Down-regulation of cyclic-nucleotide phosphodiesterase 3B in 3T3-L1 adipocytes induced by tumour necrosis factor α and cAMP

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We have used murine 3T3-L1 cells, which differentiate in culture and acquire morphological and biochemical features of mature adipocytes, as a model for studying the expression of cyclic-nucleotide phosphodiesterase (PDE) 3B activity, protein and mRNA during differentiation and during long-term treatment of the cells with tumour necrosis factor α (TNF- α), a cytokine associated with insulin resistance, and a cAMP analogue, *N*⁶,2'-*O*-dibutyryl cAMP (dbcAMP). PDE3B activity, protein and mRNA could be detected 4 days after the initiation of differentiation of 3T3-L1 preadipocytes. Treatment of 3T3-L1 adipocytes with 10 ng/ml TNF- α for 24 h produced a maximal (50 %) decrease in PDE3B activity, protein and mRNA, which was well correlated with both activation of protein kinase A (PKA) and stimulation of lipolysis, presumably reflecting an increase in intracellular cAMP concentration. To investigate the effect of cAMP on PDE3B we treated 3T3-L1 adipocytes with

dbcAMP. After 4 h with 0.5 mM dbcAMP, PDE3B activity was decreased by 80 %, which was also correlated with a decrease in PDE3B protein and mRNA. This effect was abolished in the presence of *N*-[2-(bromocinnamylamino)ethyl]-5-isoquinoline-sulphonamide (H-89), a specific PKA inhibitor. We conclude that the lipolytic effect of TNF- α involves the down-regulation of PDE3B, which is associated with increased activation of PKA, presumably owing to increased levels of cAMP. In addition, the PKA activation induced by dbcAMP resulted in the down-regulation of PDE3B. These results, which suggest that PDE3B is a novel target for long-term regulation by TNF- α and cAMP, could contribute to the understanding of the mechanisms of insulin resistance.

Key words: diabetes, gene expression, insulin, lipolysis, protein kinase A.

INTRODUCTION

Adipose tissue lipolysis is dependent on the intracellular concentration of cAMP [1], which is determined at the levels of both synthesis and degradation. Hydrolysis of cAMP is accomplished by cyclic-nucleotide phosphodiesterases (PDEs). Nine PDE families have been identified; they differ in their primary structures, affinities for cAMP and cGMP, responses to specific effectors, sensitivities to specific inhibitors and the mechanisms whereby these enzymes are regulated (reviewed in [2]). The PDE3 gene family contains two members, PDE3A and PDE3B. These isoforms are transcribed from different genes and are expressed in a tissue-specific manner. PDE3B (reviewed in [3]) is expressed in insulin-sensitive cells, including hepatocytes, adipocytes and pancreatic β cells, and has been shown to be important in regulating anti-glycogenolysis, anti-lipolysis and insulin secretion [4]. Acute hormonal regulation of PDE3B has been extensively studied in primary rat adipocytes [5]. The insulin-induced phosphorylation (on serine 302) and activation of PDE3B is believed to be the major mechanism whereby insulin decreases the intracellular level of cAMP, leading to a decrease in the activity of protein kinase A (PKA), a net dephosphorylation of hormone-sensitive lipase (HSL) and thus an inhibition of catecholamine-induced lipolysis [6]. PDE3B is also phosphorylated and activated in response to cAMP-increasing agents, which is important in the feedback regulation of cAMP and lipolysis [7]. The insulin-

activated signalling pathway leading to the activation of PDE3B involves phosphoinositide 3-kinase and an insulin-stimulated PDE3B kinase that was recently suggested to be protein kinase B [6].

Very little is known about the long-term regulation of PDE3B. Early studies in adipocytes from diabetic patients demonstrated a decreased adipocyte PDE activity that was normalized after treatment with insulin or oral anti-diabetic agents [8]. In streptozotocin-induced diabetic rats, decreased adipocyte PDE activity was restored to normal levels by administration of the oral hypoglycaemic agent glyburide [9,10]. In addition, down-regulation of PDE3 activity has been observed in spontaneous diabetic BB rats with full-blown insulin deficiency as well as in their pre-diabetic littermates [11]. Moreover, in adipose tissue of JCR:LA-cp rats, a strain that develops obesity, insulin resistance and vasculopathy, and in obese insulin-resistant diabetic KKAY mice, down-regulation of PDE3B activity and mRNA was reported recently [12,13]. Furthermore, it has been shown that in rat adipocytes from hyperthyroid animals, PDE3B activity was decreased, whereas catecholamine-induced lipolysis was increased [14]. The opposite result was obtained in adipocyte preparations from hypothyroid animals [15]. The effect of thyroid hormones on PDE activity could be mimicked in cultured adipocytes; incubation of 3T3-L1 adipocytes with high concentrations of thyroid hormones resulted in a decrease in PDE3B activity and an increase in isoprenaline (isoproterenol)-

Abbreviations used: AR, adrenergic receptor; CRE, cAMP-response element; dbcAMP, *N*⁶,2'-*O*-dibutyryl cAMP; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; NEFA, non-esterified fatty acid; H-89, *N*-[2-(Bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide; HSL, hormone-sensitive lipase; PDE, cyclic-nucleotide phosphodiesterase; PKA, protein kinase A; PPAR- γ , peroxisome-proliferator-activated receptor γ ; TNF- α , tumour necrosis factor α .

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induced activation of adenylate cyclase, accumulation of cAMP and lipolysis [16]. The opposite effects were found if the cells were exposed to low doses of thyroid hormones.

Although the precise mechanisms are not known, substantial evidence now indicates that tumour necrosis factor α (TNF- α) is important in the development of insulin resistance that is associated with obesity [17]. Overexpression of TNF- α mRNA and protein in adipose tissue has been observed in animal models for obesity [18] as well as in adipose tissue and muscle of obese humans [19,20]. This excess expression of TNF- α is believed to enhance the rate of adipose tissue lipolysis, hence increasing the concentration of circulating non-esterified fatty acids (NEFAs). Chronically raised levels of circulating NEFAs have been shown to elicit peripheral insulin resistance [21]. This phenomenon is generally accepted to be associated with stimulated hepatic gluconeogenesis and decreased insulin-stimulated glucose uptake in skeletal muscle. Furthermore, increased levels of NEFAs have also been demonstrated to result in impaired insulin secretion in β cells [22], thus aggravating the diabetic condition.

Catecholamines and other cAMP-increasing hormones have, besides their insulin-antagonistic effects, been shown to enhance insulin resistance due to stress caused by infections, trauma, surgery and smoking. Although increased levels of circulating NEFAs arising from catecholamine-induced lipolysis might influence insulin sensitivity as discussed above, the mechanism for catecholamine-elicited insulin resistance has not been fully elucidated. Taking into account that PDE3B in adipocytes has a central role in regulating lipolysis by controlling the intracellular levels of cAMP and has been implicated in the pathophysiology of diabetes [9,10,12], in the present study we examined the regulation of PDE3B gene expression in response to TNF- α and cAMP. We addressed this question with murine 3T3-L1 adipocytes, a cell line that expresses PDE3B only in the mature adipocyte [23]. Here we show that the emergence of PDE3B activity, protein and mRNA occurs 4 days after the initiation of differentiation of 3T3-L1 cells. We also suggest a novel mechanism for the TNF- α -induced lipolysis: by down-regulating PDE3B, cAMP concentrations are increased, leading to the activation of PKA, which results in phosphorylation and activation of HSL. In addition, the PKA activation induced by *N*⁶,2'-*O*-dibutyryl cAMP (dbcAMP) also resulted in the down-regulation of PDE3B.

MATERIALS AND METHODS

Materials

3T3-L1 cells [24] were from American Type Culture Collection (Manassas, VA, U.S.A.). Cell-culture media and foetal calf serum (FCS) were from Gibco BRL. dbcAMP was from Sigma (St. Louis, MO, U.S.A.). TNF- α was from Alexis Corporation (San Diego, CA, U.S.A.). *N*-[2-(Bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide (H-89) was from BioMol (Plymouth Meeting, PA, U.S.A.). OPC3911 was from Otsuka Pharmaceutical Corp. (Tokyo, Japan). Polyclonal affinity-purified HSL antibodies were raised against full-length recombinant rat HSL [25]; polyclonal antibodies against rat peroxisome-proliferator-activated receptor γ (PPAR- γ) were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Cell culture

3T3-L1 preadipocytes were distributed (30 000–40 000 per 60 mm diam. dish) in 5 ml of Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) FCS and grown at 37 °C under a humidified air/CO₂ (19:1) atmosphere. After the cells had

reached confluence, fresh DMEM containing 0.5 mM isobutylmethylxanthine, 1 μ M dexamethasone and 10 μ g/ml insulin was added. After incubation for 72 h, the medium was changed to DMEM supplemented with 10% (v/v) FCS, which was changed every 48 h. Treatment of mature 3T3-L1 adipocytes was performed 7–9 days after the initiation of differentiation. Cells were shifted to serum-free medium, pre-labelled with [³²P]P_i and treated as indicated in the Figure legends. Cells were analysed for PDE3B phosphorylation/activity, protein and mRNA or HSL and PPAR- γ protein as described below.

Analysis of PDE3B: phosphorylation, Western blot and activity measurements

After the initiation of differentiation or labelling or after treatment with effectors, the medium from one dish was aspirated. The cells were put on ice and washed twice with ice-cold PBS and scraped into 500 μ l of homogenization buffer containing 25 mM Tris/HCl, pH 7.5, 250 mM sucrose, 5 mM MgCl₂, 0.2 mM EGTA, 20 mM β -glycerophosphate, 20 mM phenylphosphate, 1 μ g/ml of pepstatin A and 10 μ g/ml of antipain and leupeptin. Cells were homogenized (ten strokes at 4 °C) and centrifuged (33 000 *g*, 45 min, 4 °C). The floating fat and the supernatant were removed carefully and the pellet was either homogenized in solubilization buffer [5] for immunoprecipitation or homogenized in homogenization buffer and assayed for PDE activity as described below. Anti-PDE3B antibodies [26] were added to the solubilized membranes and after incubation (16 h, 4 °C) PDE3B was immunisolated by the addition of Protein A–Sepharose [50% (v/v) slurry]. After incubation for 30 min at 4 °C, the immunoprecipitates were washed three times with PBS supplemented with 0.1% *N*-lauroylsarcosine, then mixed with Laemmli sample buffer [27] and subjected to SDS/PAGE. Dried gels were analysed for ³²P-PDE3B by using a Fujix Bas 2000. For the analysis of PDE3B protein, SDS/PAGE was followed by electroblotting to PVDF membrane (Millipore) at 30 V for 16 h in 5% (v/v) methanol/96 mM glycine/12.5 mM Tris/HCl. Non-specific binding was blocked with Tris-buffered saline containing 0.5% gelatin. Western blot analysis was performed with the SuperSignal chemiluminescence system (Pierce, Rockford, IL, U.S.A.) in accordance with the manufacturer's instructions. PDE3B protein was detected with a 1:1000 dilution of a polyclonal antiserum against PDE3B [26].

PDE3B activity was assayed in the presence or absence of 3 μ M OPC 3911, a selective PDE3 inhibitor, in a total volume of 300 μ l for 8 min at 30 °C, as described previously [28].

Western blot analysis of HSL and PPAR- γ

For the analysis of HSL and PPAR- γ proteins, 3T3-L1 cells were harvested and homogenized in homogenization buffer (see above). Aliquots of 40 μ g of protein were subjected to SDS/PAGE, followed by electroblotting to nitrocellulose membranes (Hybond C+; Amersham) at 30 V for 16 h in 20% (v/v) methanol/192 mM glycine/25 mM Tris/HCl. Non-specific binding was blocked with Tris-buffered saline containing 5% (w/v) non-fat dried milk. The blots were probed with either a 1:5000 dilution of HSL antibodies or a 1:2500 dilution of PPAR- γ antibodies. Detection of proteins was performed as described above.

RNA extraction and analysis

Total RNA was extracted from 3T3-L1 cells cultured in 100 mm diam. dishes according to the method by Chomczynski and Sacchi [29] or by using RNeasy B (Nordic Biosite AB, Täby,

Sweden) in accordance with the manufacturer's instructions. RNA quality and concentration were assessed by UV-absorption spectrophotometry. Approx. 10 μ g of total RNA was subjected to electrophoresis in 1% (w/v) agarose/formaldehyde gels. RNA was then transferred to nylon membranes (Amersham) and fixed by using a UV cross-linker (Stratagene). Membranes were hybridized for 1 h in ExpressHyb solution (Clontech) containing 2×10^6 c.p.m./ml cDNA labelled with [α - 32 P]dCTP (Amersham) by random priming with the Oligolabelling Kit (Amersham Pharmacia Biotech) in accordance with the manufacturer's instructions. Membranes were washed twice for 20 min at room temperature in $2 \times$ SSC [SSC is 0.3 M NaCl/0.03 M sodium citrate (pH 7.0)]/0.1% SDS and then twice for 20 min at 60 °C with $0.1 \times$ SSC/0.1% SDS. The specific cDNA probe was a 630 bp cDNA fragment from the regulatory domain of rat PDE3B. An oligonucleotide specific for the 18 S ribosomal RNA was end-labelled with 32 P and used as a control [30].

Lipolysis measurements

Differentiated 3T3-L1 adipocytes cultured in 60 mm diam. dishes were incubated in the presence of TNF- α for 24 h as indicated. The cells were washed twice with PBS and incubated at 37 °C for an additional 30 min in Krebs-Ringer Hepes buffer containing 100 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4 , 1 mM MgSO_4 , 2.5 mM CaCl_2 and 25 mM Hepes, supplemented with 1% (w/v) BSA, 2 mM glucose and 200 nM adenosine. Glycerol released into the medium was determined by the method of Dole and Meinertz [31]. Protein content was measured with the Bio-Rad protein assay (Bio-Rad, Hercules, CA, U.S.A.) in accordance with the manufacturer's instructions.

PKA assay

3T3-L1 adipocytes, incubated for 24 h in the presence of TNF- α , as indicated, were harvested, homogenized and centrifuged (12000 g, 4 °C, 10 min). A 10 μ l portion of the cytosol fraction (after the floating fat had been discarded) was removed and incubated for 20 min at 30 °C with 5 μ l of a phosphorylation mixture containing 20 mM Tris, pH 7.4, 250 mM sucrose, 50 mM MgSO_4 , 5 mM dithioerythritol, 200 μ M ATP, 10 μ g of Kemptide and [γ - 32 P]ATP (23 μ Ci) in the presence or absence of protein kinase inhibitor (25 μ M) or 16 μ M cAMP. The reactions were stopped by the addition of 10 μ l of 1% (w/v) BSA/1 mM ATP (pH 3.0) and 10 μ l of 12.5% (w/v) trichloroacetic acid, after which the samples were put on ice for 20 min and centrifuged. Aliquots (15 μ l) of the supernatants were removed and applied to phosphocellulose paper (P81 membrane). After three washes in 75 mM H_3PO_4 and one wash in acetone, the amount of 32 P incorporated was determined by scintillation counting. Corrections for non-PKA activity were made as described previously [32].

RESULTS

Expression of PDE3B during differentiation of 3T3-L1 cells

As shown in Figures 1(A) and 1(B), PDE3B activity and protein were detected 4 days after the initiation of differentiation of 3T3-L1 cells, which was well correlated with the expression of PDE3B mRNA as detected by Northern blot analysis (Figure 1C). At this time the cells started to exhibit adipocyte morphology, i.e. spherical cells with a few fat droplets (results not shown). The

Figure 1 Emergence of PDE3B activity, protein and mRNA during differentiation of 3T3-L1 cells

Crude membrane fractions and total cell homogenates from 3T3-L1 cells, harvested at the indicated times after the induction of differentiation (day 0), were prepared. (A) Membrane protein (45 μ g) was assayed for PDE3B activity. Data are expressed as percentages of maximal PDE3B activity (50 pmol/min per mg of protein at 7 days) and are means $\pm$ S.E.M. for three individual experiments. (B) Membrane protein (150 μ g) was subjected to Western blot analysis to detect PDE3B protein. (E) Total cell homogenate (40 μ g) was used for Western blot analysis of HSL protein. Total RNA from one 100 mm diam. dish of 3T3-L1 cells differentiated at the time indicated was extracted and 10 μ g of total RNA was subjected to Northern blot analysis. (C, D) PDE3B mRNA (C) and 18 S rRNA (D) were detected by sequential hybridization of the same blot with the corresponding probes. Results shown in (B–E) are each representative of three individual experiments.

expression level of PDE3B activity, protein and mRNA remained stable with time once the cells were terminally differentiated. HSL, the rate-limiting enzyme in triacylglycerol hydrolysis, has previously been reported as a late marker of differentiation [33]. As shown in Figure 1(E), the expression of HSL protein was detected shortly before the expression of PDE3B, suggesting that PDE3B belongs to the group of late markers in the differentiation process of 3T3-L1 cells.

To test the ability of PDE3B to respond acutely to hormones, fully differentiated 3T3-L1 adipocytes were prelabelled with [32 P]P $_i$ and subsequently stimulated with isoprenaline or insulin. The results from these experiments indicated increased phosphorylation and activation of PDE3B in comparison with the control (Figure 2), which are consistent with previous findings in primary rat adipocytes and 3T3-L1 adipocytes [5,34]. For all experiments discussed here, fully differentiated 3T3-L1 adipocytes were used to study the effects of TNF- α and dbcAMP on the expression of PDE3B.

Figure 2 Acute phosphorylation and activation of PDE3B by insulin and isoprenaline in 3T3-L1 adipocytes

Terminally differentiated 3T3-L1 adipocytes (one 60 mm diam. dish per condition) were serum-starved for 14 h and thereafter prelabelled for 2 h with [32 P]P_i (0.5 mCi/ml) before stimulation for 10 min with insulin (100 nM) or for 5 min with isoprenaline (300 nM). Membrane fractions were prepared. (A) Solubilized membrane protein (150 μ g) was immunoprecipitated and subjected to SDS/PAGE, followed by the detection of 32 P-PDE3B by using a Fujix Bas 2000. The results are representative of two individual experiments. (B) Membrane protein (45 μ g) was assayed for PDE3B activity. Results are expressed as percentages of control (50 pmol/min per mg of protein) and are means $\pm$ S.E.M. for three individual experiments.

TNF- α mediates the down-regulation of PDE3B expression associated with induction of PKA activity

Figure 3(A) shows that treatment of 3T3-L1 adipocytes with TNF- α induced lipolysis, in agreement with previous studies [35]. The rate of lipolysis in adipocytes is critically dependent on the intracellular concentration of cAMP, which in turn is regulated by PDE3B. To examine the possibility that the TNF- α -mediated increase in lipolysis could involve the down-regulation of PDE3B, we incubated 3T3-L1 adipocytes in the presence or absence of TNF- α . As shown in Figure 3(B), the incubation of 3T3-L1 adipocytes with different concentrations of TNF- α for 24 h resulted in a down-regulation of PDE3B activity, which was maximal (50% decrease) at 10 ng/ml (0.6 nM) TNF- α and was correlated with a decrease in PDE3B protein and mRNA levels (Figures 3C and 3D). Increasing the concentrations of TNF- α to 100 ng/ml (6 nM) did not elicit any additional decrease in the PDE3B activity. High concentrations of TNF- α have been reported to promote the delipidation and dedifferentiation of 3T3-L1 cells. Some papers have also described such an effect of TNF- α even at low doses (0.2–3 nM); it has been proposed that this dedifferentiation action implies a primary effect on PPAR- γ expression [36,37]. To verify whether long-term treatment with TNF- α could induce the dedifferentiation of 3T3-L1 adipocytes in our system, the effect of TNF- α on PDE3B expression was compared with that of PPAR- γ . Under the same conditions as those in which TNF- α produced a decrease in PDE3B expression, no down-regulation of the expression of PPAR- γ was observed (Figures 3C and 3F). These results suggest that the down-regulation of PDE3B expression by TNF- α is a specific event and does not reflect a general down-regulation of adipocyte-related proteins. As demonstrated in Figure 4, treatment with TNF- α induced a doubling of the PKA activity, most

Figure 3 Down-regulation of PDE3B activity, protein and mRNA in response to TNF- α is correlated with increased lipolysis

Fully differentiated 3T3-L1 adipocytes were incubated for 24 h in the presence of TNF- α at the indicated concentrations before the preparation of total cell homogenate, membranes or RNA. (A) 3T3-L1 adipocytes were washed twice with PBS, incubated in Krebs–Ringer Hepes buffer (30 min, 37 $^{\circ}$ C) as described in the Materials and methods section before measurement of the glycerol released into the incubation medium. The results are means $\pm$ S.E.M. for three or four experiments. (B) Membrane fractions (45 μ g) were assayed for PDE3B activity. Results are expressed as percentages of control (50 pmol/min per mg of protein) and are means $\pm$ S.E.M. for three individual experiments. (C, F) Membrane protein (60 μ g) (C) or total cell homogenate (40 μ g) (F) was subjected to Western blot analysis to detect PDE3B protein or PPAR- γ protein respectively. Total RNA from treated 3T3-L1 cells (one 100 mm diam. dish per condition) was extracted and 10 μ g of total RNA was subjected to blot transfer analysis. (D, E) PDE3B mRNA (D) and 18 S rRNA (E) were detected by sequential hybridization of the same blot with the corresponding probes. Results in (C–F) are representative of three individual experiments.

Figure 4 Increased activity of PKA in response to TNF- α

Cytosol fractions were prepared from fully differentiated 3T3-L1 adipocytes (one 60 mm diam. dish per condition) treated for 24 h with TNF- α at the indicated concentrations. Aliquots were assayed for PKA activity with Kemptide as substrate. The results are means $\pm$ S.E.M. for three individual experiments.

probably due to an increase in intracellular cAMP levels mediated, at least in part, by a decrease in the expression of PDE3B.

Treatment with dbcAMP results in the PKA-mediated down-regulation of PDE3B expression

The above findings indicate that the lipolytic effect of TNF- α involves the activation of PKA, presumably reflecting increased levels of cAMP, mediated via the down-regulation of PDE3B expression. In contrast, long-term treatment of cells with cAMP has previously been shown to induce the up-regulation of PDE3A and PDE3B in aortic smooth-muscle cells [38,39] and of PDE3B in T-cells [40].

To investigate the effect of chronically increased levels of cAMP on the expression of PDE3B, we treated 3T3-L1 adipocytes with dbcAMP. As shown in Figure 5(A), PDE3B activity was rapidly attenuated on treatment with dbcAMP, with maximal effects after 4–5 h. At this time point 80 % of PDE3B activity was down-regulated, which was well correlated with a 75 % decrease in PDE3B protein and mRNA (Figures 5B and 5C). Incubation of 3T3-L1 adipocytes with H-89, a selective and potent PKA inhibitor, completely abolished the cAMP-induced down-regulation of PDE3B (Figure 5E), suggesting that the cAMP-mediated reduction in PDE3B expression is mediated via activation of PKA.

In summary, our results show that the emergence of PDE3B activity, protein and mRNA occurs late during the differentiation of 3T3-L1 cells. Moreover, we demonstrate that treatment of 3T3-L1 adipocytes with TNF- α induces the down-regulation of PDE3B expression, which is associated with increased activation of PKA and hence increased lipolysis. In addition, chronic elevation of cAMP also resulted in the down-regulation of PDE3B expression.

DISCUSSION

Very little is known about the long-term regulation of PDE3B and its role in disease. Interestingly, recent studies have demonstrated down-regulation of PDE3B expression in adipose tissue of the JCR:LA-cp rat, a model that develops obesity, insulin resist-

Figure 5 Down-regulation of PDE3B activity, protein and mRNA in response to dbcAMP in 3T3-L1 adipocytes

Terminally differentiated 3T3-L1 adipocytes (one 60 mm diam. dish per condition) were serum-starved for 16 h, during which treatment with dbcAMP (0.5 mM) for the indicated times was performed before the preparation of membrane fractions and RNA. (A) Membrane protein (45 μ g) was analysed for PDE3B activity. Results are expressed as percentages of PDE3B activity in control (untreated) cells (50 pmol/min per ml) and are means $\pm$ S.E.M. for three individual experiments. (B) Membrane fractions (150 μ g) were subjected to Western blot analysis to detect PDE3B protein. Total RNA was extracted from treated 3T3-L1 cells (one 100 mm diam. dish per condition) and 10 μ g of total RNA was subjected to Northern blot analysis. (C, D) PDE3B mRNA (C) and 18 S rRNA (D) were detected by sequential hybridization of the same blot with the corresponding probes. Results are representative of two individual experiments. (E) Fully differentiated 3T3-L1 adipocytes, serum-starved for 16 h, were pretreated with H-89 (10 μ M, 10 min) before the addition of dbcAMP (0.5 mM). After the indicated times, membrane fractions were prepared and 45 μ g of membrane protein was analysed for PDE3B activity. Filled bars indicate cells treated with dbcAMP; hatched bars indicate cells that have been treated with H-89 and dbcAMP. Results are means for two individual experiments.

ance and arteriosclerosis [12], as well as in obese insulin-resistant diabetic KKAy mice [13]. In the present study we used 3T3-L1 adipocytes as a model system to examine the long-term regulation of PDE3B in response to TNF- α , a cytokine associated with obesity-linked insulin resistance. Characterization of PDE3B in 3T3-L1 cells revealed the simultaneous appearance of PDE3B activity, protein and mRNA 4 days after the onset of differentiation of 3T3-L1 cells (Figures 1A–1C), when the cells are spherical in shape and begin to accumulate lipid droplets (results not shown), indicating that the emergence of PDE3B occurs during the terminal phase of adipocyte differentiation. The activity, protein and mRNA levels of enzymes involved in fatty acid metabolism, including fatty acid synthetase, glycerophosphate-3-dehydrogenase, perilipin and HSL (reviewed in [41]), have been reported to increase 10–100-fold during this phase. In addition, on acute stimulation of mature 3T3-L1 adipocytes with insulin or isoprenaline, PDE3B was phosphorylated and activated (Figure 2), in agreement with previous studies of rat adipocytes and 3T3-L1 adipocytes [5,34]. Thus mature 3T3-L1 adipocytes seem to constitute an appropriate model for investigating the regulation of PDE3B expression in response to TNF- α and dbcAMP.

The observation that TNF- α induced the down-regulation of PDE3B activity, protein and mRNA concomitant with increased lipolysis suggests that PDE3B is a novel target for TNF- α in its lipolytic signalling pathway. In addition, the TNF- α -induced down-regulation of PDE3B and increase in lipolysis was observed without down-regulation of the adipocyte marker, PPAR- γ , indicating that the TNF- α -induced decrease in PDE3B expression does not reflect a general dedifferentiation of the 3T3-L1 adipocytes. We therefore suggest that TNF- α induces lipolysis, at least partly, by decreasing the expression of PDE3B with a subsequent increase in cAMP levels and an activation of PKA, which then phosphorylates and activates HSL, the rate-limiting enzyme in triacylglycerol hydrolysis. However, other signalling mechanisms for the lipolytic effect of TNF- α have been suggested, although the expression of the target enzyme, HSL, does not seem to be affected [42,43]. For example, TNF- α has recently been reported to induce the down-regulation of perilipin A and B, proteins associated with lipid droplets. The precise role for perilipins in the regulation of lipolysis is not clear but it has been suggested that the accumulation of perilipins at the surface of the fat droplet limits lipid hydrolysis [44,45]. As a result, the TNF- α -mediated decrease in perilipins at the surface of the lipid droplet is proposed to increase lipolysis. Others have suggested that the lipolytic effect of TNF- α in primary cultures of adipocytes involves the down-regulation of G $_i$ proteins [43]. The basal rate of lipolysis in isolated adipocytes is normally kept at a minimum owing to endogenous adenosine, which is released spontaneously from these cells. Adenosine, by binding to A $_1$ adenosine receptors, activates G $_i$, which by inhibiting adenylate cyclase impairs the production of cAMP and hence decreases the rate of lipolysis. Moreover, the differential regulation of β -adrenergic receptor (AR) expression in 3T3-F442A adipocytes has been reported in response to TNF- α [46]. In that study the expression of β_1 -AR and β_3 -AR were found to be decreased, whereas up-regulation of β_2 -AR was observed, suggesting that the induction of β_2 -AR by TNF- α might potentiate the lipolytic effect of the cytokine. Thus the down-regulation of perilipins, G $_i$ proteins and PDE3B and the induction of β_2 -AR by different mechanisms could co-operate to mediate TNF- α -induced lipolysis. The mechanisms whereby catecholamines and other hormones acting through the cAMP system induce insulin resistance are not clear but could involve the stimulation of lipolysis in adipose tissue, resulting in increased levels of circulating NEFAs. We found that chronic

exposure of 3T3-L1 adipocytes to dbcAMP resulted in the down-regulation of PDE3B. Several proteins involved in the regulation of lipolysis have been shown to be affected in the long term by cAMP, including the desensitization of ARs [47] and the down-regulation of HSL expression [48]. Moreover, it has been reported that increased levels of cAMP result in the up-regulation of PKA in rat Sertoli cells [49], which is consistent with our findings (Figure 5E). In addition, recent studies have shown that PDE3A and PDE3B in rat aortic smooth-muscle cells and PDE3B in T-cells are up-regulated in response to cAMP-increasing agents [38–40]. It has also been reported that chronic exposure of T-cells to cAMP and G $_s$ -coupled agonists results in the up-regulation of other PDEs including PDE4A, PDE4B and PDE4D. Such an up-regulation has been suggested to be part of the mechanisms whereby cAMP is subject to long-term feedback regulation [40]. These findings, which indicate that several isoforms of PDE3 and PDE4 are up-regulated in response to cAMP in various cell types, are in contrast with our results that an increase in cAMP levels brings about the attenuated expression of PDE3B in 3T3-L1 adipocytes. Thus the specific role for the cAMP-mediated down-regulation of PDE3B in lipolysis is difficult to predict owing to the complexity of cAMP-mediated effects on gene expression. The mechanisms involved in the transcriptional regulation of the PDE3B gene by TNF- α and cAMP have not yet been established, because the murine PDE3B gene has been only partly elucidated [3]. One can speculate that cAMP-mediated regulation involves the phosphorylation of a cAMP-response element (CRE)-binding protein by PKA, which upon binding to a CRE within the promoter region would block the transcription. Such a possible CRE has been identified within the promoter region of the mouse PDE3B gene (J.-R. Tang and V. Manganiello, unpublished work), and the effects of cAMP on the PDE3B promoter activity are currently under investigation.

In summary, here we report that the emergence of PDE3B occurs late in the differentiation process of 3T3-L1 cells. Furthermore, we suggest a novel mechanism for the TNF- α -mediated induction of lipolysis, which includes down-regulation of the expression of PDE3B, resulting in increased cAMP levels, the induction of PKA activity and the phosphorylation and activation of HSL. We also propose a role for PDE3B in the development of catecholamine-elicited insulin resistance because exposure of 3T3-L1 adipocytes to dbcAMP resulted in down-regulation of PDE3B expression.

We thank Professor Per Belfrage and Dr. Dominique Langin for helpful discussions. This work was supported by Swedish Medical Research Council Projects 3362 for Per Belfrage and 12537 for E. D.; the Swedish Society of Medicine; the Swedish Diabetes Association; Pålssons Foundation, Malmö, Sweden; the Royal Physiographic Society, Lund, Sweden; Centre of Excellence Grant from the Juvenile Diabetes Foundation, U.S.A. and Knut and Alice Wallenberg Foundation; and Novo Nordisk, Copenhagen, Denmark.

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Received 1 June 1999/18 October 1999; accepted 6 December 1999