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# Chronic TNFα and cAMP pre-treatment of human adipocytes alter HSL, ATGL and perilipin to regulate basal and stimulated lipolysis

Véronic Bézaire [a,b], Aline Mairal [a,b], Rodica Anesia [a,b], Corinne Lefort [a,b], Dominique Langin [a,b,c,*]

[a] Laboratoire de Recherches sur les Obésités, Inserm U858, Toulouse, France
[b] Université de Toulouse, UPS, Institut de Médecine Moléculaire de Rangueil, IFR150, Toulouse, France
[c] CHU de Toulouse, Laboratoire de Biochimie, Institut Fédératif de Biologie de Purpan, Toulouse, France

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ABSTRACT

**We examined the effects of chronic TNFα and dibutyryl-cAMP (Db-cAMP) pre-treatment on the lipolytic machinery of human hMADS adipocytes. TNFα decreased adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) protein content and triglycerides (TG)-hydrolase activity but increased basal lipolysis due to a marked reduction in perilipin (PLIN) protein content. Conversely, Db-cAMP increased ATGL and HSL protein content but prevented PLIN phosphorylation, the net result being accentuated basal lipolysis. In forskolin-stimulated conditions, TNFα and Db-cAMP pre-treatment decreased stimulated TG-hydrolase activity and impaired PLIN phosphorylation. Together, this resulted in a severely attenuated response to forskolin-stimulated lipolysis.**


## 1. Introduction

The regulation of human adipose tissue lipolysis is a complex multi-factorial process. Alterations of lipolysis and lipase expression have been shown in obesity and insulin resistance [1–3]. Lipolysis is governed by adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL). HSL displays in vitro affinity for both triglyceride (TG) and diglyceride (DG) molecules [4,5] while the recently discovered ATGL exerts affinity for TG only [6]. In fact, a body of evidence has emerged suggesting that despite their common capacity to hydrolyze TG, ATGL and HSL act sequentially [7–9]. To fully exert its action on lipid breakdown, ATGL requires the coactivator CGI-58, which in itself is devoid of TG-hydrolase activity [10]. Lipases access to stored lipids is dependent upon perilipin (PLIN), a member of the Perilipin, Adipophilin, TIP-47 (PAT) protein family which decorates lipid droplets (LD) of the adipocyte [11].

Acute regulation of the lipolytic machinery in fat cells occurs at the post-translational level. In human adipocytes, catecholamines and natriuretic peptides induce the activation of protein kinase A (PKA) and G, respectively [12]. The protein kinases phosphorylate HSL and PLIN [13,14] leading to CGI-58 release [15], HSL translocation [16,17] and LD fragmentation [18]. The prominent role of ATGL in PKA-stimulated lipolysis has recently been shown in murine [9,19] and human adipocytes [7] and specifically attributed to the phosphorylation of PLIN on serine 517 [20].

Determinants of long term regulation of lipolysis and lipase action remain largely unknown. Sustained activation of the sympathetic nervous system may lead to desensitization of catecholamine-stimulated lipolysis [21,22]. Moreover, TNFα has been shown to modulate human fat cell lipolysis [23,24]. Herein, we used a unique human white adipocyte cell model, termed hMADS adipocytes [7] to examine adaptations of the lipolytic machinery to prolonged TNFα exposure and sustained PKA activation with dibutyryl-cyclic AMP (Db-cAMP) [25]. The specific HSL inhibitor 4-isopropyl-3-methyl-2-[1-(3-(*S*)-methyl-piperidin-1-yl)-methanoyl]-2*H*-isoxazol-5-one

*Abbreviations:* ACS, acyl-CoA synthase; ATGL, adipose triglyceride lipase; Bay, 4-isopropyl-3-methyl-2-[1-(3-(*S*)-methyl-piperidin-1-yl)-methanoyl]-2*H*-isoxazol-5-one; DG, diglycerides; Db-cAMP, dibutyryl-cyclic AMP; FK, forskolin; hMADS cells, human adipose tissue derived-multipotent stem cells; HSL, hormone-sensitive lipase; JNK, c-*jun* $NH_2$ terminal kinase; LD, lipid droplet; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; NPRA, natriuretic peptide receptor A; OA, oleic acid; PAT, Perilipin, adipophilin, TIP-47; PDE3B, phosphodiesterase 3B; PKA, protein kinase A; PLIN, perilipin; TG, triglycerides

* Corresponding author. Address: Laboratoire de Recherches sur les Obésités, Inserm U858 – I2MR – Equipe 4, 1 Avenue Jean Poulhès, BP 84225, 31432 Toulouse Cedex 4, France. Fax: +33 561325623.
*E-mail address:* Dominique.Langin@inserm.fr (D. Langin).

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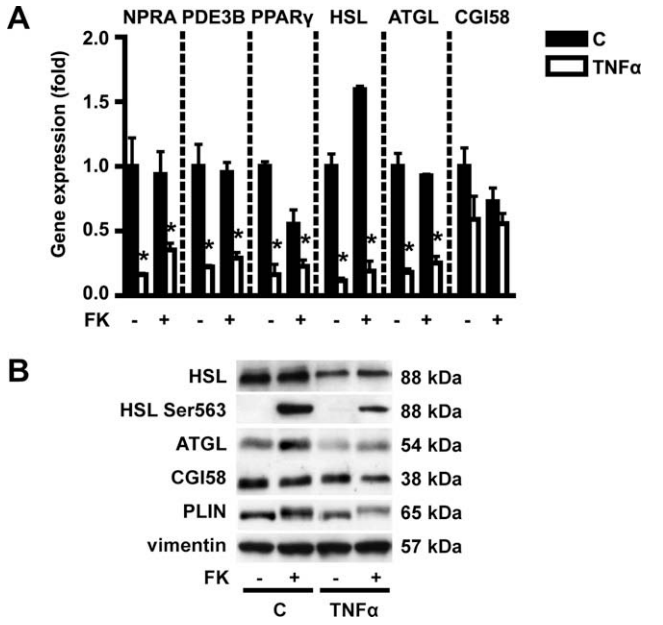


**Fig. 1.** Gene and protein expression of hMADS lipolytic machinery after chronic treatment with TNFα. Cells were pre-treated for 72 h with TNFα (100 ng/mL) from Day 12 to Day 15. On Day 15, TNFα was removed and cells were acutely treated for 3 h with or without FK (1 μM) and harvested for gene expression and Western blot analysis. (A) Gene expression of NPRA, PDE3B, PPARγ, HSL, ATGL and CGI-58 assessed by real-time RT-PCR and normalized with 18S rRNA levels. (B) Western blots of HSL, HSL $Ser^{563}$, ATGL, CGI-58 and PLIN, normalized to vimentin. The data are presented as means ± S.E.M. $N = 3–6$. * Significantly different from control condition (C).

(Bay) was used to discriminate between HSL and ATGL specific actions [2]. Consequences on the lipolytic machinery protein content, TG-hydrolase activity, and lipolysis were examined in basal and forskolin (FK)-stimulated states.

## 2. Materials and methods

### 2.1. Cell culture

Human adipose tissue derived-multipotent stem cells (hMADS cells) were cultured as previously described [7]. Experiments were held on Days 12–15 of differentiation. Pharmacological treatments of cells were both chronic and acute. Chronic pre-treatment with 100 ng/mL TNFα (72 h) or 1 mM Db-cAMP (48 h) was administered and removed prior to acute manipulations. Acute treatment (3 h) with 1 μM FK and/or 10 μM specific HSL inhibitor Bay [2] was added prior to harvesting cells or during functional measurements.

### 2.2. Determination of mRNA levels

Total RNA was extracted using the RNeasy total RNA mini kit (Qiagen) and processed as previously described [7].

### 2.3. Immunoblotting

Western blots and revelation were performed as described [7]. Total hMADS cell homogenates were prepared in extraction buffer (10 mM Tris HCl – pH 7.4, 0.25 M sucrose, 1 mM EDTA, 1 mM DTT) or Laemmli sample buffer. Primary antibodies used were anti-hHSL (1:12 000, gift from Dr. Cecilia Holm, Lund, Sweden), anti-hHSL-

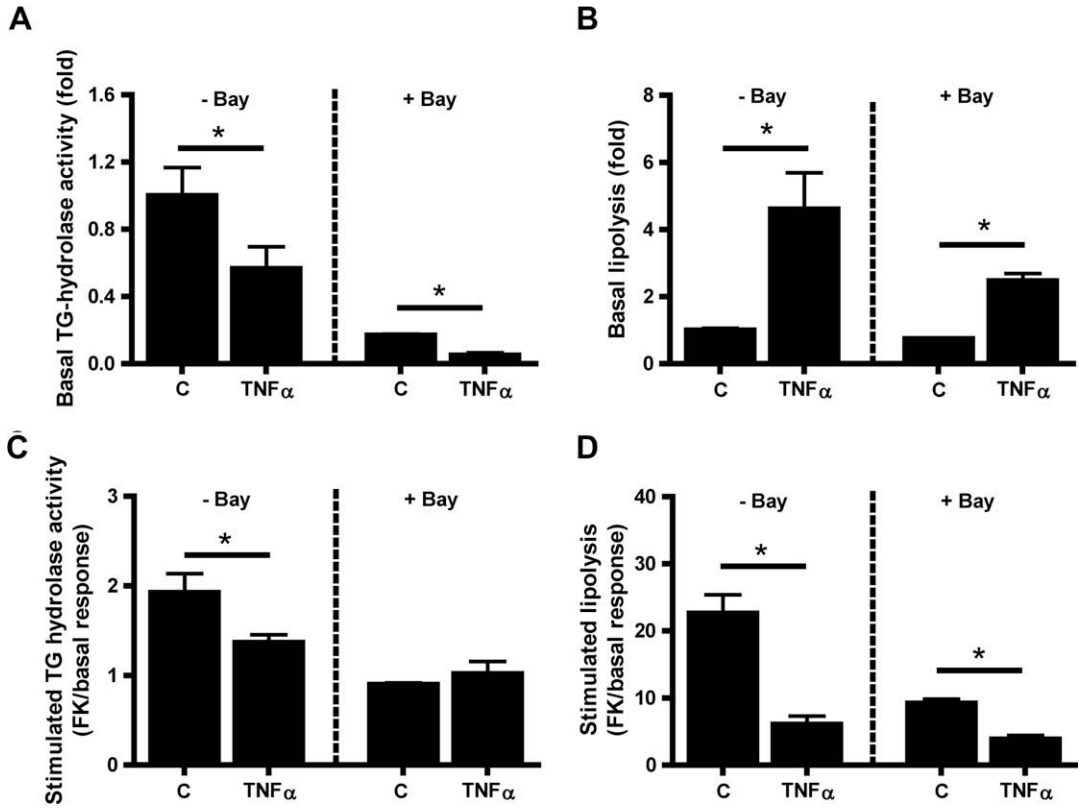


**Fig. 2.** TG-hydrolase activity and lipolysis of hMADS cells after chronic treatment with TNFα. Cells were pre-treated for 72 h with TNFα (100 ng/mL) Day 12 to Day 15. On Day 15, TNFα was removed and cells were acutely treated for 3 h with or without FK (1 μM) and/or Bay (10 μM) for TG-hydrolase activity and lipolysis. (A) TG-hydrolase activity. (B) Basal lipolysis. (C) TG-hydrolase activity in response to FK treatment. (D) Lipolytic response to FK treatment. The data are presented as means ± S.E.M. $N = 3–6$. * Significantly different from control condition (C).

Ser563 (1:1000, Cell Signaling), anti-hATGL (1:1000, Cell Signaling), anti-hCGI58 (1:1000, Tebu-Bio), anti-hPLIN (1:2000, Progen), and anti-vimentin (1:1000, Euromedex).

### 2.4. Lipolytic flux measurement

Whole cell lipolysis was investigated using radiolabeled oleic acid ([3H-9,10]-OA) and acyl-CoA synthase (ACS) inhibitor Triacsin C (Sigma, France) as previously described [7]. In these conditions, release of [3H-9,10]-OA over 3 h represents total adipocyte lipolysis in the absence of re-esterification. FK (1 μM) and/or Bay (10 μM) were added to cells during lipolysis. Following lipolysis, cells were washed and scraped in extraction buffer for protein normalization (Bio-Rad Assay) of lipolysis.

### 2.5. Lipase activity

In vitro enzymatic activities were performed as described [7]. To determine HSL-independent TG lipase activity, the selective HSL inhibitor Bay was added during the activity assay.

### 2.6. Statistical analysis

Statistical significance was determined using non-parametric Mann–Whitney tests. Differences were considered significant at $P < 0.05$.

## 3. Results and discussion

### 3.1. Chronic TNFα pre-treatment reduces protein content of HSL, ATGL, and PLIN

Human hMADS adipocytes were pre-treated for 72 h with TNFα to assess the modulation of lipolysis at transcriptional and translational levels. Gene expression of natriuretic peptide receptor A (NPRA), phosphodiesterase 3B (PDE3B), PPARγ, HSL and ATGL was severely reduced in basal and FK conditions (Fig. 1A). These data are in line with previously published data on adipose tissue gene expression [26–29]. The decrease in gene expression was associated with a concomitant decrease in HSL and ATGL proteins (Fig. 1B). Western blot analyses revealed that chronic TNFα pre-treatment decreased HSL (−42%, $P < 0.01$), ATGL (−58%, $P < 0.001$), and PLIN (−38%, $P < 0.01$) protein content. When cells were challenged by an acute FK treatment, phosphorylation of HSL and PLIN was revealed by detection of phosphorylated HSL Ser$^{563}$ and an upward electrophoretic shift in PLIN (Fig. 1B). Via a yet unknown mechanism, FK also increased ATGL protein content (+27%, $P < 0.05$) in hMADS adipocytes (Fig. 1B). The effect of FK on HSL and PLIN phosphorylation was maintained in TNFα pre-treated cells. TNFα did not influence CGI-58 gene and protein expression.

### 3.2. Downregulation of PLIN and lipase expression induce modulation of basal and stimulated lipolysis

Use of the specific HSL inhibitor Bay revealed a reduction in HSL and ATGL specific TG-hydrolase activity following chronic TNFα pre-treatment (Fig. 2A). However, basal lipolysis attributed specifically to HSL and ATGL increased with TNFα (Fig. 2B). Therefore, in basal conditions, downregulation of PLIN and PDE3B expression by TNFα showed a predominant effect over diminished lipase content and capacity, resulting in enhanced lipolysis. This is probably the result of partly antagonizing signalling pathways activated by TNFα: mitogen-activated protein kinase (MAPKs) (p44/42 and JNK) and NF-κB pathways [24]. Under acute FK condition, TNFα pre-treatment of hMADS adipocytes abrogated the expected increase in intrinsic HSL TG-hydrolase activity (Fig. 2C). Moreover, TNFα pre-treatment significantly attenuated whole cell FK-stimulated lipolysis attributed to HSL and ATGL (Fig. 2D). The impairment in whole cell stimulated lipolysis may result from several mechanisms, i.e., reduced lipase, notably ATGL, expression; diminished PLIN level, and hence total PLIN phosphorylation; and decreased stimulated HSL TG hydrolase activity. We have recently shown the critical role of ATGL in the initiation of stimulated lipolysis in hMADS adipocytes [7]. Moreover, it is likely that reduced phosphorylated PLIN proteins on LD minimize CGI-58 release into the cytosol and hence, ATGL-specific PKA-stimulated lipolysis [15,30,31]. HSL-specific contribution to PKA-stimulated lipolysis in human adipocytes is impaired by TNFα due to firstly, attenuated intrinsic PKA-stimulated TG-hydrolase activity, and secondly, reduced HSL docking on LD, a consequence of limited PLIN content and phosphorylation [32].

### 3.3. Chronic Db-cAMP pre-treatment increases HSL phosphorylation and ATGL content but abrogates PLIN phosphorylation

Next, we aimed at determining the mechanism by which sustained activation of the PKA pathway alters lipolysis in human adipocytes. Treatment of cells for 48 h with Db-cAMP significantly reduced expression of NPRA and PDE3B, both involved in early lipolysis signalling. Gene expression of hMADS adipocytes lipolytic machinery was unaltered (Fig. 3A) but total HSL (+30%, $P = 0.06$) and ATGL protein content (+64%, $P < 0.05$) increased in basal conditions (Fig. 3B). Phosphorylation of HSL residue Ser$^{563}$ was also induced ($P < 0.01$). As shown in Fig. 1B, acute FK stimulation also led to HSL Ser$^{563}$ phosphorylation and accentuated ATGL content (+51%, $P < 0.05$). As expected, the effects of Db-cAMP pre-treatment and acute FK stimulation, both converging on the PKA pathway, were not additive. The FK-induced upward electrophoretic shift

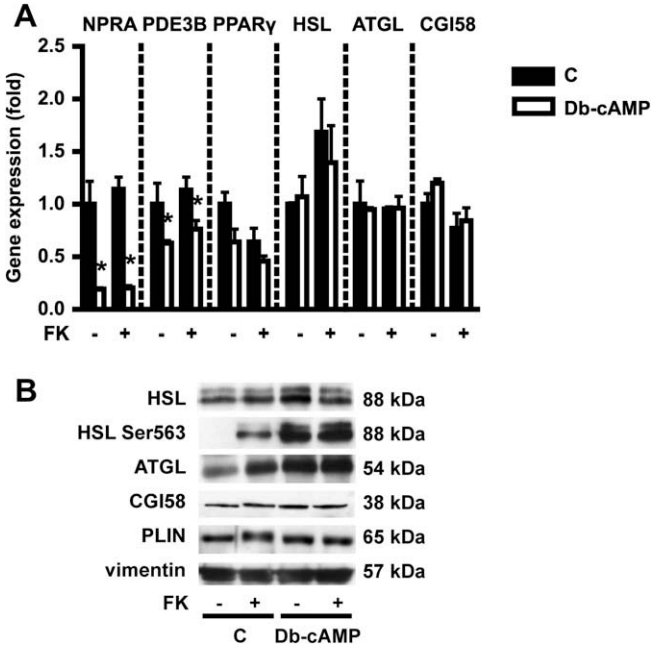


**Fig. 3.** Gene and protein expression of hMADS lipolytic machinery after chronic treatment with Db-cAMP. Cells were pre-treated for 48 h with Db-cAMP (1 μM) from Day 13 to Day 15. On Day 15, Db-cAMP was removed and cells were acutely treated for 3 h with or without FK (1 μM) and harvested for gene expression and Western blotting. (A) Gene expression of NPRA, PDE3B, PPARγ, HSL, ATGL and CGI-58 assessed by real-time RT-PCR and normalized with 18S rRNA levels. (B) Western blots of HSL, HSL Ser$^{563}$, ATGL, CGI-58 and PLIN, normalized to vimentin. The data are presented as means ± S.E.M. $N = 3–6$. * Significantly different from control condition (C).

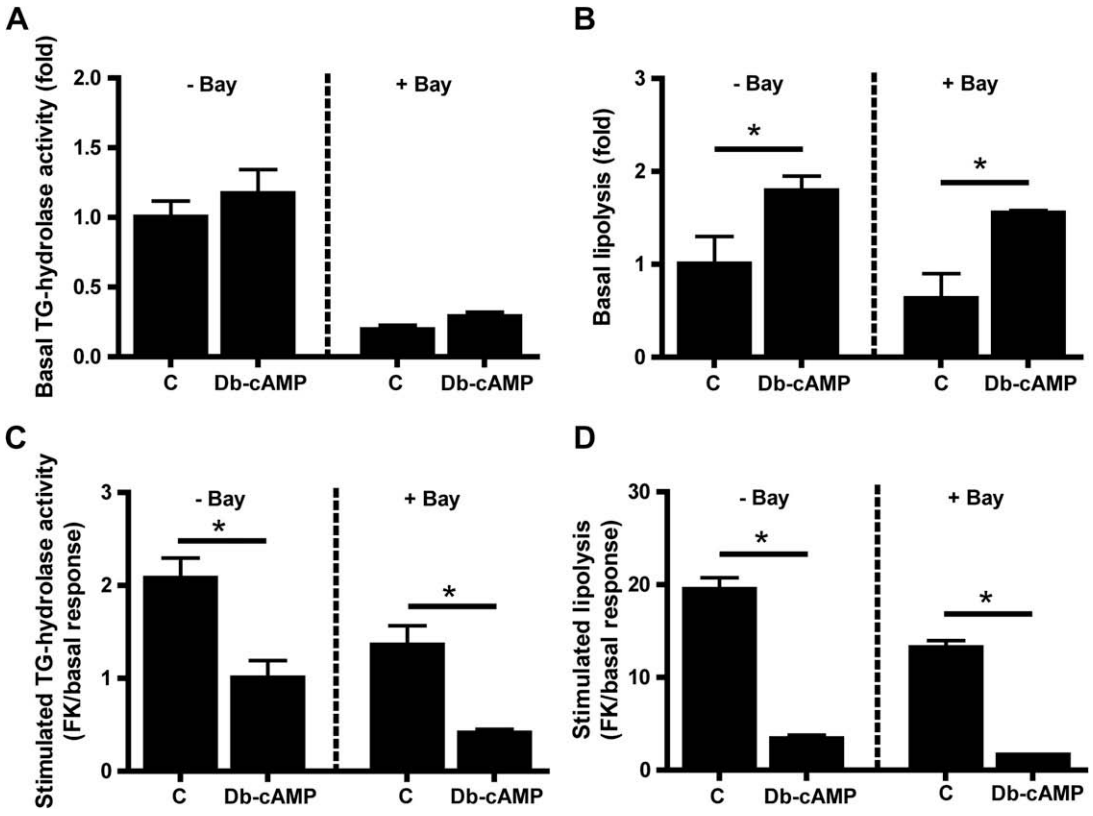


**Fig. 4.** TG-hydrolase activity and lipolysis of hMADS cells after chronic treatment with Db-cAMP. Cells were pre-treated for 48 h with Db-cAMP (1 μM) from Day 13 to Day 15. On Day 15, Db-cAMP was removed and cells were acutely treated for 3 h with or without FK (1 μM) and/or Bay (10 μM) for TG-hydrolase activity and lipolysis. (A) TG-hydrolase activity. (B) Basal lipolysis. (C) TG-hydrolase activity in response to FK treatment. (D) Lipolytic response to FK treatment. The data are presented as means ± S.E.M. $N = 3–6$. * Significantly different from control condition (C).

in the PLIN band was observed in control condition, but abolished by chronic Db-cAMP pre-treatment (Fig. 3B). Therefore, chronic Db-cAMP pre-treatment results in HSL, but not PLIN, phosphorylation suggesting a specific desensitization mechanism for the PAT protein.

### *3.4. Chronic Db-cAMP pre-treatment impairs normal FK response in TG-hydrolase activity and lipolysis*

The increase in HSL and ATGL did not translate into elevated TG-hydrolase activity (Fig. 4A) suggesting post-translational control of lipase activity. However, basal lipolysis was nearly doubled with chronic Db-cAMP pre-treatment (Fig. 4B). This increase could be the combined consequence of reduced PDE3B gene expression and increased HSL phosphorylation. Db-cAMP has previously been shown to down-regulate PDE3B expression and activity [33]. The expected FK-induced increase in intrinsic HSL activity was abrogated, and ATGL intrinsic activity was reduced below basal levels with chronic Db-cAMP pre-treatment (Fig. 4C). Similarly, specific HSL and ATGL FK-stimulated lipolysis was drastically reduced (Fig. 4D). Impaired stimulated lipolysis could be attributed to both reduced ATGL intrinsic activity, reduced FK-stimulated HSL TG hydrolase activity and the lack of PLIN phosphorylation. Whether resistance to PKA-stimulated lipolysis is a defect or a protective mechanism to prevent excessive hydrolysis is unclear and warrants further investigation.

### *3.5. Concluding remarks*

Through different molecular mechanisms, our work shows that chronic exposure of human fat cells to TNFα or sustained activation of the PKA pathway result in increased basal lipolysis and a markedly blunted response to stimulated lipolysis.

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