Cyclic AMP-mediated EPAC activation is involved in the regulation of adipogenic genes during 3T3-L1 fibroblasts differentiation

Journal:	*Development Growth and Differentiation*
Manuscript ID:	DGD-00081-2013.R1
Manuscript Type:	Original Research Article
Date Submitted by the Author:	13-Nov-2013
Complete List of Authors:	Gabrielli, Matías; University of Buenos Aires, Biochemistry Martini, Claudia; University of Buenos Aires, Biochemistry Brandani, Javier; University of Buenos Aires, Biochemistry Raiger Iustman, Laura; University of Buenos Aires, Biochemistry Romero, Damian; University of Mississippi Medical Center, Biochemistry Vila, Maria; University of Buenos Aires, Biochemistry
Key Words:	3T3-L1 Cells, Adipogenesis, C-EBP beta, cyclic AMP, PPAR gamma

SCHOLARONE™
Manuscripts

For Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Cyclic AMP-mediated EPAC activation is involved in the regulation of adipogenic genes during 3T3-L1 fibroblasts differentiation

Matías Gabrielli*, Claudia N. Martini*, Javier N. Brandani*, Laura Judith Raiger Iustman*, Damián G. Romero# and María del C. Vila *°

* Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón 2, Ciudad Universitaria, 1428, Buenos Aires, Argentina.

Dept. of Biochemistry, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216, USA

° To whom correspondence should be addressed. Phone 541145763300 Ext: 443, Fax: 541145763342, e-mail: mvila@qb.fcen.uba.

For Peer Review

Author Contributions

Conceived and designed the experiments: DGR, MCV. Performed the experiments: MG, CNM, JNB, DGR, LJRI. Analyzed the data: MG, CNM, JNB, DGR, MCV. Wrote the paper: MCV.

Running title: EPAC involvement in adipogenesis

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

ABSTRACT

Adipogenesis is stimulated in 3T3-L1 fibroblasts by a combination of insulin, dexamethasone and isobutylmethylxanthine, IBMX, (I+D+M). Two transcriptional factors are important for the acquisition of the adipocyte phenotype, C/EBP beta (CCAT enhancer-binding protein beta) and PPAR gamma (peroxisome proliferator-activated receptor gamma). IBMX increases cAMP content, which can activate protein kinase A (PKA) and/or EPAC (exchange protein activated by cAMP). To investigate the importance of IBMX in the differentiation mixture, we first evaluated the effect of the addition of IBMX on the increase of C/EBP beta and PPAR gamma and found an enhancement of the amount of both proteins. IBMX addition (I+D+M) or its replacement with a cAMP analog, dibutyryl-cAMP or 8-(4-chlorophenylthio)-2-O'-methyl-cAMP (8CPT-2-Me-cAMP), the latter activates EPAC and not PKA, remarkably increased PPAR gamma mRNA. However, neither I+D nor each of the inducers alone, increased PPAR gamma mRNA to a similar extent suggesting the importance of the presence of both, IBMX and I+D. It was also found that the addition of IBMX or 8CPT-2-Me-cAMP was able to increase the content of C/EBP beta with respect to I+D. In agreement with these findings, a microarray analysis showed that the presence of either 8CPT-2-Me-cAMP or IBMX in the differentiation mixture was able to up-regulate PPAR gamma and PPAR gamma-activated genes as well as other genes involved in lipid metabolism.

Our results prove the involvement of IBMX-cAMP-EPAC in the regulation of adipogenic genes during differentiation of 3T3-L1 fibroblasts and therfore contributes to elucidate the role of cyclic AMP in this process.

Keywords: 3T3-L1 Cells, adipogenesis, C-EBP beta, cyclic AMP, PPAR gamma

For Peer Review

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

1. INTRODUCTION

Obesity which is due to an increase in size and number of adipocytes (Gregoire *et al.*, 1998) and is associated with several frequent diseases such as diabetes, hypertension and cardiovascular disease (Lean, 2000), has importantly increased in the last decades all over the world.

3T3-L1 fibroblasts are useful tool in the study of adipocyte differentiation (Rubin *et al.*, 1978). After the addition of a differentiation mixture containing: insulin, dexamethasone and isobutylmethylxanthine (IBMX), post-confluent 3T3-L1 fibroblasts re-enter the cell cycle (Student *et al.*, 1980). This proliferation step is called mitotic clonal expansion (MCE) (Qiu *et al.*, 2001) and precedes the adipogenic gene expression program leading to adipocyte differentiation.

During the differentiation program two early transcriptional factors CCAT enhancer-binding protein beta (C/EBP beta) and C/EBP delta are induced during the first hours and then they trigger the expression of two key transcription factors in adipogenesis: peroxisome proliferator-activated receptor gamma (PPAR gamma) and C/EBP alpha which are involved in the induction of proteins associated with adipocyte phenotype (Farmer, 2006).

IBMX, one of the inducers in the differentiation mixture, is an inhibitor of phosphodiesterase, thus it is used to increase intracellular cAMP which is the physiological activator of PKA. PKA was initially believed to be the only protein activated by cAMP. However, in the last years, PKA-independent cAMP responses were reported (Holz *et al.*, 2006; Chin & Abayasekara, 2004; Kanda & Watanabe, 2007; Yin *et al.*, 2006). It has been reported that cAMP activates EPAC (exchange proteins activated by cAMP), which is a newly discovered family of GTP exchanging factors for

For Peer Review

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

the small GTP-binding proteins Rap1 and Rap2 (Kawasaki *et al.*, 1998; Rehmann *et al.*, 2006).

We have previously shown that cAMP signals through PKA for MCE and through EPAC for adipogenesis and that EPAC but not PKA signaling is necessary for differentiation to occur (Martini *et al.*, 2009). In agreement, other reports show the involvement of EPAC signaling in the differentiation of 3T3-L1 fibroblasts to adipocytes (Petersen *et al.*, 2008; Ji *et al.*, 2010). However Petersen *et al.* (2008) reported that both EPAC and PKA signaling are necessary for adipogenesis.

In the absence of IBMX in the differentiation mixture no significant adipogenesis is obtained (Martini *et al.*, 2009; Qiu *et al.*, 2001; Liu *et al.*, 2005) which indicates the importance of IBMX signaling for 3T3-L1 fibroblasts differentiation to adipocytes. In this paper we analyzed the role of IBMX in the differentiation mixture as well as the requirement of insulin and dexamethasone and further investigated the ability of EPAC to mediate IBMX action.

For Peer Review

2. **MATERIALS AND METHODS**

2.1. Chemicals

DMEM, trypsin and insulin were obtained from Invitrogen (Carlsbad, CA), 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, dibutyryl-cAMP, 8-(4-chlorophenylthio)-2-O’-methyl-cAMP (8CPT-2-Me-cAMP). 3T3-L1 fibroblasts were obtained from Asociación Banco Argentino de Células (origin: ATCC).

2.2. Differentiation induction of 3T3-L1 fibroblasts

3T3-L1 fibroblasts were cultured in DMEM + 10% fetal bovine serum (FBS) with 0.1 mg/ml streptomycin, 100 U/ml penicillin and 250 ng/ml fungizone (DMEM +

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

10% FBS). To induce differentiation, two days post-confluent cells were treated with a mixture containing: 10 µg/ml insulin, 0.5 mM isobutylmethylxanthine (IBMX) and 100 nM dexamethasone in DMEM + 10% FBS. In some experiments, differentiation mixtures containing insulin and dexamethasone alone or in combination with dibutyryl-cAMP (1 mM) or 8CPT-2-Me-cAMP (0.2 mM) or containing each inducer alone were used. In the experiments with cAMP analogs treatments were repeated after 24 h. Three days after the induction of differentiation medium was replaced with DMEM + 10% FBS supplemented with insulin. Then medium was changed every 2 days with DMEM + 10% FBS. For the treatments with 50 µM Rp-cAMPS and 50 µM Rp-8-Br-cAMPS (Biolog), cells were treated with a mixture of both inhibitors 1 h prior to the addition of the differentiation mixture and the inhibitors were also present in the fresh medium supplemented with insulin added 3 days after induction

2.3. Oil-Red-O staining

3T3-L1 adipocyte monolayers (usually on day 8) were washed three times with phosphate-buffered saline (PBS) and then fixed for 30 min with 10% formaldehyde in PBS. Oil-Red-O (0.4%) in isopropanol was diluted with two volumes of water, filtered, and added to the fixed cell monolayers for 30 min at room temperature. Cell monolayers were then washed with water, and the stained triglyceride droplets in the cells were visualized and photographed.

2.4. Cell counting

Two days post-confluent 3T3-L1 fibroblats were treated with: DMEM + 10% FBS alone (C), or with the addition of differentiation mixture in the absence (I+D+M) or presence of Rp-cAMPS (50 µM) and Rp-8-Br-cAMPS (50 µM) which were added 1

For Peer Review

h prior to the addition of the differentiation mixture (I+D+M+Rps). Three days after the induction of differentiation, cells were trypsinized, resuspended in DMEM medium and an aliquot was counted using a Neubauer chamber.

2.5. Western-blot determination

After treatment of two days post-confluent 3T3-L1 fibroblasts for the times indicated in each case (24 or 30 h for C/EBP beta and 8 days for PPAR gamma), cells were harvested in PBS, vortexed and before the addition of Laemmli sample buffer (Laemmli, 1970) an aliquot was obtained for protein determination using the method of Bradford with serum albumin as standard (Bradford, 1976). After SDS-PAGE using equal amount of protein in each lane (5-30 μg), gels were transferred to nitrocellulose membrane, blocked with 5% milk for an hour and then treated overnight with the corresponding antibodies (anti-C/EBP beta (C-19) from Santa Cruz and PPAR gamma (C26H12) from Cell Signaling) followed by HRP-conjugated secondary antibody for 1 h. Finally, chemiluminiscence reagent was used for detection.

2.6 Immunofluorescence microscopy for PPAR gamma detection:

Fibroblasts were cultured on coverslips in 24 well-plates and 8 days after induction of differentiation, cells were fixed in 4% formaldehyde, permeabilized in PBS-0.025% Tween 20 and blocked in 5% BSA and then incubated with an anti-PPAR gamma followed by a Cy2-conjugated secondary antibody.

2.7 RNA extraction and qPCR analysis

3T3-L1 fibroblasts cultured in 12 wells-plates were lysed in TRI Reagent (Molecular Research Center, Inc.) after treatments for the indicated times. RNA

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

extraction was performed according to standard protocol and pellets were resuspended in nuclease free water. RNA was quantified with NanoDrop 2000 spectrophotometer (Thermo Scientific), and 2 μg were used for reverse transcription using MMLV Reverse Transcriptase (Promega). Relative expression of cDNAs was determined by quantitative real-time PCR using a Bio-Rad MyIQ2 thermal cycler. Each PCR reaction was performed in a final volume of 25 μl containing 5 μl of a 1:10 dilution of first-strand cDNA, 12.5 μl of Mezcla Real (Biodynamics) and 15 pmol of each primer. All reactions were performed using the following cycling conditions: 94°C for 2 min, followed by 40 cycles of 95 °C for 20 s, 58 °C for 30 s and 72 °C for 40 s. Target gene mRNA expression was normalized to acidic ribosomal protein (Rplp0) as reference gene. The following primers were used: PPAR gamma 2, Fwd: CCAGAGCATGGTGCCTTCGCT, Rev: CAGCAACCATTGGGTCAGCTC; and Rplp0, Fwd: GAGGAATCAGATGAGGATATGGGA, Rev: AAGCAGGCTGACTTGGTTGC.

For Peer Review

2.8 Microarray Analysis

Total RNA was extracted from 3T3-L1 fibroblasts induced with I+D, I+D+IBMX or I+D+8CPT-2-Me-cAMP using TRI Reagent (MRC) according to the manufacturer's instructions. Quality of the samples was checked with 2100 Bioanalyzer (Agilent Technologies). Microarray analysis was performed at the Northwestern University Genomics Core using Illumina MouseRef-8 v2.0 Expression BeadChip (Illumina). Acquired data was analyzed with GeneSpringGX software (Agilent).

2.9 Statistical Analysis

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

The experiments were carried out three times unless otherwise stated. All data were expressed as mean ± SD. Statistical analysis was performed by One-Way ANOVA, followed by Dunnett's post-hoc test. A p value below 0.05 was considered statistically significant.

3. RESULTS

Effect of the presence of IBMX in the differentiation mixture on C/EBP beta and PPAR gamma

Since differentiation of 3T3-L1 fibroblasts to adipocytes was obtained after 8-10 days of induction with a mixture that contains insulin, dexamethasone and IBMX (I+D+M), we first wanted to evaluate the contribution of IBMX on the protein level of C/EBP beta and PPAR gamma, which are known as important early and late genes in adipogenesis, respectively. As it is shown in figure 1A, after 24 h treatment, I+D slightly increased C/EBP beta with respect to control cells, but this increase was lower than the one observed in the presence of IBMX in the differentiation mixture. Similar results were obtained with PPAR gamma (Fig. 1B).

Taking into account that PPAR gamma, is considered the master gene in adipogenesis, we further analyzed the role of IBMX on PPAR gamma intracellular localization by immunofluorescence. We observed an exacerbation of nuclear PPAR gamma in I+D+M vs. I+D after 8 days of treatment when terminal differentiation was attained (Fig. 2).

We also evaluated the effect at the level of PPAR gamma mRNA. We found that 8 days after induction of differentiation PPAR gamma mRNA in I+D+M was remarkably increased with respect to control cells and cells treated with I+D alone (Fig.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

3). This is in keeping with the extent of differentiation obtained with each of those treatments (Fig. 4).

Effect of the replacement of IBMX with 8CPT-2-Me-cAMP on cell differentiation and PPAR gamma

IBMX, an inhibitor of phosphodiesterase, is a usefeul tool to increase intracellular cAMP. At present, several reports (Petersen *et al.*, 2008; Ji *et al.*, 2010) including ours (Martini *et al.*, 2009), have shown the importance of EPAC signaling in the differentiation of 3T3-L1 fibroblasts. However, Petersen *et al*. (2008) proposed that EPAC is necessary but not sufficient to mediate cAMP response, since PKA activation is also required.

On the contrary, other reports, showed that PKA is not necessary for differentiation (Kato *et al*., 2007; Ji *et al*., 2010). In keeping with this, we previously reported (Martini *et al.*, 2009), that inhibition of PKA is able to block MCE but not differentiation of 3T3-L1 fibroblasts and that the replacement of IBMX in the differentiation mixture with 8CPT-2-Me-cAMP, a cAMP analog that activates EPAC and not PKA, was able to induce differentiation but not MCE, which indicates that MCE is a PKA-dependent process but adipogenesis is PKA-independent.

To further investigate the role of PKA in 3T3-L1 fibroblasts differentiation, herein, we analyzed the effect on MCE and differentiation of inhibitors of PKA different from H89 that we had previously tested (Martini *et al.*, 2009). We used a mixture containing Rp-cAMPS and Rp-8-Br-cAMPS, which has been previously used in 3T3-L1 fibroblasts to inhibit PKA activity (Petersen *et al*., 2008**)**. We found that inhibition of PKA with Rp-cAMPS and Rp-8-Br-cAMPS, inhibited MCE but not

For Peer Review

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

differentiation of 3T3-L1 cells (Fig. 5) which supports that MCE, but not differentiation, is a PKA-dependent process.

To further test the importance of EPAC activation in differentiation, two cyclic AMP analogs, dibutyryl-cAMP (db-cAMP) or 8CPT-2-Me-cAMP, were used to replace IBMX in the differentiation mixture, the differentiation obtained with only one addition of any of the analogs at 0 h was lower than that observed with IBMX. However, differentiation was improved by the addition of fresh differentiation mixture containing the cell permeable cAMP analog, 24 or 48 h after induction of differentiation. The best differentiation, which is similar to that obtained with IBMX, was attained with addition of the cAMP analogs at 0 and 24 h (Fig. 4). Interestingly no effect was found when cAMP analogs were added 48 h after induction of differentiation with I+D alone (data not shown), suggesting the importance of their presence during the first 48 h.

Taking this into account, we replaced IBMX in the differentiation mixture with db-cAMP or 8CPT-2-Me-cAMP added at 0 and 24 h to measure PPAR gamma mRNA. A noticeable increase was found in samples treated with the cAMP analogs with respect to control or I+D. However, neither I+D nor each of the inducers alone, were able to increase PPAR gamma mRNA in a similar extent (Fig. 3). This is in keeping with the ability of each of those treatments to differentiate 3T3-L1 fibroblasts.

For Peer Review

Effect of the replacement of IBMX with 8CPT-2-Me-cAMP on C/EBP beta

To evaluate the involvement of EPAC signaling in IBMX-mediated C/EBP beta increase, we also investigated the effect of 8CPT-2-Me-cAMP on the level of this early adipogenic gene. I+D+M or I+D+8CPT-2-Me-cAMP increased C/EBP beta protein level more than I+D after 24 h (Fig. 6A). Similar results were obtained when

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

C/EBP beta was analyzed at 30 h, after the addition of a second dose of 8CPT-2-Me-cAMP at 24 h (Fig. 6B).

Effect of the replacement of IBMX with 8CPT-2-Me-cAMP on adipogenic genes

To confirm the ability of 8CPT-2-Me-cAMP to replace IBMX in the differentiation mixture, 3T3-L1 fibroblasts were differentiated with mixtures containing: I+D, I+D+M or I+D+8CPT-2-Me-cAMP, RNA was extracted from differentiated cells to compare the pattern of adipogenic genes expressed with the different treatments by microarray analysis. This analysis confirmed the ability of I+D+M_or I+D+8CPT-2-Me-cAMP to up-regulate PPAR gamma with respect to I+D (Table 1).

Consistently, the presence of 8CPT-2-Me-cAMP in the differentiation mixture was able to regulate genes involved in adipogenesis which are known to be activated by PPAR gamma like perilipin (Nagai *et al.*, 2004), G0/G1 switch gene 2 (Zandbergen *et al.*, 2005), hormone sensitive lipase (Yajima *et al.*, 2007) and lipoprotein lipase (Schoonjans *et al.*, 1996).

In addition, other genes reported to be regulated during adipogenesis, were also regulated in the presence of 8CPT-2-Me-cAMP, such as acyl-CoA synthetase long-chain family member 1, stearoyl-Coenzyme A desaturase 1, 1-acylglycerol-3-phosphate O-acyltransferase 2, diacylglycerol O-acyltransferase 2, diacylglycerol O-acyltransferase 1, solute carrier family 25 member 10 (dicarboxylate transporter), 1-acylglycerol-3-phosphate O-acyltransferase 9 (Table 1).

These results further prove the ability of cAMP-EPAC to mimic IBMX response in adipogenesis.

For Peer Review

4. DISCUSSION

It is well known the importance of C/EBP beta and PPAR gamma in adipogenesis. In the present paper we report that the increase in C/EBP beta and PPAR gamma in 3T3-L1 fibroblasts treated with differentiation mixture (I+D+M) is higher than that obtained with I+D alone. Taking into account that differentiation of 3T3-L1 fibroblasts is impaired in the absence of IBMX, these results suggest that not only the increase of two important adipogenic genes, C/EBP beta and PPAR gamma, but also the extent of this increase seems to be relevant for differentiation to occur.

When IBMX was replaced by db-cAMP or 8CPT-2-Me-cAMP, we found that a second addition of differentiation mixture containig I+D with these cAMP analogs at 24 h is required to observe a differentiation similar to the one with I+D+M. Hydrolysis of both cAMP analogs by phosphodiesterases might explain these results, as well as, the previously reported lack of differentiation of 3T3-L1 fibroblasts in the presence of I+D with only the addition at 0 h of 8CPT-2-Me-cAMP at a concentration similar to the one used in our experiments (Petersen *et al.*, 2008; Ji *et al.*, 2010).

The ability of 8CPT-2-Me-cAMP to replace IBMX in the differentiation mixture, as well as the lack of effect of Rp-cAMPS in adipogenesis, are both in agreement with other reports that show that PKA activity is not necessary for 3T3-L1 differentiation (Ji *et al.*, 2010; Kato *et al.*, 2006; Martini *et al.*, 2009; Wang *et al.*, 1997) or may even inhibit differentiation (Li *et al.*, 2008).

On the other hand, no differentiation was found when cAMP analogs were only added 48 h after the induction of differentiation with I+D alone, indicating the importance of their presence during the first 48 h. In agreement, it has been reported that IBMX-cAMP is necessary for the generation of PPAR gamma ligands which occurs during the first 48 h after induction of differentiation (Tzameli *et al.*, 2004).

For Peer Review

Herein, we found that the presence of IBMX in the differentiation mixture induced a noticeable increase in PPAR gamma mRNA content with respect to I+D alone and similar results were obtained when IBMX was replaced by db-cAMP or 8CPT-2-Me-cAMP. Furthermore, similar extent of differentiation was obtained with IBMX or either of the cAMP analogs. These results indicate that IBMX or the cAMP analogs, including an EPAC specific activator, are necessary to obtain an important increase in PPAR gamma which was not reached with I+D.

Either I+D or IBMX alone were unable to increase PPAR gamma mRNA and differentiation to a similar extent as the I+D+M. In keeping with these results, which indicates that not only IBMX but also I+D is necessary for differentiation, we found that insulin but not IBMX alone or in the presence of I+D contributes to activate PKB (data not shown), a kinase required for 3T3-L1 differentiation (Xu & Liao, 2004; Martini *et al.*, 2009, Mauri *et al.*, 2010).

We confirmed by microarray analisys the ability of 8CPT-2-Me-cAMP to replace IBMX in the differentiation mixture and to up-regulate PPAR gamma as well as genes which has peroxisome proliferator-response element (PPRE) upstream of the promoter such as perilipin, a lipid droplet associated protein crucial for storage and mobilization of lipids in adipocytes (Sun *et al.*, 2013; Takahashi *et al.*, 2013), and G0/G1 switch 2 which is associated with growth arrest required for adipogenesis in 3T3-L1 fibroblasts (Zandbergen *et al.*, 2005). In addition, several genes involved in lipid metabolism and transport were up-regulated both by IBMX and 8CPT-2-Me-cAMP. Several of the genes that we found regulated by IBMX are in agreement with a previous report where differences in gene expression in 3T3-L1 fibroblasts differentiated in the presence or absence of IBMX were analyzed by microarray (Liu *et al.,* 2005).

For Peer Review

Our results further prove the ability of cAMP-EPAC signaling to mimic IBMX action in the differentiation of 3T3-L1 fibroblasts to adipocytes. The importance of EPAC signaling is in keeping with a previous report that shows that EPAC1 knockdown in 3T3-L1 fibroblasts inhibits PPAR gamma mRNA expression and differentiation (Ji *et al.*, 2010).

In conclusion, our findings indicate that IBMX-cAMP-EPAC is involved in the regulation of adipogenic genes during differentiation of 3T3-L1 fibroblasts and that cAMP signaling through EPAC in the presence of insulin and dexamethasone is necessary and sufficient for adipogenesis. Thus, this report contributes to elucidate the role of cyclic AMP in this process.

ACKNOWLEDGEMENTS

This work was supported by research grants from Agencia Nacional de Promoción Científica y Tecnológica and Consejo Nacional de Investigaciones Científicas y Técnicas of Argentina. The funders had no role in study design, data and analysis, decision to publish, or preparation of the manuscript.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

REFERENCES

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye-binding. *Anal. Biochem*. **72**, 248–254.

Chin, E.C. & Abayasekara, D.R.E. 2004. Progesterone secretion by luteinizing human granulosa cells: a possible cAMP-dependent but PKA-independent mechanism involved in its regulation. *J. Endocrinol*. **183**, 51–60.

Farmer, S.R.. 2006. Transcriptional control of adipocyte formation. *Cell. Metab*. **4**, 263-273.

Gregoire, F.M., Smas, C.M. & Sul, H.S., 1998. Understanding adipocyte differentiation. *Physiol. Rev*. **78**, 783–809.

Holz, G.G., Kang, G., Harbeck, M., Roe, M.W. & Chepurny, O.G. 2006. Cell physiology of cAMP sensor Epac. *J. Physiol*. **577** (Pt 1), 5–15.

Ji, Z., Mei, F.C. & Cheng, X. 2010. EPAC, not PKA catalytic subunit, is required for 3T3-L1 preadipocytes differentiation. *Front. Biosci*. **2**, 392-398.

Kanda, Y. & Watanabe, Y. 2007. Adrenaline increases glucose transport via a Rap1-p38MAPK pathway in rat vascular smooth muscle cells. *Br. J. Pharmacol*. **151**, 476–482.

Kato, Y., Ozaki, N., Yamada, T.,Miura, Y. & Oiso, Y. 2007. H-89 potentiates adipogenesis in 3T3-L1 cells by activating insulin signaling independently of protein kinase A. *Life Sci*. **80**, 476–483.

Kawasaki, H., Springett, G.M., Mochizuki, N., Toki, S., Nakaya,M., Matsuda, M., Housman, D.E. & Graybiel, A.M. 1998. A family of cAMP-binding proteins that directly activate Rap1. *Science*. **282**, 2275–2279.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227**: 680-685.

Lean, M.E. 2000. Pathophysiology of obesity. *Proc. Nutr. Soc*. **59**, 331–336.

Li, F., Wang, D., Zhou, Y., Zhou, B., Yang, Y., Chen, H. & Song, J. 2008. Protein Kinase A suppresses the differentiation of 3T3-L1 preadipocytes. *Cell Res*. **18**, 311-323.

Liu, J., DeYoung, S.M., Zhang, M., Zhang, M., Cheng, A. & Saltiel, A.R. 2005. Changes in integrin expression during adipocyte differentiation. *Cell Metabolism*. **2**, 165-177.

Martini, C.N., Plaza, M.V. & Vila, M.C. 2009. PKA-dependent and independent signaling in 3T3-L1 fibroblasts differentiation. *Mol. Cell. Endocrinol*. **298**, 42-47.

Mauri, T., Ho, J. & Stambolic, V. 2010. Regulation of adipocyte differentiation by distinct subcellular pools of protein kinase B (PKB/AKT). *J. Biol. Chem*. **285**, 15038-15045.

Nagai, S., Shimizu, C., Umetsu, M., Taniguchi, S., Endo, M., Miyoshi, H., Yoshioka, N., Kubo, M. & Koike, T. 2004. Identification of a functional peroxisome proliferator-activated receptor responsive element within the murine perilipin gene. *Endocrinology* **145**, 2346-2356.

Petersen, R.K., Madsen, L., Pedersen, L.M., Hallenborg, P., Hagland, H., Viste, K., Doskeland, S.O. & Kristiansen, K. 2008. Cyclic AMP (cAMP)-mediated stimulation of adipocyte differentiation requires the synergistic action of EPAC- and cyclic AMP-dependent protein kinase-dependent processes. *Mol. Cell. Biol***. 28**, 3804-3816.

Qiu, Z., Wei, Y., Chen, N., Jiang, M., Wu, J. & Liao, K. 2001. DNA synthesis and mitotic clonal expansion is not a required step for 3T3-L1 preadipocytes into adipocytes. *J. Biol. Chem*. **276**, 11988–11995.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Rehmann, H., Das, J., Knipscheer, P., Wittinghofer, A. & Bos, J.L. 2006. Structure of the cyclic-AMP-responsive exchange factor Epac2 in its auto-inhibited state. *Nature.* **439**, 625–628.

Rubin, C.S., Hirsch,A., Fung, C. & Rosen, O.M. 1978. Development of hormone receptors and hormonal responsiveness in vitro. Insulin receptors and insulin sensitivity in the preadipocyte and adipocyte forms of 3T3-L1 cells. *J. Biol. Chem***. 253**, 7570–7578.

Schoonjans, K., Peinado-Onsurbe, J., Lefebvre, A.M., Heyman, R.A., Briggs, M., Deeb, S., Staels, B. & Auwerx, J. 1996. PPAR alpha and PPAR gamma activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. *EMBO J*. **15**, 5336-5348.

Sun, Z., Gong, J., Wu, H., Xu, W., Wu, L., Xu, D., Gao, J., Wu, J.W., Yang, H., Yang, M. & Li, P. 2013. Perilipin1 promotes unilocular lipid droplet formation through the activation of Fsp27 in adipocytes. *Nat. Commun*. **4**, 1594.

Student, A.K., Hsu, R.Y. & Lane, M.D. 1980. Induction of fatty acid synthetase synthesis in differentiating 3T3-L1 preadipocytes. *J. Biol. Chem*. **255**, 4745–4750.

Takahashi, Y., Shinoda, A., Furuya, N., Harada, E., Arimura, N., Ichi, I., Fujiwara, Y., Inoue, J. & Sato, R. 2013. Perilipin-mediated lipid droplet formation in adipocytes promotes sterol regulatory element-binding protein-1 processing and triacylglyceride accumulation. *PLoS One*. **8**(5):e64605.

Tzameli, I., Fang, H., Ollero, M., Shi, H., Hamm, J.K., Kievit, P., Hollenberg, A.N. & Flier J.S. 2004. Regulated production of a peroxisome proliferator-activated receptor-gamma ligand during an early phase of adipocyte differentiation in 3T3-L1 adipocytes. *J. Biol. Chem*. 279, 36093-36102.

For Peer Review

Wang, H., Goligorsky, M.S. & Malbon, C. C. 1997. Temporal activation of Ca2+-Calmodulin-sensitive protein kinase type II is obligate for adipogenesis. *J. Biol. Chem.* 272, 1817-1821.

Xu, J. & Liao, K. 2004. Protein kinase B/AKT plays a pivotal role in insulin-like growth factor-1 receptor signaling induced 3T3-L1 adipocyte differentiation. *J. Biol. Chem*. **279**, 35914-35922.

Yin, F., Wang, Y.Y., Du, J.H., Li, C., Lu, Z.Z., Han, C. & Zhang, Y.Y. 2006. Noncanonical cAMP pathway and p38 MAPK mediate beta2-adrenergic receptor-induced IL-6 production in neonatal mouse cardiac fibroblasts. *J. Mol. Cell. Cardiol.* **40**, 384–393.

Yajima, H., Kobayashi, Y., Kanaya, T. & Horino, Y. 2007. Identification of peroxisome-proliferator responsive element in the mouse HSL gene. *Biochem. Biophys. Res. Commun.* **352**, 526-531.

Zandbergen, F., Mandard, S., Escher, P., Tan, N.S., Patsouris, D., Jatkoe, T., Rojas-Caro, S., Madore, S., Wahli, W., Tafuri, S., Müller, M. & Kersten S. 2005. The G0/G1 switch gene 2 is a novel PPAR target gene. *Biochem J.* **392**, 313-324.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

LEGENDS TO FIGURES

Figure 1: Evaluation of the amount of C/EBP beta and PPAR gamma by western-blot. Two days post-confluent 3T3-L1 fibroblasts were treated with: DMEM + 10% FBS alone (C), or with the addition of: insulin + dexamethasone (I+D), or insulin + dexamethasone + IBMX (I+D+M). After induction of differentiation the amount of C/EBP beta at 24 h **(A)** and PPAR gamma at 8 days **(B)** were analyzed by western-blot.

Figure 2: Evaluation of PPAR gamma content by immunofluorescence. Two days post-confluent 3T3-L1 fibroblasts were treated with: DMEM + 10% FBS alone (C), or with the addition of: insulin + dexamethasone (I+D), or insulin + dexamethasone + IBMX (I+D+M). Eight days after induction of differentiation the amount of PPAR gamma was analyzed by immunofluorescence assay as indicated in methods.

Figure 3: Quantification of PPAR gamma mRNA. Two days post-confluent 3T3-L1 fibroblasts were treated with: DMEM + 10% FBS alone (C), or with the addition of: insulin + dexamethasone (I+D), insulin + dexamethasone + IBMX (I+D+M), insulin + dexamethasone + dibutyryl-cAMP (I+D+db-cAMP), insulin + dexamethasone + 8CPT-2-Me-cAMP (I+D+8CPT), insulin (I), dexamethasone (D) or IBMX (M). Eight days after induction of differentiation the amount of PPAR gamma mRNA was analyzed by qPCR as indicated in methods (data expressed as mean ± SD, n=3). * Significantly different from C, $p < 0.05$ (ANOVA, Dunnett's Test).

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 4: Oil-Red-O staining of lipid droplets. Two days post-confluent 3T3-L1 fibroblasts were treated with: DMEM + 10% FBS alone (C), or with the addition of: insulin + dexamethasone (I+D), insulin + dexamethasone + IBMX (I+D+M), insulin + dexamethasone + dibutyryl-cAMP (I+D+db-cAMP), or insulin + dexamethasone + 8CPT-2-Me-cAMP (I+D+8CPT), db-cAMP (1 mM) and 8CPT-2-Me-cAMP (0.2 mM) were added at 0 and 24 h. Eight days after induction of differentiation lipid droplets were stained with Oil-red-O as indicated in methods.

For Peer Review

Figure 5: Effect of the addition of inhibitors of PKA on MCE and differentiation of 3T3-L1 fibroblasts. **A**) Two days post-confluent 3T3-L1 fibroblats were treated with: DMEM+ 10% FBS alone (C), or with the addition of: differentiation mixture in the absence (I+D+M) or presence of Rp-cAMPS (50 μM) and Rp-8-Br-cAMPS (50 μM) which were added 1 h prior to the addition of the differentiation mixture (I+D+M+Rps). After 3 days of induction of differentiation, cells were trypsinized and counted as indicated in methods. Results are expressed relative to control which is set to 1 and represent mean ± S.E. (n=2). * Significantly different from C, $p < 0.05$ (ANOVA). **B**) Two days post-confluent 3T3-L1 fibroblats were treated with the addition of differentiation mixture in the absence (I+D+M) or presence of Rp-cAMPS (50 μM) and Rp-8-Br-cAMPS (50 μM) which were added 1 h prior to the addition of the differentiation mixture (I+D+M+Rps). The inhibitors were also supplemented in the fresh medium that was added 3 days after induction. Eight days after induction of differentiation lipid droplets were stained with Oil-red-O as indicated in methods.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 6: Evaluation of the amount of C/EBP beta. Two days post-confluent 3T3-L1 fibroblasts were treated with: DMEM + 10% FBS alone (C), or with the addition of: insulin + dexamethasone (I+D), insulin + dexamethasone + IBMX (I+D+M) or insulin + dexamethasone + 8CPT-2-Me-cAMP (I+D+8CPT). 24 h **(A)** or 30 h **(B)** after induction of differentiation the amount of C/EBP beta was analyzed by western-blot as indicated in methods.

For Peer Review

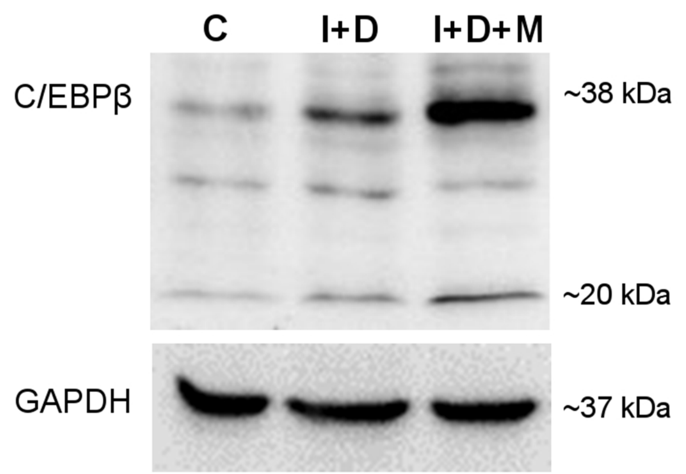
Table 1. mRNA changes induced by the presence of IBMX or 8CPT-2-Me-cAMP in the differentiation mixture

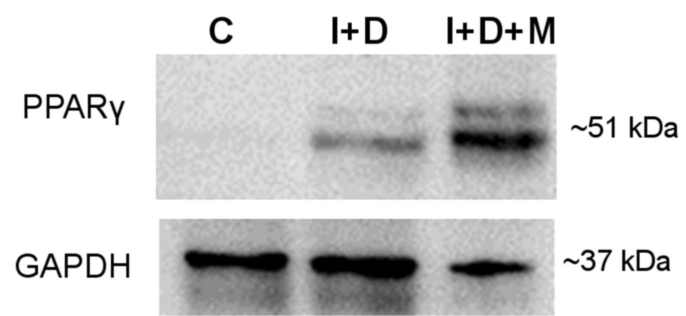
Gene Symbol	Gene Name	Accession Number	Fold Change (I+D+M/I+D)	Fold Change (I+D+8CPT/I+D)
PPAR gamma target genes				
Pparg	Peroxisome proliferator activated receptor gamma	NM_011146	4.51	3.25
Srebf1	Sterol regulatory element binding factor 1	NM_011480	8.23	14.20
Ppargc1b	Peroxisome proliferator activated receptor gamma, coactivator 1 beta	NM_133249	6.58	3.23
Lipe	Lipase, hormone sensitive	NM_010719	10.10	12.14
Lpl	Lipoprotein lipase	NM_008509	2.40	2.73
Scd1	Stearoyl-Coenzyme A desaturase 1	NM_009127	8.23	14.20
G0s2	G0/G1 switch gene 2	NM_008059	15.61	37.80
Plin	Perilipin	NM_175640	34.28	57.49
Lipid metabolism				
Acsl1	Acyl-CoA synthetase long-chain family member 1	NM_007981	47.64	22.59
Hsd11b1	Hydroxysteroid 11-beta dehydrogenase 1	NM_008288	8.12	10.88
Agpat2	1-acylglycerol-3-phosphate O-acyltransferase 2	NM_026212	10.52	7.45
Dgat1	Diacylglycerol O-acyltransferase 1	NM_010046	7.83	5.39
Dgat2	Diacylglycerol O-acyltransferase 2	NM_026384	35.47	24.29
Slc25a10	Solute carrier family 25 member 10	NM_013770	5.96	5.46
Fabp5	Fatty acid binding protein 5	NM_010634	19.83	17.92

I, insulin; D, dexamethasone; M, isobutilmethylxanthine; 8CPT, 8-pCPT-2-O'-Me-cAMP

For Peer Review

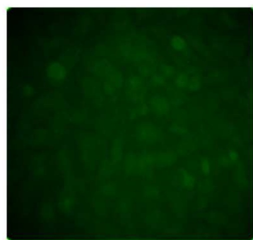
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

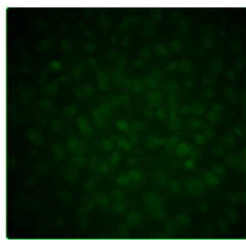


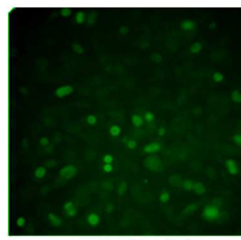


73x106mm (300 x 300 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



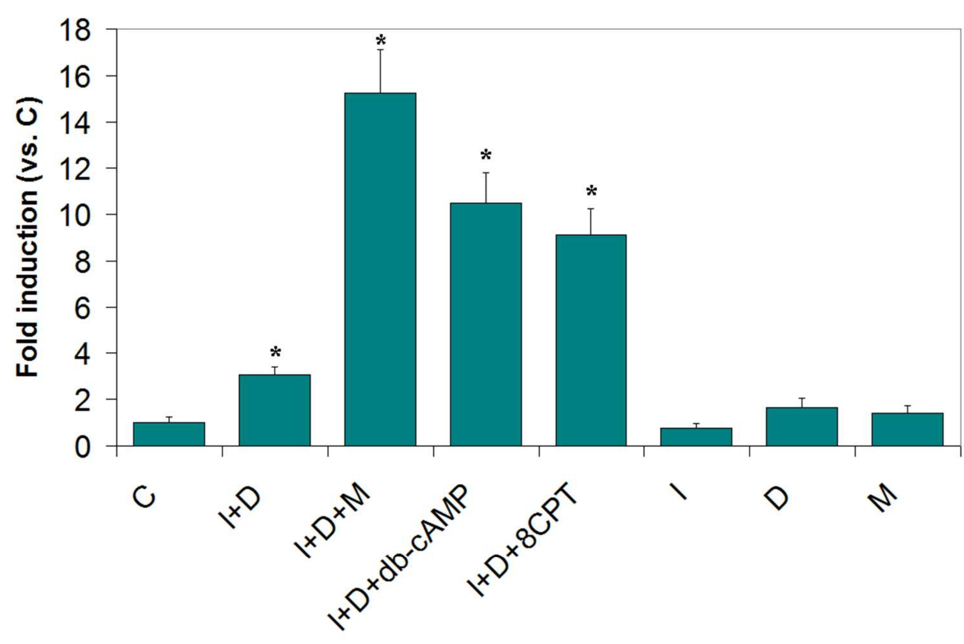




118x37mm (300 x 300 DPI)

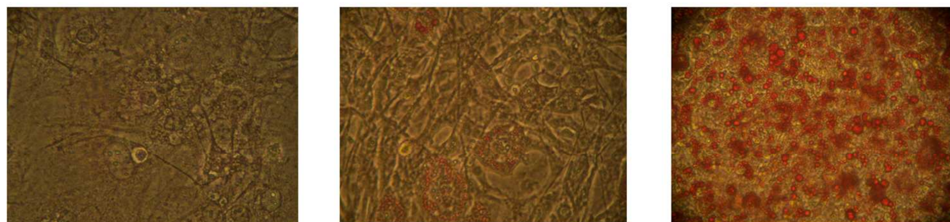
For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

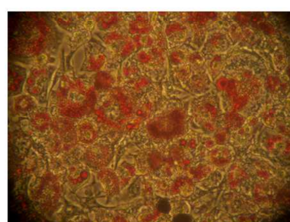


90x59mm (300 x 300 DPI)

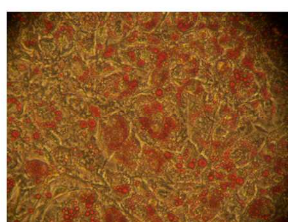
Review



C I+D I+D+M



I+D+db-cAMP



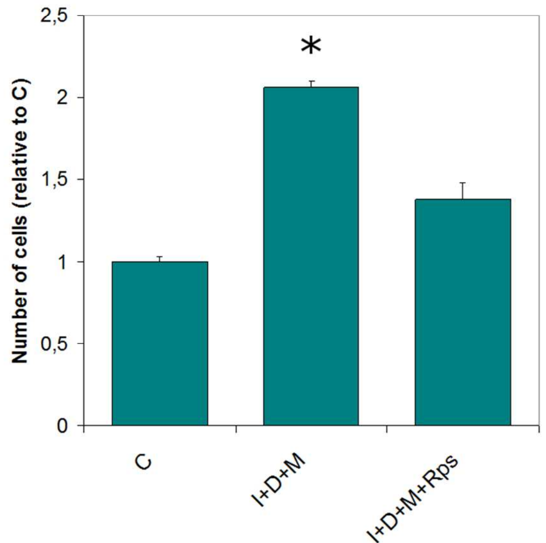
I+D+8CPT

140x94mm (300 x 300 DPI)

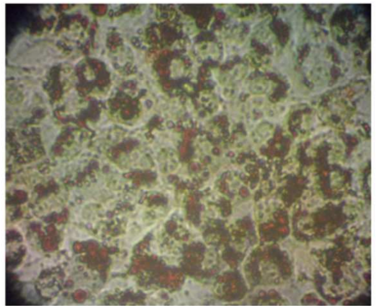
Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

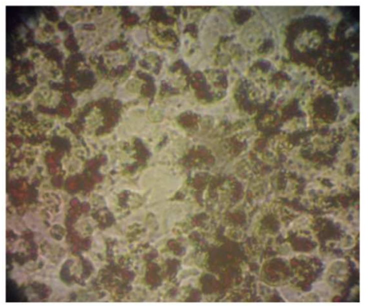
A



B



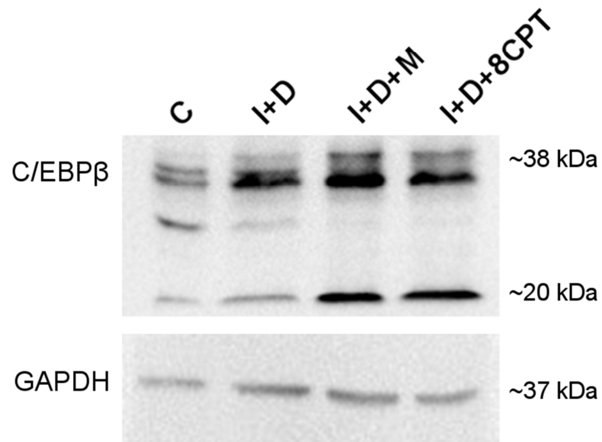
I+D+M

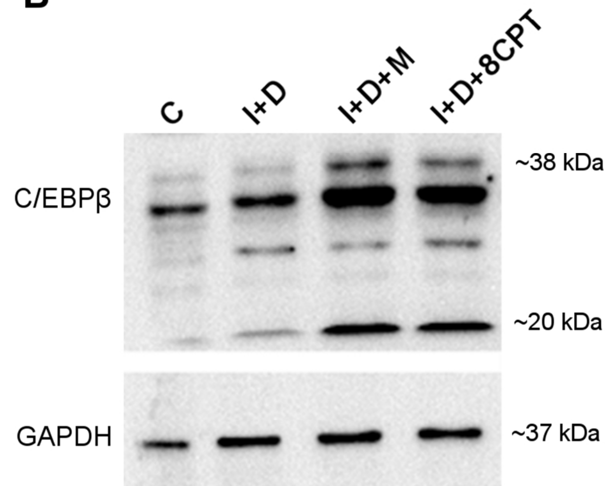


I+D+M+Rps

90x117mm (300 x 300 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60





80x128mm (300 x 300 DPI)