rats. Activities were estimated in the absence (basal PDE activity) or in the presence of selective inhibitors or activators (for details, see Section 2). Data are means ± S.E.M. of four experiments, they are expressed as percentages of the level detected in basal conditions (A) per mg protein for isolated cells and (B) per whole tissue. *, $P < 0.05$, Fa/fa versus fa/fa rats.

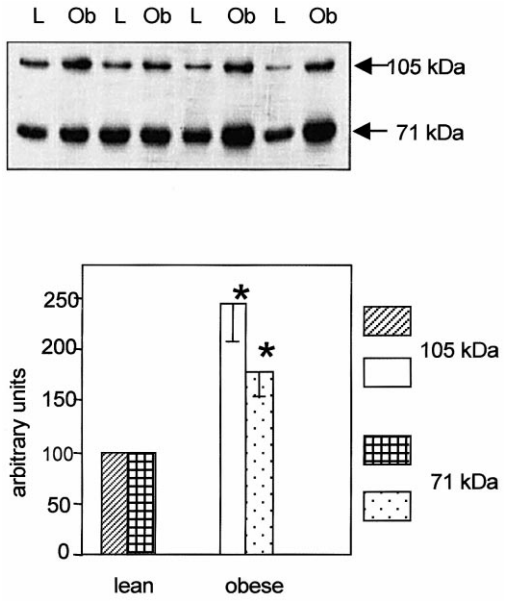

Fig. 2. Quantitative analysis of the PDE2 protein isoform in BAT of 14 days old Zucker rats. A representative Western blot is shown in the upper panel. The intensity of the bands was measured by scanning densitometry. Data are means ± S.E.M., they are expressed as percentages of the level detected in lean Fa/fa rats. *, $P < 0.05$, 10 Fa/fa versus 10 fa/fa rats.

4. Discussion

4.1. Isoforms of PDE in BAT

The expression of PDE(s) has been poorly investigated in BAT. However, the presence of PDE3 mRNA in this tissue was first demonstrated by in situ hybridisation [22] and the regulation of this isoform is thought to be similar to that in white adipose cells [14]. The data provided in this study confirm that PDE3 is present in BAT but they also show that activity of this isoform represents only half of the total PDE activity, thus suggesting that other PDE(s) play a role in the degradation of cAMP. As it has been shown in white adipose tissue [17], PDE3 in BAT is mainly located in the particulate fraction of homogenate. Our results also show that at least three forms of PDE were active in brown adipocytes of rat. The fact that the slight increase in PDE4 activity found in tissue of fa/fa rats could not be evidenced in isolated brown fat cells suggests that a little part of PDE4 activity in tissue

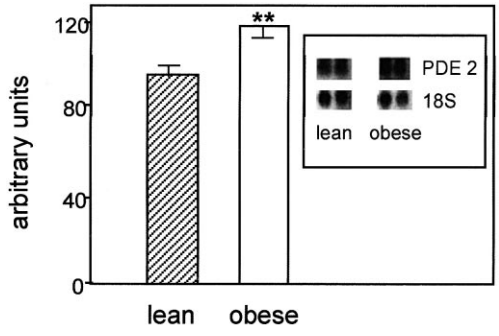

Fig. 3. PDE2 mRNA levels in BAT of 14 days old Zucker Fa/fa and fa/fa rats. Data are means ± S.E.M. of analysis from 13 Fa/fa and 15 fa/fa. PDE2 mRNA levels were normalised to the 18S RNA content and are expressed as percentages of the level detected in lean Fa/fa rats. **, $P < 0.01$, fa/fa versus Fa/fa.

homogenates resulted from contamination by other cell types. However, due to the great difference in both the size and lipid content of brown adipose cells between obese and lean rats, floating adipocytes after collagenase treatment of the tissue did not reflect whole tissue cellularity. Thus, the expression of PDE4 could not be accurately investigated in BAT of Zucker pups and we focused our attention on that of PDE2 whose activity measured either in isolated cells or in tissue was significantly increased in fa/fa compared to Fa/fa rats.

4.2. Effect of the fa gene on PDE2 expression

The present study reveals for the first time that PDE2 is overexpressed in BAT of pre-obese fa/fa rats. The increase in PDE2 mRNA content of BAT is proportional to the increase in both the PDE2 protein concentration and the PDE2 activity. Therefore, in the mutant fatty rat, the control of PDE2 gene expression is exerted at a pre-translational level.

Of the major classes of PDEs, the PDE2 is unique in exhibiting positively co-operative kinetics for hydrolysis of both cAMP and cGMP [11]. We have previously shown that the capacity for cAMP production was significantly decreased in BAT of fa/fa pups as compared with their lean Fa/fa littermates [10]. This study provides evidences that in fa/fa rats, a significant increase in the expression of PDE2 was well-correlated with a decrease of the cGMP concentration, but the biological function of PDE2 in BAT is nevertheless not defined. Most of the actions of cGMP are believed to be mediated by protein kinase G whose expression in brown fat has never been investigated. In brown adipocytes differentiated in culture, addition of a cGMP analogue suppresses lipoprotein lipase activity [23] and it could be expected that a fall in the concentration of cGMP would increase the enzyme activity. However, lipoprotein lipase expression is significantly reduced in BAT of fa/fa compared to Fa/fa pups [8], thus strongly suggesting that variations of the cGMP concentration in the physiological range did not affect lipoprotein lipase activity in BAT.

It has been shown that PDE2 could account for a substantial portion of total cAMP hydrolysis in several bovine tissues [24]. In BAT of fa/fa pups, the overexpression of PDE2 could also play a role in regulation of the intracellular cAMP concentration and therefore could contribute to the defective activation of thermogenesis in this tissue [4].

In summary, this study shows that in BAT from rats, activities of both the PDE2 and PDE4 are comparable to that of PDE3. In pre-obese Zucker pups, PDE2 expression is increased and consequently, the cGMP concentration is decreased. These data together with previous results [10] clearly demonstrate that production of cyclic nucleotides is strongly altered in BAT of 14 days old Zucker fa/fa rats. However, the connection between mutation in the gene encoding the leptin receptor and the regulation of PDE2 expression is not clear and further investigations are needed to study the factors involved in this process.

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