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AIDS 2004, **18**:959–968

Uncoupling protein 1 gene expression implicates brown adipocytes in highly active antiretroviral therapy-associated lipomatosis

Highly active antiretroviral therapy (HAART) in AIDS patients often results in partial lipodystrophy. A subset of patients under HAART also develop lipomatosis, often characterized by enlarged dorsocervical fat pads referred to as 'buffalo hump' [1]. A recent study estimated a 6% prevalence of 'buffalo hump' lipomas among HAART-treated patients [2]. The pathophysiological mechanisms underlying lipodystrophy and lipomatosis are poorly understood. The anatomical distribution of HAART-associated lipomas is reminiscent of that in multiple symmetric lipomatosis. This unusual syndrome has diverse origins including mitochondrial dysfunction [3]. The mitochondrial toxicity associated with HAART and the similar anatomical distribution has led to the hypothesis that HAART-associated 'buffalo hump' syndrome and multiple symmetric lipomatosis could have a common aetiopathology [4]. Lipomas in patients with mitochondrial DNA mutations contain a mixture of univacuolated and multivacuolated fat cells that resemble brown fat rather than white fat [5]. However, the phenotype of the adipose cells developing in HAART-associated lipomas is unknown.

Brown adipose tissue is specialized in thermogenesis and is formed by multivacuolated adipocytes. It is almost absent in adults, although some brown adipocytes are interspread within the white fat depots [6]. The unequivocal cell identity of brown adipocytes can be monitored only by determining uncoupling protein 1 (UCP-1) gene expression, a unique molecular marker that distinguishes brown from white adipocytes [7]. Understanding the cellular basis of lipomatosis may contribute to the knowledge of the aetiopathology of the overall adipose alterations associated with HAART in AIDS patients. We therefore analysed UCP-1 gene expression in HAART-associated lipomas to identify the potential implication of brown adipocytes in HAART-associated lipomatosis.

We studied three HIV-positive patients (L1–L3) undergoing liposuction for the removal of the dorsocervical fat-pad and a fourth HIV-positive patient undertaking the surgical removal of a lipoma in the forearm (L4). They had been on HAART composed of three antiretroviral drugs: stavudine plus two other transcriptase inhibitors (L2–L4), or plus a transcriptase inhibitor and a protease inhibitor (L1). The controls (C1–C5) were HIV-positive patients on HAART with a pattern of drug composition and duration similar to the patients showing lipomatosis. They had clinical characteristics consistent with HAART-associated lipodystrophy (fat wasting from the face, buttocks, limbs and central adiposity), but they did not show lipomatosis. The controls were similar to patients with lipomas with respect to their mean age (41.0 versus 41.8 years, $P = 0.86$), serum CD4 cells/μl (697 versus 438, $P = 0.35$), serum cholesterol (5.9 versus 4.8 + 0.8 mmol/l, $P = 0.86$) and triglyceride levels (1.7 versus 1.8 mmol/l, $P = 0.90$). Controls and patients had achieved virological control of HIV infection (1.43 versus 1.77 $\log_{10}$ for HIV RNA, $P = 0.38$). Biopsy samples of subcutaneous adipose tissue from controls were taken from the abdominal area. A sample of perirenal fat from an unrelated patient already used as a positive control for UCP-1 gene expression [8] was analysed in parallel. UCP-1 messenger RNA was determined by a reverse transcriptase–polymerase chain reaction plus Southern blot assay specifically designed for highly sensitive analysis of UCP-1 gene expression in humans, whereas cyclophilin-A mRNA was assayed as an internal control using reverse transcriptase–polymerase chain reaction, as already reported [8].

The results indicated that the lipomas showed a clear signal for UCP-1 mRNA, somewhat weaker than that in the positive control (Fig. 1). No signal or faint signals were detected in the non-lipoma subcutaneous adipose tissue of lipodystrophic patients. These results

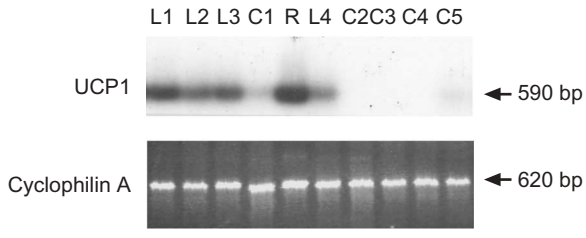

Fig. 1. Analysis of uncoupling protein 1 messenger RNA expression in lipomas (L1–L4) compared with non-lipoma subcutaneous adipose tissue (C1–C4) from patients treated with highly active antiretroviral therapy, and a positive control for uncoupling protein 1 mRNA expression (R). Southern blot analysis of reverse transcriptase–polymerase chain reaction products of uncoupling protein 1 (UCP-1) mRNA amplification is shown in the upper panel. Equal amplification of the cyclophilin-A mRNA, as an internal control of the RNA samples, is shown in the lower panel.

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demonstrate that HAART-associated lipomas express the UCP-1 gene, and therefore cells of the brown adipocyte lineage are implicated in HAART-associated lipomatosis.

Residual brown adipocytes or preadipocytes have already been implicated in lipomatosis of unknown aetiology and in patients bearing mtDNA mutations [4,8]. The fact that lipomas with aetiopathology as diverse as mtDNA mutations or HAART share UCP-1 gene expression reinforces the hypothesis that mitochondrial disturbances, of either genetic or pharmacological origin, cause a dysregulation of adipocyte biology leading to the appearance of highly proliferative brown adipocyte-like cells and lipomas. Brown adipocytes could be preferential targets of HAART-induced mitochondrial toxicity as these cells have a high mitochondrial content, in contrast with white adipocytes. Impaired mitochondrial function may thus elicit a compensatory response based on brown adipocyte proliferation. Brown adipocytes have a high capacity to proliferate and differentiate under the effects of pathophysiological stimuli such as, for instance, phaeochromocytoma [7]. Moreover, a dual 'white and brown' fat morphology appears in the subcutaneous adipose tissue from lipoatrophic areas of HAART-treated patients [9]. A comparison of UCP-1 mRNA levels in the adipose depots of lipodystrophic HAART patients will be required to establish whether a dysregulation of brown versus white adipocyte biology is also implicated in the overall development of lipodystrophy, apart from the specific involvement in lipomatosis reported here.

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Tenofovir-related nephrotoxicity in HIV-infected patients

Tenofovir disoproxil fumarate (TDF) is the first nucleotide analogue approved for the treatment of HIV infection. Its potential for mitochondrial dysfunction is lower than when using nucleoside analogues [1,2], but other nucleotide analogues, such as cidofovir and adefovir, may induce kidney damage as a result of mitochondrial DNA depletion in kidney tubular cells [3]. As the structure of TDF is closely related to those molecules, concern has been raised about its potential nephrotoxicity. Cell culture studies, however, have demonstrated that TDF is only minimally toxic for renal tubular cells [4], even though the use of supratherapeutic doses of TDF in animals may cause renal toxicity [5]. Although human trials with TDF have not shown clinically relevant renal dysfunction [6,7], up to 22 cases of TDF-related nephrotoxicity have been reported up to August 2003 (Table 1) [8–17]. We review these cases here, and describe a new patient with acute renal failure associated with TDF.

A 46-year-old man with HIV infection and a previous history of arterial hypertension, hypercholesterolemia and high alcohol intake, was seen at our outpatient clinic in March 2003. He CD4 cell count was 819 cells/μl and his plasma HIV-RNA level was less than 50 copies/ml. His weight was 81 kg and he had no previous history of renal dysfunction. He had initiated treatment with stavudine, didanosine, and efavirenz 3 years earlier, switching to didanosine, lamivudine and nelfinavir one year later because of liver toxicity.

Table 1. Main features of 22 cases of tenofovir-related nephrotoxicity.

No.	Concomitant drugs	CD4 (cells/μl)	Creat (μmol/l)	[P]p	[U]p	Lactate	Met acidosis	[Prot]u	[Haem]u	[P]u	[Glu]u	Ref.
1	ddI+LPV+RTV+TMP+Flu+Val+Ome+Met	NA	194.5	↓	NA	N	NA	+	NA	NA	NA	[8]
2	NA	NA	↑	NA	NA	N	NA	NA	NA	NA	NA	[9]
3	3TC+EFV+LPV+RTV	822	99.9	↓	↓	N	NA	+	NA	NA	+	[10]
4	3TC+EFV+LPV+RTV	252	70.6	↓	↓	N	NA	+	NA	NA	+	[10]
5	3TC+ddI+APV+RTV	64	100.7	↓	↓	N	NA	+	NA	NA	+	[10]
6	NA	NA	NA	↓	NA	N	NA	NA	NA	NA	NA	[11]
7	NA	NA	NA	↓	NA	N	NA	NA	NA	NA	NA	[11]
8	NA	NA	NA	↓	NA	N	NA	NA	NA	NA	NA	[11]
9	ddI+d4T+ATZ+RTV	318	↑	NA	↓	NA	+	+	+	+	+	[12]
10	LPV+RTV+ABC+ramipril	< 50	689.5	↓	NA	NA	+	+	–	NA	+	[13]
11	ddI+3TC+RTV+APV+T20	< 50	150.3	NA	NA	NA	NA	+	NA	NA	+	[13]
12	3TC+ABC+LPV+RTV	< 50	238.7	↓	NA	NA	+	+	–	NA	NA	[13]
13	ddI+APV+RTV+bumetanide+hydrocortisone	35	↑	NA	NA	↑	+	NA	NA	NA	NA	[14]
14	NA	NA	↑	NA	NA	N	NA	NA	NA	NA	NA	[15]
15	NA	NA	↑	NA	NA	N	NA	NA	NA	NA	NA	[15]
16	NA	NA	↑	NA	NA	N	NA	NA	NA	NA	NA	[15]
17	NA	NA	↑	NA	NA	N	NA	NA	NA	NA	NA	[15]
18	NA	NA	↑	NA	NA	N	NA	NA	NA	NA	NA	[15]
19	NA	NA	↑	NA	NA	N	NA	NA	NA	NA	NA	[15]
20	ddC+LPV+RTV+Met+Fen+Pir	168	1760	NA	NA	↑	+	NA	NA	NA	NA	[16]
21	3TC+LPV+RTV+TMP+Itr	0	443	NA	NA	NA	+	NA	NA	NA	NA	[16]
22	3TC+d4T+LPV+RTV+TMP	80	310	NA	N	NA	–	+	–	NA	–	[17]

ABC, Abacavir; APV, amprenavir; ATZ, atazanavir; Creat, creatinine plasma levels; ddC, zalcitabine; ddI, didanosine; d4T, stavudine; EFV, efavirenz; Fen, fenofibrate; Flu, fluconazol; [Glu]u, glycosuria; [Haem]u, haematuria; Itr, itraconazole; LPV, lopinavir; Met, metformin; Met. acidosis, metabolic acidosis; NA, not available; N, normal; Ome, omeprazol; Pir, pirimetamine; [P]p, plasma phosphorus levels; [Prot]u, proteinuria; [P]u, phosphaturia; RTV, ritonavir; 3TC, lamivudine; TMP, cotrimoxazole; T20, enfuvirtide; [U]p, uric acid plasma levels; Val, valproic acid.

Nineteen months later he was invited to switch to a simplified regimen based on didanosine, TDF and efavirenz. Concomitant medications were enalapril and pravastatin.

One month after initiating this antiretroviral regimen, the patient complained of cough, rhinorrhea and vomiting, and metoclopramide was prescribed. The next day he returned to the hospital complaining of abnormal perioral sensations. Renal dysfunction was diagnosed, and he was admitted to hospital. His plasma creatinine level was 159 μmol/l (normal < 115 μmol/l). Sodium, potassium, amylase and uric acid levels were within normal levels. Urinoanalysis showed three to 10 white cells per high-power field, glucosuria, hyaline and granulose casts, and mild proteinuria. Urine culture was sterile. Creatinine clearance was slightly reduced (77 ml/min).

Antiretroviral drugs were discontinued, clinical symptoms improved and creatinine levels returned to normal values within 48 h. One month after discharge, the patient resumed treatment with didanosine, lamivudine and efavirenz. He has remained asymptomatic, and blood biochemistry and urinoanalysis were normal for the following 4 months, except for mild proteinuria (480 mg/day), which disappeared thereafter.

The incidence of TDF-induced nephrotoxicity is not well known. Significant creatinine elevations have been reported in 5–7% of patients taking TDF for at least 6 months [6,15]. However, many cases may have been misdiagnosed, as renal damage may occur despite normal creatinine levels. Only 1–4% of patients discontinue TDF because of nephrotoxicity during the first year of treatment [10,13,15].

TDF-related kidney toxicity seems to be more frequent in patients with CD4 cell counts of less than 150 cells/μl. Therefore, immunosuppression might increase the risk of nephrotoxicity in patients exposed to TDF, as occurs with many other adverse events associated with antiretroviral drugs [18]. In addition, many patients with TDF nephrotoxicity were underweight and received low doses of ritonavir [10–12]. In four cases, renal insufficiency was present before TDF was initiated. Therefore, factors leading to a greater exposure to TDF might also facilitate kidney toxicity. This hypothesis is supported by animal studies, in which nephrotoxicity was the main TDF dose-limiting adverse event [5]. With respect to the co-administration of ritonavir, it may increase TDF plasma levels by 30% [5], which might be clinically relevant [18].

Several facts support the involvement of TDF in the development of renal dysfunction in our patient. First, acute renal failure occurred soon after TDF was initiated. Second, the patient had no previous renal dysfunction and TDF was the only new agent added before renal abnormalities appeared. Third, blood and urine analyses were similar to those reported in other cases from the literature. The most frequent manifestation of TDF renal toxicity is proximal tubulopathy, occasionally leading to Fanconi's syndrome (proximal tubular acidosis, hypophosphoremia, hypouricemia, glucosuria and proteinuria). As expected, the discontinuation of TDF led to the normalization of renal function.

Our case differs from others in several features. The patient had high CD4 cell counts and was neither underweight nor receiving interacting drugs. TDF is eliminated by the kidney by both filtration and mainly active tubular secretion via human organic anion transporter 1, as are cidofovir and adefovir. Once the drug is accumulated within the tubular cells, its high intracellular concentration may interfere with cell function. This susceptibility of the kidney to the cytotoxic effect of nucleotides is caused by the high expression of human organic anion transporter 1 in kidney cells [19]. Hypothetically, TDF nephrotoxicity in patients without any other predisposing factors might be genetically determined by a higher expression of this transporter.

In conclusion, physicians should be aware of the potential nephrotoxicity of TDF, even in patients lacking any predisposing factor. In all patients receiving TDF, routine biochemical monitoring should include urinoanalysis and plasma phosphorus, lactate and uric acid levels. If any sign of tubulopathy develops, TDF should be stopped as soon as possible. The contribution of weight, concomitant drugs, CD4 cell counts, and genetic predisposition requires further investigation.

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Mitochondrial DNA and sperm quality in patients on antiretroviral therapy – response

We read with interest the observations of Diehl *et al.* [1], but would question the validity of their conclusion that 'sperm are not useful in monitoring mitochondrial toxicity'. We have previously published in this journal, which they did not refer to, evidence of multiple mitochondrial DNA deletions in three patients who were on highly active antiretroviral therapy, including stavudine or didanosine, for longer than 18 months [2]. Diehl *et al.* [1] did not, however, state how long their patients had taken highly active antiretroviral therapy nor the nucleosides taken. Even if mtDNA depletion does not occur, mitochondrial function could still be affected by multiple mtDNA deletions. Whether or not sperm can be used to monitor mitochondrial toxicity therefore remains unclear and deserves further study.

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Long-term infant health outcomes critical in feeding decisions by HIV-positive mothers

A recent paper by Brahmbhatt and Gray [1] attempted to answer the question of whether breastfeeding is best for HIV-positive mothers.

The authors implied that formula feeding is better than breastfeeding for infants of HIV-positive mothers in less developed countries. They based this on their finding that, in less developed countries, outside the HIV context, babies of mothers who chose to formula feed because of preceding morbidity in either mother or infant had lower mortality rates than babies of mothers who chose to formula feed for other reasons. This

conclusion was drawn despite their finding that mortality, in all cases in which infants were artificially fed, was at least 3.7 times higher than among breastfed babies who were voluntarily weaned.

There are several sources of confusion in this analysis. These include combining previous maternal morbidity with previous infant morbidity, and combining data from countries with different levels of wealth and access to healthcare systems. The high death rates from formula may be partly caused by pre-existing morbidity, partly by a lack of access to healthcare (to prevent formula-related morbidity turning into mortality), and partly by the use of formula in a particular environment. The weight of each component may vary from place to place. No estimates were given in their paper.

The authors assumed that the higher mortality levels would not occur among the babies of HIV-positive women because they would not be formula feeding because of preceding morbidity. However, this assumes that all HIV-positive women are healthy (i.e. that HIV infection does not constitute 'morbidity'). Even if none of them has AIDS, many HIV-positive women will have other forms of morbidity, such as low CD4 cell counts [2] or vitamin A deficiency [3]. Even those with no observable symptoms may have an abnormally high death rate [4]. The authors also neglected to define breastfeeding properly, and did not distinguish between exclusive breastfeeding for 6 months (as recommended by the World Health Organization and United Nations Children's Fund) and mixed and short-term breastfeeding. This led to general statements about breastfeeding which blurred the very different health outcomes associated with exclusive and various types of non-exclusive breastfeeding.

The authors assumed that HIV-positive mothers who choose to formula feed are comparable with those outside the HIV/AIDS context who formula feed for reasons other than preceding mother or child morbidity. If this is incorrect, mortality among babies of formula feeding HIV-positive mothers could be much higher.

It is invalid to compare rates of HIV infection, which will have health consequences throughout the life of a child, with the negative effects of formula feeding, which can also have health consequences throughout the life of a child, but that are only measured for a short time period. What HIV-positive mothers need to help them decide whether breastfeeding or formula feeding is the best feeding alternative is to have information on long-term health outcomes.

Expectant mothers should be made aware of the extremely high infant mortality rate among babies who were formula fed because of preceding morbidity (326.8/1000 over 2 years), and also be told that exclusive breastfeeding may minimize HIV transmission as well as maximizing overall health outcomes [5]. Women who are fully informed about the issues around HIV transmission and maternal and child health issues relating to infant feeding strategies would be in a better position to select the feeding method that will be best for their individual situation.

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In the HIV era, is breast always best? Response to 'Long-term infant health outcomes critical in feeding decisions by HIV-positive mothers' by Crowe *et al.*

We thank Drs Crowe and colleagues for their comments.

The authors state that our paper claims that 'formula feeding is better than breastfeeding for infants of HIV-positive mothers in less developed countries' because 'babies of mothers who chose to formula feed because of preceding morbidity in either mother or infant had lower

mortality rates than babies of mothers who chose to formula feed for other reasons'. This is incorrect, we clearly showed that mortality associated with formula feeding as a result of preceding morbidity is higher than formula feeding for voluntary reasons. We repeatedly reported higher child mortality rates in children who were never breastfed, compared with children who had been weaned. Our fundamental point is that the reason for not breastfeeding or weaning is important because mortality was consistently higher among children who were never breastfed or were weaned because of a preceding illness. There is clear evidence of reverse causality, whereby pre-existing morbidity may cause mothers not to breastfeed or to wean, and the resultant higher mortality risk arises from the preceding illness, rather than the mode of feeding *per se*. Such self-selection and reverse causality is also evident in other studies.

The authors also suggested confusion as a result of conflation between previous infant with maternal illness, and a combination of data across countries at different levels of development and healthcare access. The majority of reasons for not breastfeeding or weaning were a result of the child's preceding illness. Omitting cases of maternal illness did not change our findings. The country-specific estimates demonstrated similar patterns, and were therefore combined to get an aggregate measure. They suggest that we disaggregate the excess mortality into components such as health access, as well as formula-related risks. Unfortunately, the DHHS survey data do not provide this information and we have stated this as a limitation of the study. However, it is striking that the findings were consistent in all countries examined, which indicates a consistent effect, irrespective of the level of development or healthcare. They also suggest that maternal immune status, micronutrient deficiencies and maternal symptomatology could increase the risk to the infant via maternal morbidity. We cannot speculate on this question because no data are available. They also recommend that we differentiate between exclusive and partial breastfeeding, but again, DHHS data are not available to address this issue.

The authors also question whether our findings can be applied to children of HIV-infected mothers who voluntarily decide not to breastfeed. We suggest that counselling HIV-positive mothers on methods of feeding should recognize that current child mortality estimates for non-breastfeeding are biased overestimates, because they do not adjust for the reverse causality described above. Although breastfeeding results in lower infant and child mortality rates than formula feeding in non-HIV-infected women, this risk/benefit is reversed with HIV because of the high rates of mother-to-child HIV transmission via breast milk. Although we agree with the authors that a more specific definition of breastfeeding (i.e. mixed or exclusive) is important, this information is not available in cross-sectional surveys such as the DHHS and we suggest that prospective studies are needed on methods of feeding and reasons for weaning or the non-initiation of breastfeeding.

Finally, it is argued that it is invalid to compare rates of HIV infection, which have long-term consequences for survival with the possible adverse effects of formula feeding, which are likely to be short term. We profoundly disagree with this view. The median survival time of HIV-infected children in Rakai, Uganda, is 2 years [1]. In the absence of antiretroviral therapy, all pediatric HIV infections are likely to be fatal. Given the high rates of mother-to-child transmission via breast milk, and the unconfirmed efficacy of exclusive breastfeeding for the prevention of mother-to-child transmission, based on one observational study [2], we believe that it is only reasonable to offer women the alternative of not breastfeeding in order to select the safest method for feeding infants.

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Intentional self-inoculation with HIV-positive blood: a case series from the Centers for Disease Control and Prevention HIV/AIDS Surveillance System

Up to December 2001, 816 149 patients with AIDS were reported to the Centers for Disease Control and Prevention (CDC) and 256 cases with an unusual transmission mode have been documented [1]. These

data provide the largest population-based collection of HIV risk data available. Most individuals initially reported without risk information are subsequently found to have been exposed through sex or needle-sharing. Some individuals remain categorized as no identified risk because of incomplete health department investigations as a result of the patient's death, refusal to grant an interview, or loss to follow-up. Individuals whose HIV exposure category may be unusual have always been a high priority for follow-up because of the potential public health implications (e.g. occupational transmission) [2]. In this report, we document seven cases of intentional self-inoculation with HIV-contaminated needles.

State and local health departments collect surveillance information regarding patient demographics and HIV risk factors and report them to the CDC, based on a mutually exclusive hierarchy of risks [1]. Cases with no identified risk or suspected unusual transmission are further investigated by local or state health departments [2]. These investigations include a review of the available medical records, and when possible, interviews with healthcare providers, case patients or their proxies.

We defined intentional self-inoculation as a self-report from an HIV-infected individual of intentionally inoculating oneself with HIV-contaminated blood, serum or body fluids at least once, before receiving a diagnosis of HIV or AIDS and regardless of other transmission risks. This includes intentionally sticking oneself with a needle used to draw blood or other body fluids from an individual known to be HIV positive. State and local health departments collected information on the circumstances surrounding exposure by reviewing medical records and interviewing patients or their proxies. The protocol for follow-up investigations was reviewed and approved by the Institutional Review Board of the CDC.

We identified seven cases of intentional self-inoculation with HIV-positive material (Table 1). Of these, six are known to have died, and the median time from HIV diagnosis to death was 71 months. All were white and four were women. Five worked in healthcare settings with access to contaminated sharps; one was a caregiver for family members with AIDS, and one had access to HIV-contaminated material through an HIV-positive friend. Five out of the seven had been diagnosed with depression before the inoculation event, and most had other diagnosed psychiatric disorders based on medical record review or patient self-report.

The routine follow-up of cases initially reported with no HIV risk information has allowed documentation of unusual circumstances of HIV transmission [2–4]. However, it is unlikely that HIV/AIDS surveillance data routinely detect self-inoculation cases because individuals are reluctant to disclose or may refuse an interview that could identify self-inoculation as a possible exposure mode. Because these investigations were initiated after the cases were reported to the surveillance system, direct follow-up with patients to allow a more detailed psychological profile was difficult. This is of concern, given that healthcare workers with a diagnosis of depression were highly represented in this case series. Although DNA sequencing was not available to confirm strain relatedness, the self-report and access to infectious material of all affected individuals makes self-inoculation a plausible transmission mode.

Although these may be extreme examples of individuals who deliberately put themselves at risk of HIV, they also illustrate an important challenge for HIV prevention efforts. More common than self-inoculation may be the deliberate practice of risky sexual or drug-use behaviors among those who seek out HIV infection or are indifferent to its consequences [5]. HIV prevention efforts, like those offered during counselling and testing sessions, or during prevention case management for high-risk HIV-negative individuals, should ideally include an appraisal of psychiatric co-morbidities that are known to impact high-risk behaviors [6]. As HIV-positive individuals are not generally interviewed by HIV surveillance staff, surveillance data cannot document how widespread this phenomenon may be, but all cases of unusual transmission will continue to be a high priority for follow-up investigation.

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Table 1. Characteristics of HIV-infected individuals who reported intentional self-inoculation with HIV-contaminated material, United States 1981–2001.

Case[a]	Age decade	Sex	Marital status	Occupation	CD4 cell count nearest the first HIV-positive test result (date of CD4 cell count)	Last HIV-negative test result	Inoculation date	Inoculation material	First HIV-positive test	AIDS diagnosis date	Psychiatric co-morbidity[b]	Death date
A	30s	Female	Single	Medical technician	151 (8–90)	March 1988	June 1988	IM injection with serum, twice over 2 weeks	June 1990	October 1992	Substance abuse; depression	November 1994
B	40s	Female	Married	Medical technician	110 (6–91)	Unknown	January 1989–January 1990	IM injection with serum, up to 20 times over 6 months	March 1989	June 1991	Psychiatric hospitalization; suicide attempts	December 1995
C	50s	Female	Married	Nurse	400 (4–92)	October 1991	October 1991	IM injection with whole blood from heparin lock, 3 times over 1 month	April 1992	NA	Substance abuse; suicide attempts	March 1998
D	20s	Male	Married	Caregiver to HIV-positive family members	311 (6–94)	October 1992	October 1992	IM injection with whole blood, 1 or more times	October 1993	April 1996	Depression	June 1999
E	30s	Male	Single	Respiratory therapist	42 (6–92)	Unknown	> 1 year before first HIV-positive test	Route unknown	June 1992	June 1992	Depression	November 1995
F	20s	Female	Single	Health aide	347 (9–94)	June 1990	Unknown	Repeated sticking self with needles used by a known HIV-positive friend	October 1990	August 1995	Suicide attempt; depression	August 1998
G	20s	Male	Single	Unknown	Unknown	None	1987	IM injection with whole blood, 1 or more times	December 1987	September 1992	Depression	Unknown

IM, Intramuscular.
[a]All cases were white.
[b]Diagnoses made before self-inoculation.

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Regulation of gene expression for mitochondrial transcription factors B1 and B2 in brown adipocytes. Opposite effects of differentiation and noradrenaline.

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ABSTRACT

Gene expression of the novel mitochondrial transcription factors B1 and B2 (TFB1M and TFB2M) is highly regulated in association with the mitochondrial biogenesis process that takes place during brown adipocyte differentiation, both in vivo and in cell culture. Positive agonists of differentiation such as insulin and rosiglitazone induce TFB1M and TFB2M gene expression in brown adipocytes. This contrasts with the housekeeping behavior of mitochondrial transcription factor A gene expression in brown adipose tissue, and suggests that TFB1M and TFB2M play a major role in the regulation of mitochondrial DNA expression in brown adipocytes. Noradrenaline down-regulates TFB1M and TFB2M gene expression in differentiated brown adipocytes , in a β-adrenergic-mediated, protein synthesis-independent, process. Thus, noradrenergic stimuli may act differentially on mitochondrial DNA gene expression depending on the differentiation status of brown adipocytes. It is concluded that changes in the expression of TFB1M and TFB2M may mediate the regulation of mitochondrial DNA expression in response to the cell differentiation status and extracellular signalling.

Key words:
mitochondria, mitochondrial DNA, brown adipose tissue, transcription factor, adipogenesis

INTRODUCTION

The control of the gene expression of components of the respiratory chain/oxidative phosphorylation system (OXPHOS) is an essential process associated with mitochondrial biogenesis and the adaptive response of mammalian cells and tissues to energy requirements. This requires coordinate regulation of the expression of nuclear-encoded and mitochondrial DNA (mtDNA)-encoded genes. The control of the expression of mtDNA is believed to be mediated by regulatory events acting on nuclear genes encoding for components of the mtDNA replication and transcription machinery. Among them, mitochondrial transcription factor A (TFAM) has been proposed to play a major role. It is a nuclear genome-encoded protein, member of the high-mobility group proteins, which binds mtDNA and is capable of activating mtDNA transcription "in vitro" [1] and “in vivo” [2] . Moreover, TFAM is involved in packaging mtDNA [3] and may also be involved in mtDNA replication [4]. In recent years, major advancements on the role of TFAM in mtDNA replication and transcription processes “in vivo” have been obtained, essentially by the use of animal models of targeted ablation of the TFAM gene and over-expression of TFAM in mice. These studies have demonstrated that TFAM is required for the maintenance of mtDNA during development [5] and is involved in the control of mtDNA copy number [6]. Whether TFAM behaves as a constitutive component of the mtDNA transcription (and perhaps replication) machinery or also a mediator of regulatory mechanisms, thus eliciting changes in mtDNA expression in response to developmental or environmental changes, is unknown.

The brown adipocyte is the mammalian cell specialized in non-shivering thermogenesis. Accordingly, brown adipocyte differentiation from precursor cells is associated with an enriched content of mitochondria, which are naturally uncoupled owing to the presence of the uncoupling protein-1 (UCP-1). Thus, there is remarkable plasticity in brown adipose tissue mitochondrial content according to the physiological status, i.e. thermal environment, dietary conditions and reproductive cycle [7,8], and thus brown adipose tissue (BAT) constitutes an optimal tissue model to study the molecular mechanisms involved in the regulation of OXPHOS gene expression in mammals.

Throughout the process of differentiation of brown adipocytes in primary culture from precursor cells, the relative mtDNA content and mtDNA expression are enhanced, in coordination with an increase in the expression of nuclear-encoded OXPHOS

components [9,10]. Similar events take place during brown adipose tissue differentiation in the last fetal period of mice development [10,11]. They are associated with the induction of the expression of master transcription factors for mitochondrial biogenesis such as nuclear respiratory factor-2 (NRF2/GABP) and the transcriptional co-activator PPARγ-coactivator-1α (PGC-1α) [10].

To our knowledge, the role of TFAM in mediating nuclear-gene regulation of mtDNA expression in brown fat has not been directly assessed. However, several observations indicate that TFAM is expressed in a constitutive manner during the process of brown adipocyte differentiation during development and, in C/EBPα-null mice, a model of impaired mitochondrial biogenesis and reduced mtDNA expression without changes in mtDNA content, TFAM expression is unaltered [12]. These observations suggest that changes in TFAM expression are not a major mechanism determining changes in the regulation of mtDNA transcription in association with BAT differentiation.

In 2002, two novel proteins required for mtDNA transcription, named mitochondrial transcription factors B1 and B2 (TFB1M and TFB2M), were identified in humans [13] and subsequently in mice [14]. They were reported to be required for the efficient transcription of mtDNA together with TFAM and mitochondrial RNA polymerase. Their expression was higher in tissues with high oxidative activity, like heart, liver and skeletal muscle, but no data is available on their expression in adipose tissues. TFB1M and TFB2M may play a relevant role in regulatory processes related to the control of mtDNA expression during mitochondrial biogenesis associated with brown adipocyte differentiation. In the present study we determined the regulation of TFB1M and TFB2M mRNA expression in several animal and cell models of physiological, genetic and pharmacological regulation of mitochondrial biogenesis in brown adipose tissue. This was compared with the analysis of TFAM mRNA expression and subunit II of cytochrome c oxidase (COII) mRNA, as a transcript product of mtDNA expression, and UCP-1 mRNA, the specific gene marker of brown adipocyte differentiation

MATERIALS AND METHODS

Insulin, 3,5,3'-triiodothyronine, noradrenaline, isoproterenol, propanolol, prazosin, CL316243, actinomycin D and cycloheximide were from Sigma (S. Louis,USA). Rosiglitazone was a gift from Dr.L.Casteilla (University of Toulouse, France).

For studies of gene expression in mouse tissues, Swiss adult male mice (two month-old) were killed by decapitation and skeletal muscle (gastrocnemius), liver, intercapular BAT and epididymal white adipose tissue (WAT) were removed and frozen in liquid nitrogen. For studies in C/EBPα-null mice, heterozygous female mice carrying the targeted deletion [15] were mated with heterozygous males. The day of pregnancy was determined by the presence of vaginal plugs as described above, and fetuses were obtained by Caesarean section of pregnant mice on day 17 of gestation. Neonates were studied at 2-4h after birth. Identification of fetuses and pups carrying the homozygous deletion of C/EBPα respect to wild-type littermates was performed by Southern blot analysis as described elsewhere [12]. The care and use of mice were in accordance with the European Community Council Directive 86/609/EEC and approved by the *Comitè Ètic d'Experimentació Animal of the University of Barcelona*

Brown adipocytes were differentiated in culture from precursor cells (stromal vascular fraction) as described elsewhere [10]. Stromal vascular cells were isolated from the interscapular, cervical and axilar depots of BAT of three-week-old Swiss mice. They were plated (5000 cells/cm2) and grown in Dulbecco's modified Eagle's medium: Ham's F12 medium (1:1, v/v) supplemented with 10% (v/v) fetal calf serum, 20 nM insulin, 2 nM 3,5,3'-triiodothyronine and 100 μM ascorbate ("differentiating medium"). Cells were harvested after 3, 5, 7, 9 or 11 days of culture. When indicated, after day 3, culture medium was replaced by Dulbecco's modified Eagle's medium: Ham's F12 medium (1:1, v/v) supplemented with 10% (v/v) delipidated fetal calf serum (Sigma) ("depleted medium"). When indicated, this medium was supplemented with either 20 nM insulin or 10 μM rosiglitazone. Treatment of cells with noradrenaline was performed by exposure of cells to 0.5 μM arterenol bitartrate. When indicated, differentiated brown adipocytes were treated with 1 μg/ml actinomycin D and, for studies on the role of protein synthesis, with 5 μg/ml cycloheximide, in accordance with previous studies using these chemicals in brown adipocyte cultures [9,16].

For transmission electron microscopy analysis, BAT samples or cell pellets

obtained after harvest in phosphate-buffered saline and centrifugation at 500g. for 5 min, were fixed in 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and post-fixed in 1% osmium tetroxide and 0.8% FeCNK in phosphate buffer. After dehydration in a graded acetone series, they were embedded in Spurr resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a Hitachi H600AB transmission electron microscopy at 75kV.

RNA was extracted by an affinity-column based method (Qiagen). For Northern blot analyses 25 μg of total RNA was denatured, electrophoresed on 1.5% formaldehyde-agarose gels and transferred to nylon membranes (N+, Boehringer Mannheim, Germany). Equivalent amounts of ribosomal RNA in the samples were checked by ethidium bromide u.v. visualization. Prehybridization and hybridization were performed at 55ºC in a 0.25M Na_2HPO_4 (pH 7.2), 1 mM EDTA, 20 % SDS, 0.5% blocking reagent (Boehringer Mannheim, Germany) solution. Blots were hybridized using the rat cDNA for TFAM [17], murine cDNAs for TFB1M and TFB2M [14], and the cDNA for rat COII [18] and rat UCP-1 [19]. The DNA probes were labelled using [α-32P]dCTP by the random oligonucleotide-priming method. Hybridization signals were quantified by densitometric analysis (Phoretics 1D software, Phoretics International LTD, Newcastle, UK). Statistical analysis was performed by the Student's t test.

Results

TFB1M and TFB2M mRNAs are highly expressed in brown adipose tissue.

The expression of the mRNAs for TFB1M, TFB2M and TFAM were analyzed in BAT and WAT and compared with skeletal muscle and liver, tissues which have been reported to express these genes at a high level. TFAM mRNA expression was similar in brown fat, liver and muscle. TFB1M mRNA was more expressed in BAT than in muscle but similarly to liver, whereas TFB2M mRNA expression was higher in BAT than in the other tissues. The expression of TFB2M mRNA paralleled that of COII mRNA, a marker gene of mitochondrial genome expression (Fig 1A). TFAM mRNA was slighlty more expressed in BAT than in WAT, while TFB1M and TFB2M mRNAs expression was dramatically higher in BAT. The high expression of TFB1M and TFB2M mRNAs in BAT was similar to that observed for COII mRNA but differed

from the expression of a marker gene of overall adipocyte differentiation, aP2/FABP mRNA, which was slightly more expressed in WAT than in BAT (Fig 1B).

Impaired mitochondrial biogenesis due to C/EBPα gene disruption is associated with a down-regulation of TFB1M and TF2BM mRNAs

In order to ascertain the relationship between TFB1M and TF2BM expression and mitochondrial biogenesis in BAT, the C/EBPα gene disruption model (C/EBPα-null mice) was used. In agreement with a previous report [12], C/EBPα-null mice showed impaired mitochondrial biogenesis during late fetal development (Fig 2A). Size, overall structure and cristae organization were massively disrupted in C/EBPα-null fetuses on day 17. At birth, mitochondrial structure improved, although there was not full normalization. TFAM mRNA expression was not significantly altered in C/EBPα-null mice compared with wild types at any development period. TFB1M and TFB2M mRNAs were expressed at higher levels in fetal respect than in neonatal BAT in wild type mice and, in contrast with TFAM mRNA, they were strongly down-regulated in C/EBPα-null fetuses. This was associated with impaired COII mRNA expression in the fetal period but not in neonates, in agreement with previous reports using other mitochondrial genome expression markers [12]. UCP-1 mRNA, a late differentiation marker of BAT, was markedly reduced both in C/EBPα-null fetuses and neonates.

TFB1M and TFB2M expression in brown adipocytes throughout differentiation in primary culture

Primary culture of brown adipocyte precursor cells recapitulate the process of differentiation of the brown adipocyte “in vivo”, including lipid accumulation, mitochondrial biogenesis and expression of gene markers corresponding to these processes, as described elsewhere [9, 10, 20] . Changes in the expression of TFAM, TFB1M and TFB2M mRNAs were determined throughout the differentiation process of pre-adipocytes to brown adipocytes and compared with other gene markers of differentiation (Fig 3). TFAM mRNA expression was poorly regulated, whereas TFB1M mRNA and TFB2M mRNA were dramatically up-regulated during the differentiation process. TFB1M was progressively induced during the culture, quite similarly to the profile of COII mRNA, whereas TFB2M mRNA expression showed a more sudden induction after the first days of culture like UCP-1 mRNA. In any case, the expression of either TFB1M mRNA or TF2BM mRNA in pre-adipocytes (day 3 of

culture) was less than 20% of that in differentiated brown adipocytes (days 7-9 of culture).

TFB1M and TF2BM mRNA expression depends on the brown adipocyte differentiation and mitochondrial biogenesis status. Effects of insulin and rosiglitazone.

To establish whether the high TFB1M and TFB2M mRNA expression in brown adipocytes was due to the process of differentiation itself or to the arrest of proliferation associated with the acquisition of a brown adipocyte-specific differentiated status, a second model of cell culture was used in which pre-adipocytes were cultured in a lipid-depleted medium not permissive for differentiation ("depleted medium") [21]. This leads to pre-adipocyte proliferation until confluence and at the end of the culture (day 8), cells remain non-differentiated as evidenced by the lack of lipids accumulation and fibroblast-like morphology (Fig 4, left). Together with the lack of lipids, mitochondrial biogenesis is severely impaired in these cells, as reflected by the small size and poor development of cristae (Fig 4 right).

TFAM mRNA levels in non-differentiated cells were not significantly altered compared with those in differentiated brown adipocytes. TFB1M mRNA and TFB2M mRNA expression was dramatically reduced, as observed for COII and UCP-1 mRNAs (Fig 5).

The expression of TFB1M mRNA and TF2M mRNA was analyzed when insulin or the PPARγ agonist rosiglitazone, which can favor differentiation, were added to the depleted medium. The addition of insulin resulted in partial acquisition of brown adipocyte morphology at day 8 of culture and cells showed lipid accumulation but to a lower extent than controls with differentiating medium. Rosiglitazone led to a fully differentiated brown adipocyte morphology, similar to that achieved with the differentiating medium (not shown). Accordingly, insulin partially restored the expression of marker genes for brown adipocyte differentiation (COII mRNA and UCP-1 mRNA). However, rosiglitazone fully normalized COII mRNA but still reduced UCP-1 mRNA levels (Fig 5). The pattern of expression of TFB1M and TFB2M mRNAs closely followed that of COII mRNA: insulin and rosiglitazone restored mRNA expression levels partially and completely, respectively, respect to differentiated brown adipocytes

Noradrenaline down-regulates TFB1M and TF2M mRNA expression in differentiated brown adipocytes. Effects of insulin and rosiglitazone.

The effects of noradrenaline, the main activator of the thermogenic activity of brown adipocytes, on TFB1M and TFB2M mRNA expression was determined. In differentiated brown adipocytes (day 8 of culture in differentiating medium), noradrenaline treatment moderately but significantly reduced TFAM mRNA expression, whereas TFB1M and TFB2M mRNA levels were down-regulated to around 20-30% of control values (Fig 6). TFAM mRNA levels were not significantly affected when noradrenaline was added either to pre-adipocytes (day 4 of culture) or to non-differentiated cells cultured in depleted medium (day 8). The same occurred for TFB2M mRNA. TFB1M was down-regulated by noradrenaline in pre-adipocytes but not in non-differentiated cells on day 8. When differentiation was restored by supplementation of depleted medium with insulin or rosiglitazone, the sensitivity to down-regulate the mRNAs of TFAM, TFB1M and TFB2M in response to noradrenaline was also restored. COII mRNA levels revealed a similar pattern of behavior but a less intense down-regulation by noradrenaline. In contrast, noradrenaline dramatically up-regulated the UCP-1 mRNA levels in differentiated brown adipocytes, as reported elsewhere [20]. Noradrenaline also induced UCP1 mRNA levels in pre-adipocytes but to a very low extent. In cells cultured in depleted medium, either alone or supplemented with insulin, noradrenaline induced UCP1 mRNA expression but to a minor extent than in differentiated cells. Only the addition of rosiglitazone completely restored the responsiveness of UCP-1 mRNA to noradrenaline.

TFB1M and TFB2M mRNA levels are down-regulated by noradrenaline through a β-adrenergic pathway.

To establish the pathways involved in the inhibitory effects of noradrenaline on mitochondrial transcription factors compared with the activating effects on UCP-1 mRNA, differentiated brown adipocytes were exposed to several drugs capable of mimicking or inhibiting adrenergic pathways (Fig 7). The β-adrenergic agonist isoproterenol mimicked all the effects of noradrenaline, i.e. the down-regulation of the mRNAs for mitochondrial transcription factors and COII mRNA and the induction of UCP-1. A similar behavior was observed after exposure to the β3 adrenergic activator CL316243. The capacity of propanolol (a β-adrenergic inhibitor) or prazosin (an α-adrenergic inhibitor) to influence the responsiveness to noradrenaline was also determined. Propanolol abolished the down regulation of TFAM, TFB1M and TFB2M mRNAs elicited by noradrenaline, and significantly reduced the extent of induction of

UCP-1 mRNA. In contrast, the effects of noradrenaline, either down-regulating mitochondrial transcription factors and COII mRNA or up-regulating UCP-1 mRNA, were unaffected by prazosin. This indicates that β-adrenergic pathways involving β3-mediated regulation are responsible for the down-regulation of mitochondrial transcription factors as they are for most of the induction of UCP-1 mRNA expression.

The down-.regulation of TFB1M and TF2BM mRNAs by noradrenaline occurs through the inhibition of gene transcription.

To check the involvement of gene transcription in the observed effects of noradrenaline on TFB1M mRNA and TFB2M mRNA expression, cells were treated with the inhibitor of transcription actinomycin D and exposed or not to noradrenaline (Fig 8) The half-life of TFB1M and TF2BM mRNAs followed as their mRNA decay after actinomycin D treatment was around 2h and 8h, respectively. Treatment of control brown adipocytes with noradrenaline caused a time-dependent decrease in TFB1M mRNA and TF2BM mRNA levels similar to that elicited by actinomycin D. When cells had been treated with actinomycin D, noradrenaline did not further decrease TFB1M and TF2BM mRNA levels. This indicates that gene transcription is required for the inhibitory effects of noradrenaline on TFB1M and TF2BM mRNA expression. The same requirement for transcription was observed for the COII mRNA decrease in response to noradrenaline.

The effects of noradrenaline on TFB1M mRNA and TFB2M mRNA do not depend on protein synthesis .

To test whether the inhibitory action of noradrenaline on TFB1M mRNA and TFB2M mRNA expression depends on protein synthesis, cells were treated with the protein synthesis inhibitor cycloheximide and exposed to noradrenaline (Fig 9). The presence of cycloheximide alone had minor effects on TFB1M mRNA, TFB2M mRNA and COII mRNA expression. In the presence of cycloheximide, the decay in TFB1M mRNA and TF2BM mRNA elicited by noradrenaline was almost identical to that elicited in the absence of cycloheximide. In contrast, treatment with cyclohexime abolished the reduction in COII mRNA caused by noradrenaline. This indicates that noradrenaline effects on COII mRNA are dependent on protein synthesis unlike its effects on TFB1M and TF2BM mRNA expression.

Discussion

The present study was undertaken to characterize the gene expression regulation of two novel mitochondrial transcription factors, TFB1M and TFB2M, in association with mitochondrial biogenesis in BAT. It constitutes the first report on the regulation of the TFB1M and TFB2M genes and indicates that their expression is highly regulated in association with brown adipocyte differentiation and in response to extracellular signaling. This contrasts with the expression of the other mitochondrial transcription factor, TFAM, which shows a housekeeping behavior in brown adipose tissue. Moreover, changes in gene expression occurred similarly for TFB1M and TFB2M and no major differences were observed for the responsiveness of these two genes to physiological and experimental manipulations of brown adipose tissue and brown adipocytes.

The close association between TFB1M and TFB2M gene expression and mitochondrial biogenesis in BAT is evidenced in experimental models "in vivo" (when mitochondrial biogenesis is impaired, as in C/EBPα-null mice) and "in vitro" (when mitochondrial biogenesis is induced throughout brown adipocyte differentiation in cell culture). BAT from C/EBPα-null mice does not show reduced amounts of mtDNA, and impaired mitochondrial biogenesis is associated with a specific reduction in mtDNA-encoded mRNAs [12]. The down-regulation of TFB1M and TFB2M expression, early in C/EBPα-null fetuses, is consistent with their playing a major role in mtDNA transcription during BAT differentiation "in vivo". On the other hand, although the differentiation of brown adipocytes in cell culture is associated with a slight increase in mtDNA abundance, the induction of mtDNA-encoded transcripts is of higher magnitude [9], and TFB1M and TFB2M up-regulation may enhance mtDNA gene expression through activation of mtDNA transcription.

Although the molecular signals inducing TFB1M and TFB2M gene expression in association with the mitochondrial biogenesis during brown adipocyte differentiation have not been established here, several observations suggest the involvement of master transcription factors of brown adipocyte differentiation, such as CCAAT enhancer-binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ). The positive action of rosiglitazone, a specific PPARγ activator, on TFB1M and TFB2M gene expression points to PPARγ as a regulator candidate, and C/EBPα also

deserves further research in the light of the present observations in C/EBPα-null mice. In fact, computer assisted analysis (Matinspector program) of the 5' non-coding regions of the TFB1M and TFB2M murine genes (accession numbers NT039636 and NT039188) reveals the presence of putative C/EBP regulatory sites in both genes (unpublished observations). Similarly, the potential regulation of TFB1M and TFB2M gene transcription by the nuclear respiratory factor NRF2/GABP should be analyzed in the future, considering the presence of consensus binding sites for this transcription factor in the 5' non-coding regions of murine and human TFB1M and TFB2M genes [13,14] and the relevance of NRF2/GABP in the control of OXPHOS gene expression in brown adipocytes [9]. Finally, PGC-1α, as a co-activator involved in brown adipocyte differentiation and mitochondrial biogenesis [22] may be involved in the induction of TFB1M and TFB2M gene expression through co-activation of PPARγ or the induction of NRF2/GABP.

Whereas the regulation of TFB1M and TFB2M gene expression is consistent with the mitochondrial biogenesis process and the associated enhancement of mtDNA expression, a more complex relationship between TFB1M and TFB2M and brown adipocyte function is evidenced by the observed down-regulation of TFB1M and TFB2M mRNA expression in response to noradrenaline in differentiated brown adipocytes. This effect involves β3-adrenergic activation and occurs through mechanisms that do not require protein synthesis but rather gene transcription. This suggests the presence of negative responsive elements, sensitive to adrenergic effects, in the transcriptional regulatory regions of the TFB1M and TFB2M genes.

Noradrenaline enhances brown adipose tissue thermogenesis, but its action on mitochondrial biogenesis is unclear. In vivo, the long-term exposure of rodents to noradrenaline increases BAT mass and mitochondrial content, which is mainly due to the stimulation of differentiation of the pre-adipocytes present in the tissue into mature brown adipocytes [23]. The noradrenergic stimulus of brown pre-adipocytes in culture is a positive signal for differentiation, including mitochondrial biogenesis [24]. These observations are consistent with the present findings, indicating that noradrenaline does not down-regulate TFAM, TFB2M or COII gene expression in brown pre-adipocytes although they contrast with the down-regulation of the TFB1M gene.

These findings indicate that noradrenaline exerts its major effects down-regulating the expression of components of the mtDNA expression machinery when

brown adipocytes are already differentiated, even when differentiation is achieved by culture with regular medium or under the stimulus of rosiglitazone. In differentiated brown adipocytes, noradrenaline slighlty but significantly down-regulates the levels of the mtDNA-encoded COII mRNA. The marked down-regulation of TFB1M and TFB2M gene expression in response to noradrenaline may be responsible for such effect, especially considering that COII mRNA down-regulation is protein synthesis-dependent, unlike TFB1M and TFB2M reduction. The distinction between the biological effects of noradrenaline in pre-adipocytes and in differentiated adipocytes is consistent with the β3-adrenergic mechanism observed for TFB1M and TFB2M down-regulation in response to noradrenaline. It has been established that during differentiation there is a shift from the prevalence of β1-adrenergic receptors in brown pre-adipocytes to a major presence of β3-adrenergic receptors when brown adipocytes are already differentiated [25] and noradrenaline may repress TFB1M and TFB2M preferentially in cells with an active β3-adrenergic pathway, i.e. differentiated brown adipocytes. However, the concomitant occurrence of the β1-dependent pathway cannot be excluded, especially considering that TFB1M gene expression retains its responsiveness to noradrenaline in pre-adipocytes.

The biological significance of TFB1M and TFB2M down-regulation by noradrenaline in differentiated brown adipocytes is unknown. Differentiated brown adipocytes are specialized in relatively short-term thermogenic response. Thus, the acute effects of noradrenaline may be more relevant for a rapid enrichment of UCP-1 in existing mitochondrial through the activation of UCP-1 gene transcription than for the complex and long-lasting process of mitochondrial biogenesis. However, this does not explain why noradrenaline not only does not induce TFB1M and TFB2M gene expression but it markedly down-regulates these genes. Moreover, the effects of noradrenaline down-regulating components of the OXPHOS system appear not to be restricted to mtDNA since the expression of, at least, the nuclear-encoded subunit IV of cytochrome c oxidase is also down-regulated (unpublished observations). It should also be noted that noradrenaline induces PGC-1α gene expression in brown adipocytes [26] and this co-activator is claimed to play a role in mitochondrial biogenesis in brown adipocytes, at least regarding the differentiation process. The rise in PGC-1α in response to noradrenaline in differentiated brown adipocytes, when their mitochondrial content is already high, may be more related to the specific induction of UCP-1

transcription, as proposed recently [27], rather than to mitochondrial biogenesis. Finally, the present data on the down-regulation of the TFB1M and TFB2M genes by noradrenaline should be added to a recent report showing that the expression of PPARγ, a master transcription factor for adipogenesis, is also down-regulated in response to noradrenaline in brown adipocytes, whose functional significance is also unknown [28].

In conclusion, our results suggest that the regulation of gene expression for TFB1M and TFB2M can be a major mechanism for the control of mtDNA gene transcription during the mitochondrial biogenesis process associated with brown adipocyte differentiation. Accordingly, positive stimuli of brown adipocyte differentiation, such as those elicited by insulin and PPARγ activation, up-regulate TFB1M and TFB2M gene expression. In contrast, noradrenaline represses TFB1M and TFB2M gene expression in brown adipocytes, especially when these are already differentiated, thus pointing to a differential role of noradrenergic pathways in the control of mitochondrial biogenesis and mtDNA expression depending on the differentiated status of brown adipocyte cells.

In recent years, alterations in mtDNA function due to genetic alterations or pharmacological treatments have been identified as responsible for several human adipose tissue disturbances such as lipodystrophies and lipomatosis [29,30]. The identification of the novel mitochondrial transcription factors TFB1M and TFB2M as pivotal actors in the control of mtDNA expression machinery may open new directions in the understanding of the pathophysiology of adipose tissues disturbances due to mitochondrial dysfunction and the identificaction of novel therapeutic targets.

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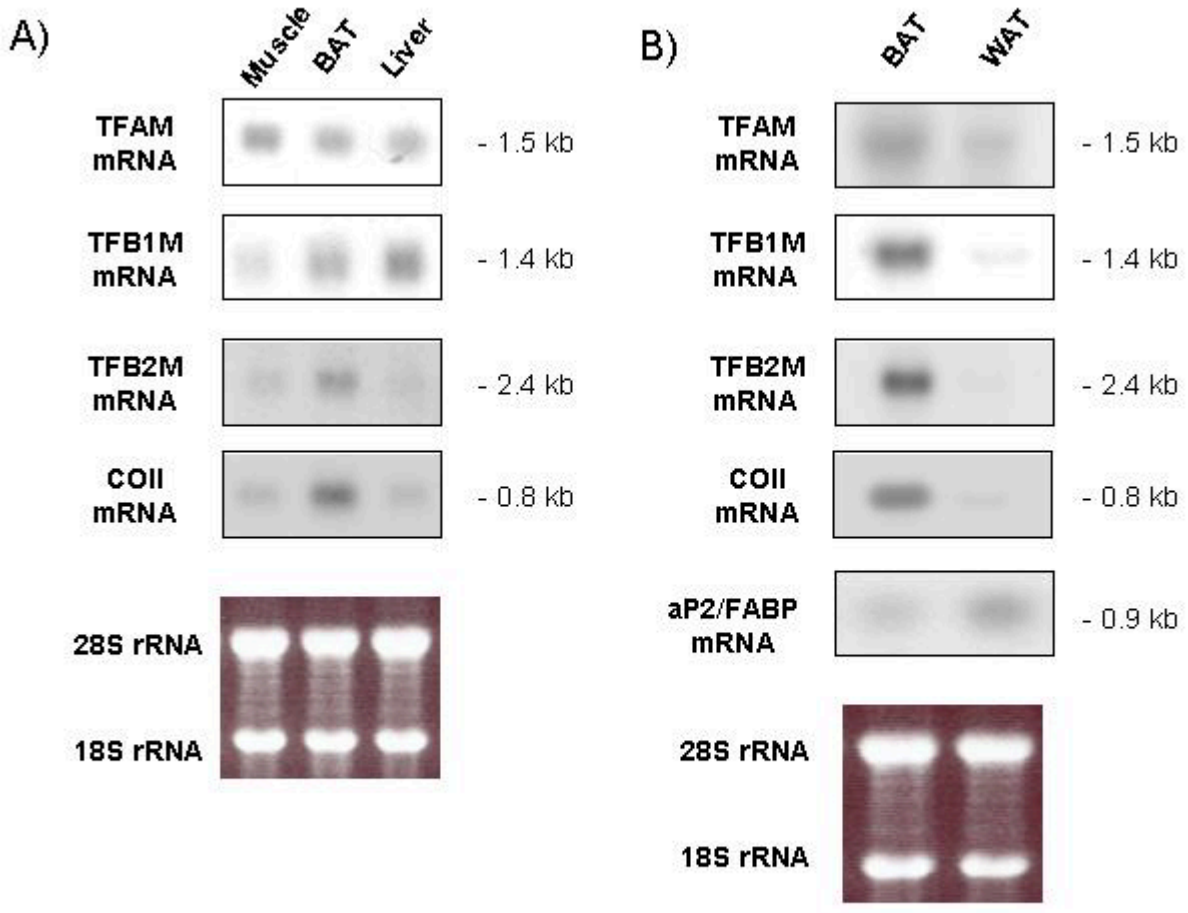
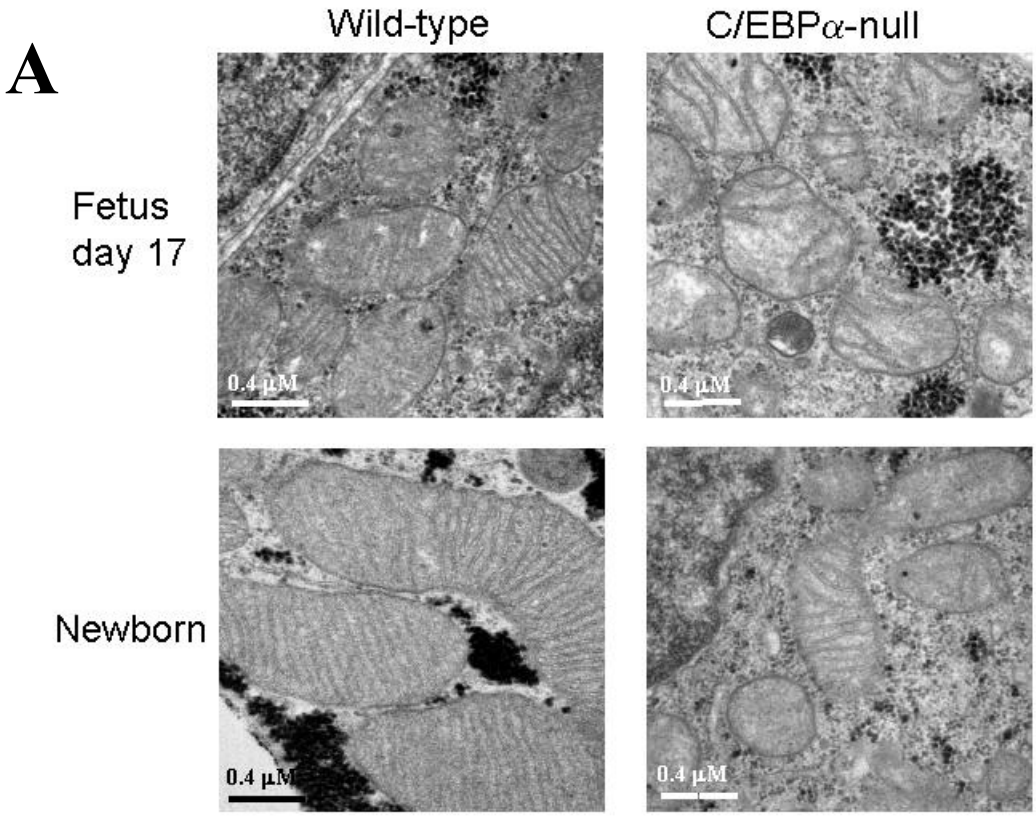


Figure 1

TFAM, TFB1M and TFB2M mRNA expression in brown adipose tissue. Examples of Northern blot analysis (25 μg RNA/lane) of TFAM, TFB1M and TFB2M transcripts in BAT compared with other murine tissues (A) or to WAT (B). Expression of COII mRNA and aP2/FABP mRNA is shown for comparison. Transcript sizes are shown on the right. Controls of equal loading of RNA were established by ethidium bromide staining.


A
Wild-type
C/EBPα-null
Fetus
day 17
Newborn
0.4 µM
0.4 µM
0.4 µM
0.4 µM

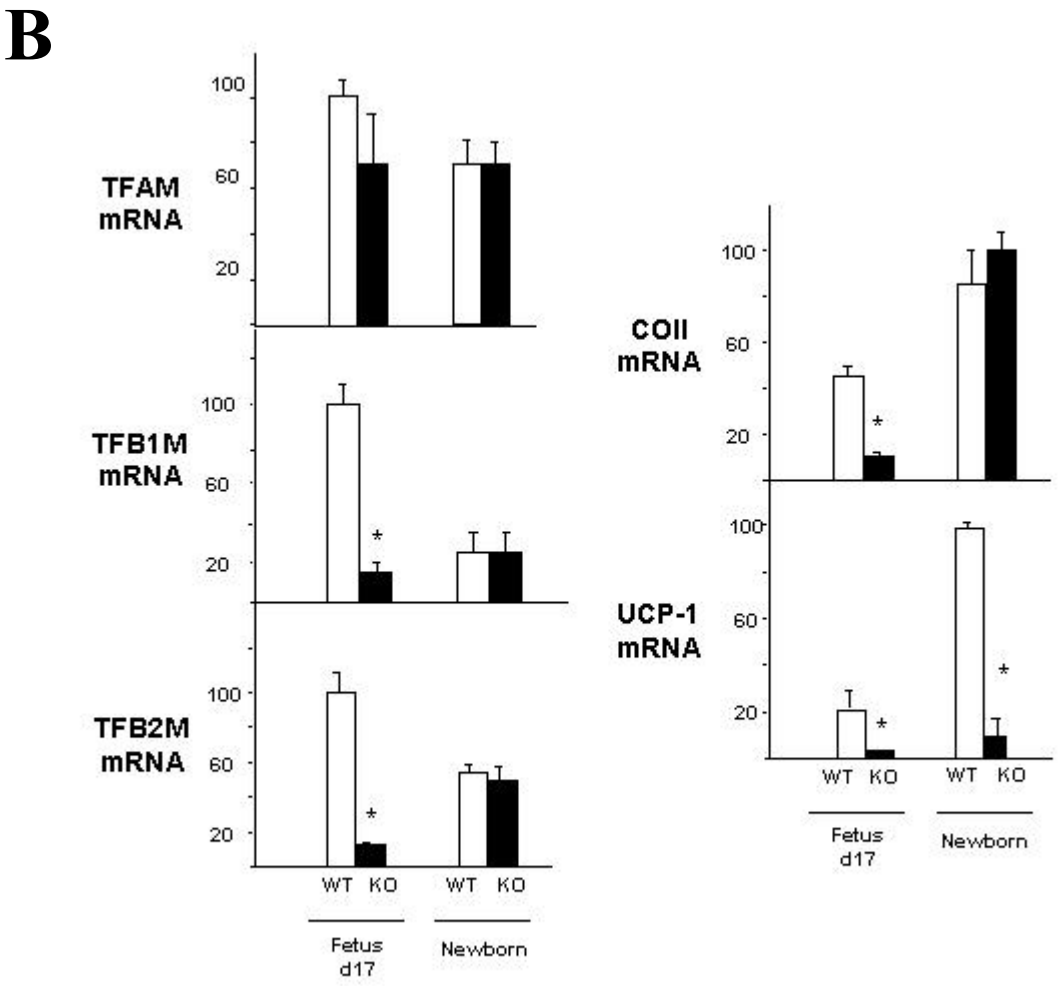


Figure 2

Mitochondrial morphology and TFAM, TFB1M and TFB2M mRNA expression in brown adipose tissue from C/EBPα-null mice. A, Example of transmission electron microscopy analysis of mitochondria morphology in brown adipose tissue from wild-type and C/EBPα-null mice. Scale bars show magnification. B, Bars are means ± SEM of three independent experiments for every age and genotype and are expressed as percentages relative to the mean value at the time of maximum levels of expression which was set to 100 (arbitrary units). Relative abundance of the mitochondrial transcripts TFAM, TFB1M and TFB2M mRNAs was determined by Northern blot analysis of 25 μg RNA and compared with expression of COII and UCP-1 mRNAs Controls of equal loading of RNA by ribosomal ethidium bromide staining were established as in Fig 1. Statistical differences ($p < 0.05$) between wild-type and C/EBPα-null mice are indicated as *.

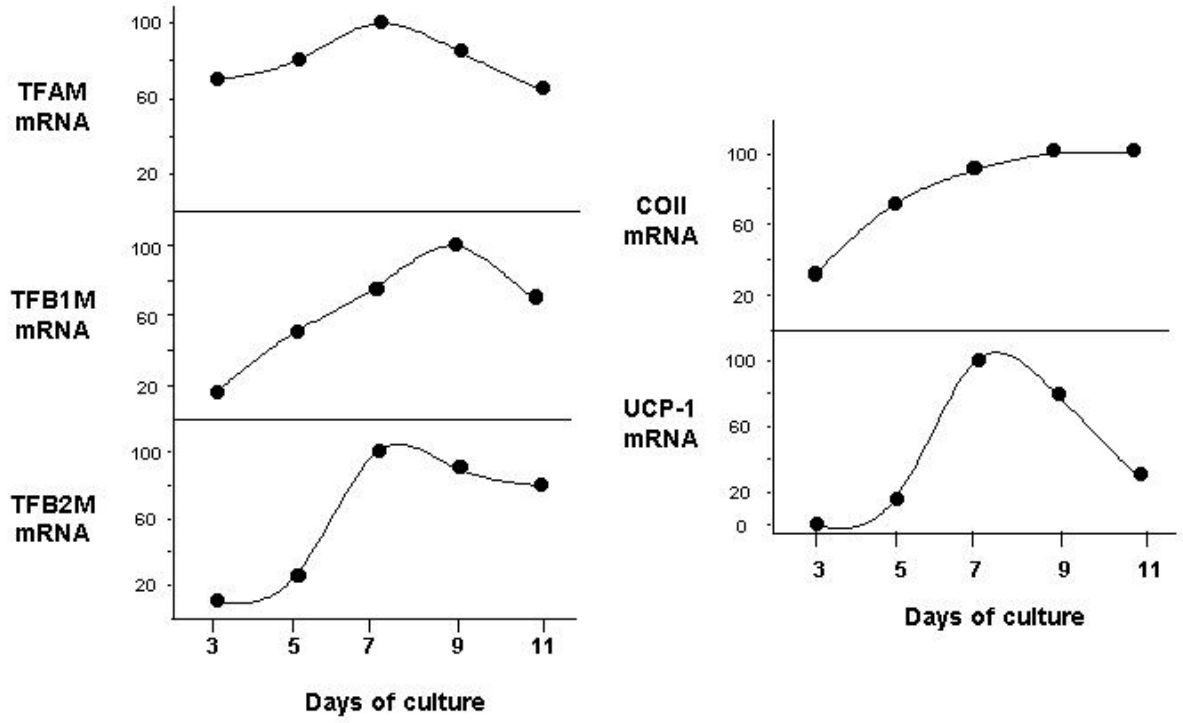


Figure 3

Time-course of TFAM, TFB1M and TFB2M mRNA expression during brown adipocyte differentiation in culture. Points are means of two-three independent experiments from every time of culture and are expressed as percentages relative to the mean value at the time of maximum levels of expression which was set to 100 (arbitrary units). Relative abundance of TFAM, TFB1M and TFB2M mRNAs was determined by Northern blot analysis of 25 μg RNA and compared with the expression of COII and UCP-1 mRNAs. Controls of equal loading of RNA by ribosomal ethidium bromide staining were established as in Fig 1.

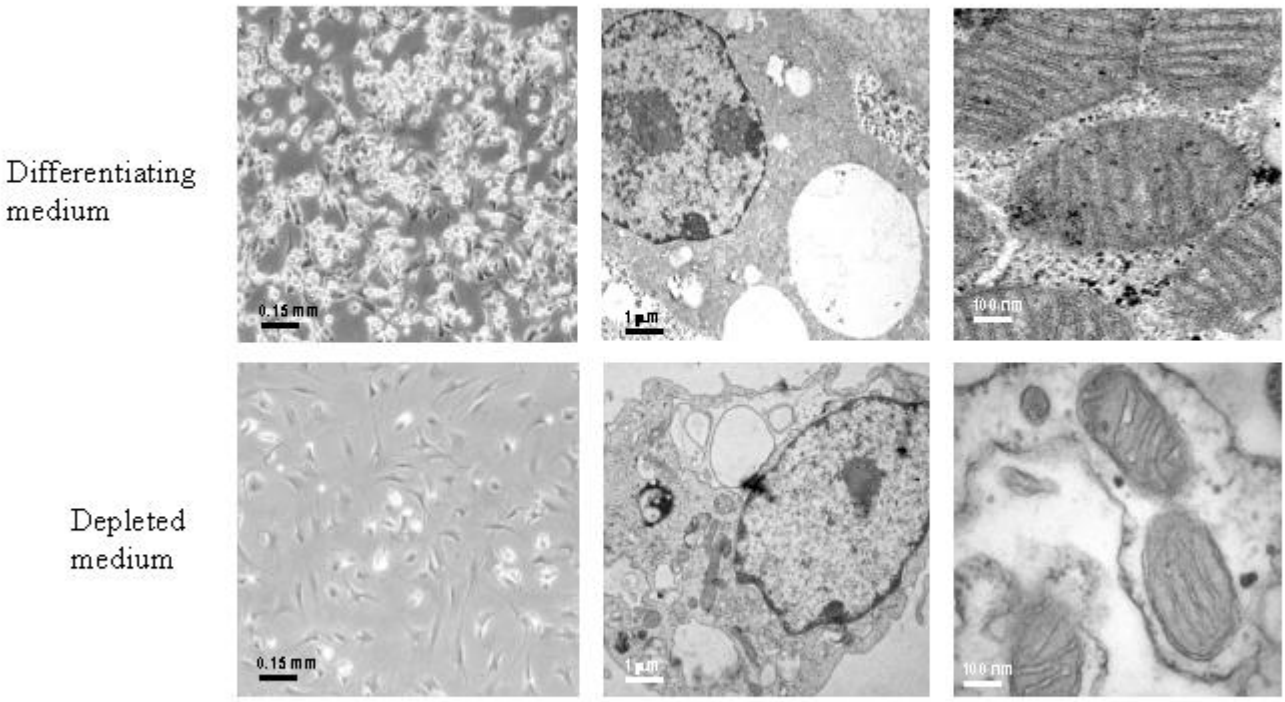


Figure 4
Differentiation-dependent mitochondrial biogenesis in brown adipocytes. Brown adipocyte precursor cells were cultured for 8 days in differentiating medium or in depleted medium, and analyzed for overall morphology (phase-contrast photomicrographs in the left panels) and transmission electron microscopy for mitochondrial morphology (central and right panels). Scale bars show magnification.

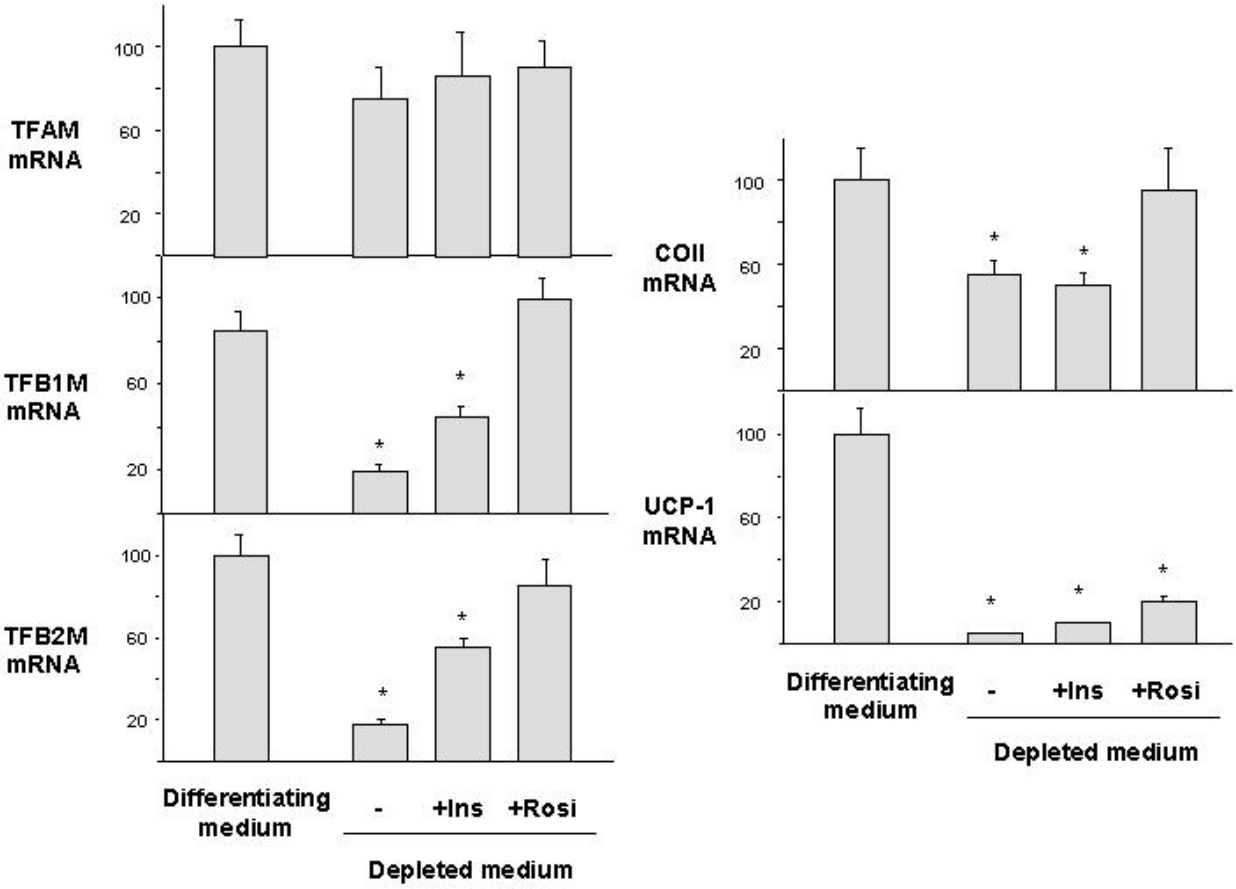


Figure 5

Differentiation-dependent expression of TFAM, TFB1M and TFB2M mRNAs. Effects of insulin and rosiglitazone. Brown adipocyte precursor cells were cultured for 8 days in differentiating medium or in depleted medium supplemented or not with 20 nM insulin or 10 μM rosiglitazone (see Methods section). Relative abundance of TFAM, TFB1M and TFB2M mRNAs was determined by Northern blot analysis of 25 μg RNA and compared with the expression of COII and UCP-1 mRNAs. Bars are means ± SEM of three independent experiments and are expressed as percentages relative to the mean value in brown adipocytes cultured in differentiating medium which was set to 100 (arbitrary units). Controls of equal loading of RNA by ethidium bromide staining were established as in Fig 1. Statistical differences ($p < 0.05$) from the "differentiating medium" group are indicated as *.

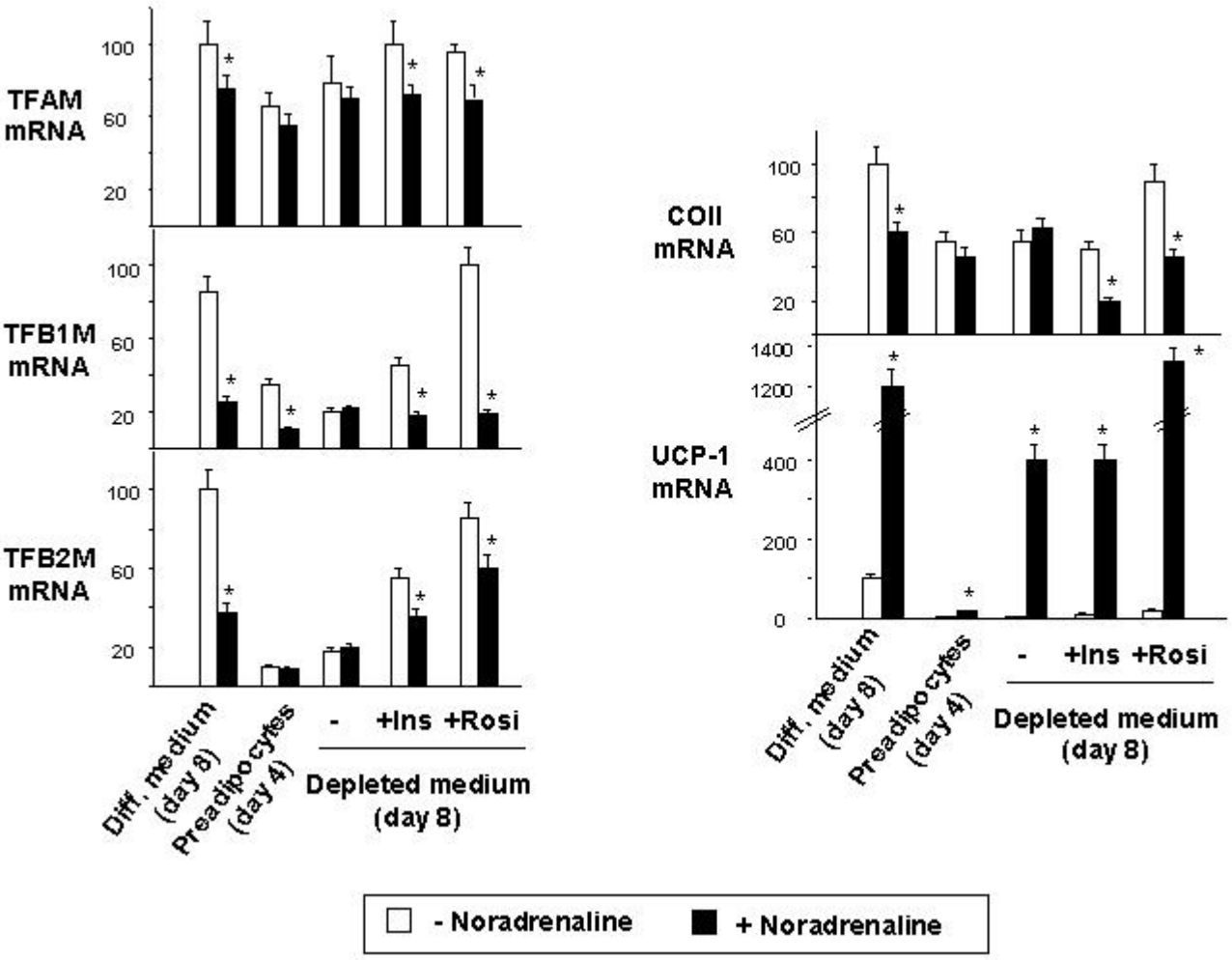


Figure 6

Effects of noradrenaline on TFAM, TFB1M and TFB2M mRNAs in brown adipocytes at various stages of differentiation. Brown adipocyte precursor cells were cultured for 8 days or for 4 days (pre-adipocytes) in differentiating medium or for 8 days in depleted medium supplemented or not with 20 nM insulin or 10μM rosiglitazone (see Methods section), and treated or not with 0.5 μM noradrenaline for 5 h. Relative abundance of TFAM, TFB1M and TFB2M mRNAs was determined by Northern blot analysis of 25 μg RNA and compared with the expression of COII and UCP-1 mRNAs. Bars are means ± SEM of three independent experiments and are expressed as percentages relative to the mean value in brown adipocytes cultured in differentiating medium, which was set to 100 (arbitrary units). Controls of equal loading of RNA by ethidium bromide staining were established as in Fig 1. Statistical differences ($p < 0.05$) due to noradrenaline are indicated as *.

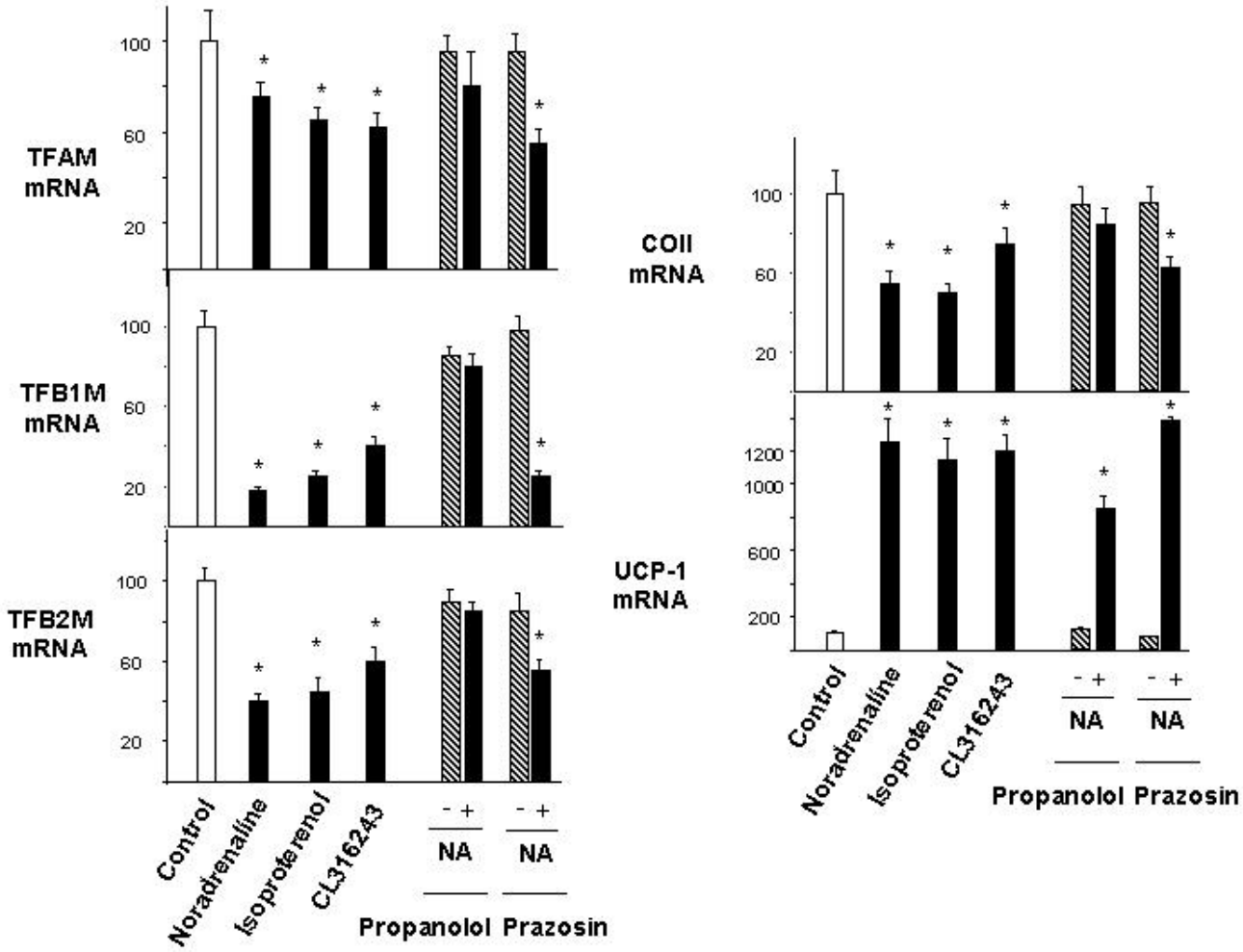


Figure 7

Characterization of adrenergic pathway regulating TFAM, TFB1M and TFB2M mRNA levels in brown adipocytes. Differentiated brown adipocytes (8 days of culture in differentiating medium) were treated for 5h with 0.5µM noradrenaline, 1µM isoproterenol, 1µM CL-316243, 0.5µM noradrenaline plus 10µM propanolol or 0.5µM noradrenaline plus 10µM prazosin. Relative abundance of TFAM, TFB1M and TFB2M mRNAs was determined by Northern blot analysis of 25 µg RNA and compared with the expression of COII and UCP-1 mRNAs. Bars are means ± SEM of three independent experiments and are expressed as percentages relative to the mean value in brown adipocytes cultured in differentiating medium which was set to 100 (arbitrary units). Controls of equal loading of RNA by ethidium bromide staining were established as in Fig 1. Statistical differences ($p < 0.05$) from the controls are indicated as *.

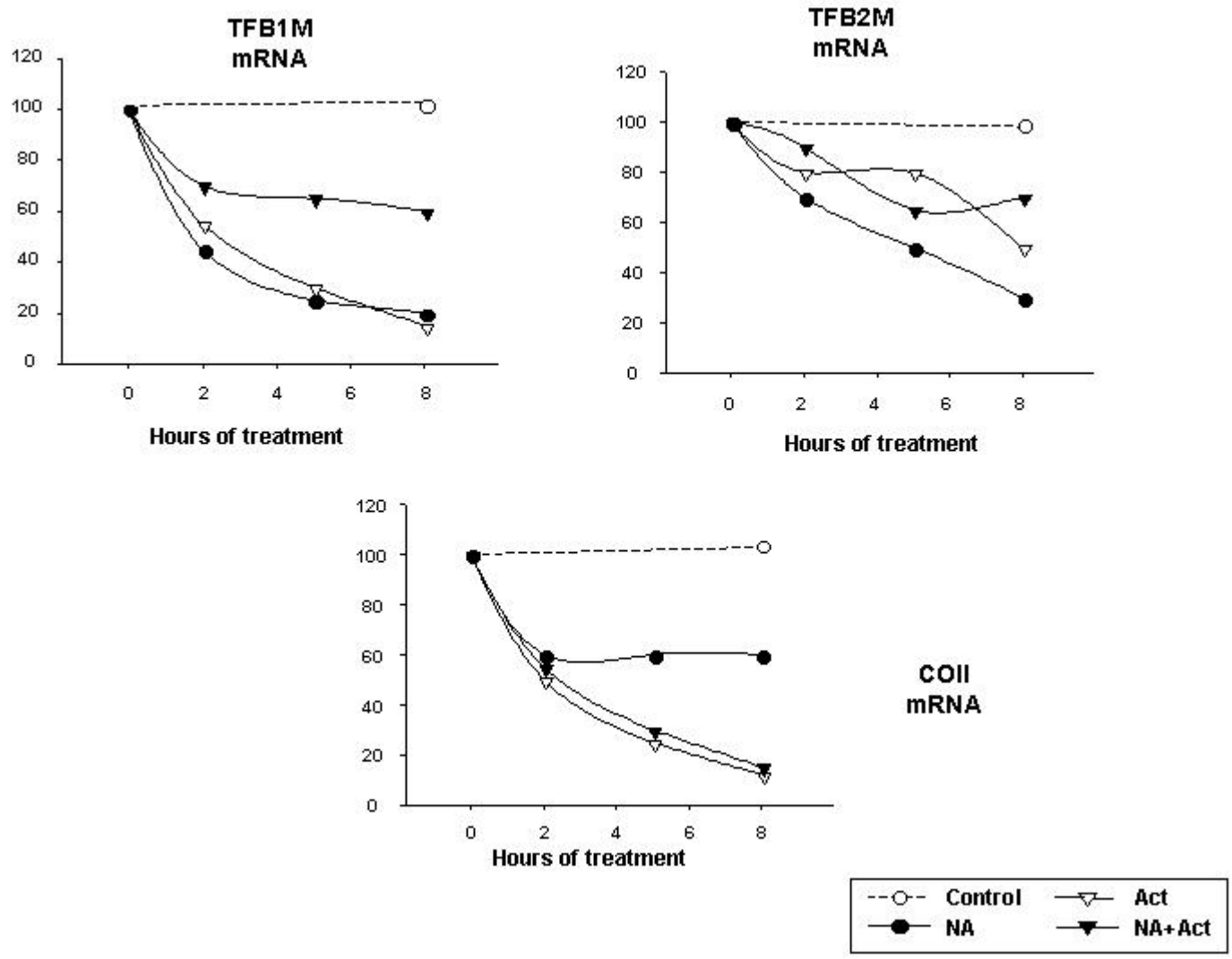


Figure 8

Effects of transcription inhibition on the action of noradrenaline on TFB1M, TFB2M and COII mRNA levels. Differentiated brown adipocytes (8 days of culture in differentiating medium) were exposed to 1 μg/ml actinomycin D (Act) and treated with 0.5 μM noradrenaline (NA). Relative abundance of TFB1M and TFB2M mRNAs was determined by Northern blot analysis of 25 μg RNA and compared with the expression of COII mRNA at various times after treatments. Points are means of two-three independent experiments from every time of culture and are expressed as percentages relative to the mean value before treatments (time 0) which was set to 100 (arbitrary units). Controls of equal loading of RNA by ribosomal RNA staining were established as in Fig 1.

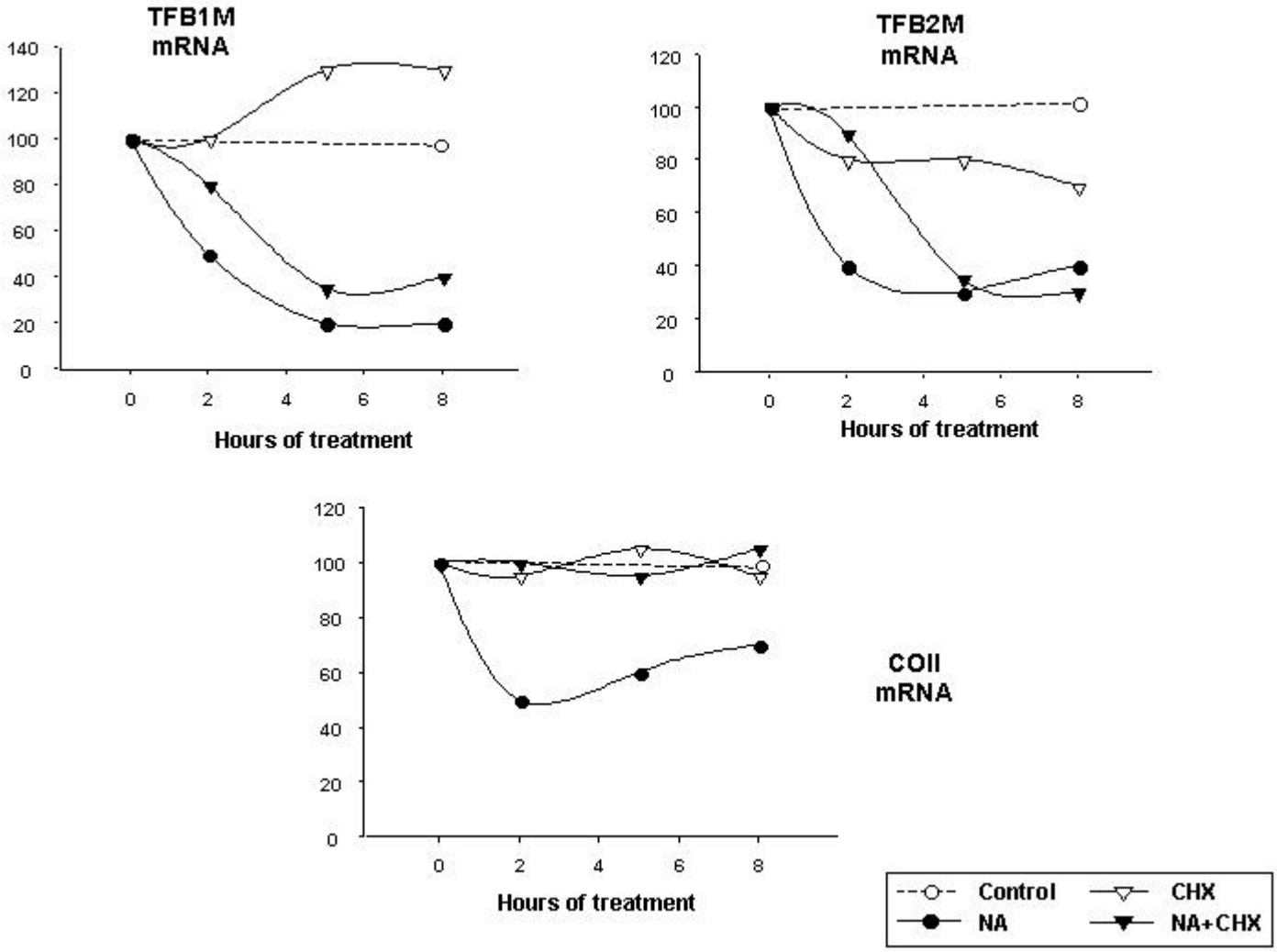


Figure 9

Effects of protein synthesis inhibition on the action of noradrenaline on TFB1M, TFB2M and COII mRNA levels. Differentiated brown adipocytes (8 days of culture in differentiating medium) were exposed or not to 5 μg/ml cycloheximide (CHX) and treated with 0.5 μM noradrenaline (NA). Relative abundance of TFB1M and TFB2M mRNAs was determined by Northern blot analysis of 25 μg RNA and compared with the expression of COII mRNA at various times after treatments. Points are means of two-three independent experiments from every time of culture and are expressed as percentages relative to the mean value before treatments (time 0) which was set to 100 (arbitrary units). Controls of equal loading of RNA by ribosomal RNA staining were established as in Fig 1.

ESTUDIO DE LA EXPRESIÓN DEL GEN PGC-1α EN ADIPOCITOS MARRONES MURINOS EN CULTIVO PRIMARIO. IDENTIFICACIÓN DE UNA NUEVA VÍA DE REGULACIÓN POR AGONISTAS PPAR Y RETINOIDES

INTRODUCCIÓN Y PLANTEAMIENTO EXPERIMENTAL

PGC-1α es un coactivador transcripcional que se ha descrito como un regulador de numerosas respuestas celulares como la termogénesis adaptativa, la biogénesis mitocondrial y la homeostasis energética, entre otras (1). La gran variedad de procesos biológicos que PGC-1α es capaz de controlar radica en su capacidad de interaccionar específicamente con una gran variedad de factores de transcripción como los receptores nucleares de hormonas, factores de respiración nucleares y factores de transcripción específicos del músculo.

Una de las funciones más relevantes y mejor estudiadas de PGC-1α es el papel que este coactivador está jugando en el proceso de la termogénesis adaptativa, donde actúa como un enlace entre los receptores nucleares y el programa transcripcional específico de la termogénesis adaptativa (2). En animales homeotermos, el gasto energético global es el resultado del metabolismo basal, la actividad física y la termogénesis adaptativa, siendo esta última la responsable de los cambios en la disipación de calor como respuesta a la temperatura ambiental, al estado nutricional y a las infecciones. En cada una de estas situaciones se activan mecanismos fisiológicos que inducen la producción de calor en diferentes tejidos, particularmente en el músculo esquelético y el tejido adiposo marrón (TAM). Se ha observado que la expresión de PGC-1α es altamente inducida en respuesta al frío en el TAM y el músculo esquelético (2) y esta inducción se lleva a cabo a través de la vía adrenérgica, en el TAM (3;4).

El TAM es un tejido con una función fundamental en el proceso de la termogénesis adaptativa, en mamíferos de pequeño tamaño, y esto es gracias a la presencia de la proteína UCP-1 y de una alta capacidad oxidativa en los adipocitos marrones. Se ha observado que PGC-1α es un coactivador muy importante en la expresión de UCP-1 (5) y un regulador esencial en el proceso de la biogénesis mitocondrial (2;6-8).

Uno de los objetivos de este capítulo es el estudio de la expresión del gen PGC-1α en el contexto de la célula adiposa marrón. El modelo de adipocitos marrones murinos en cultivo primario presenta un modelo de estudio óptimo para analizar la expresión de PGC-1α y su regulación ya que el proceso de diferenciación

que llevan a cabo los adipocitos marrones en cultivo, así como las respuestas frente a determinados estímulos, se asemejan bastante a los del tejido *in vivo* (6;9;10). En este trabajo se ha profundizado en el estudio de la vía adrenérgica responsable de la activación de PGC-1α por la noradrenalina, así como también se describen otras vías que están regulando la expresión de PGC-1α en el adipocito marrón.

MATERIALES Y MÉTODOS

La noradrenalina (NA), el isoproterenol, el CL316243, el propanolol, el prazosín, la insulina, la 3,5,3'-triiodotironina (T_3), la actinomicina D, la cicloheximida, el ácido ascórbico, el ácido all-*trans* retinoico, el ácido 9*cis* retinoico, el Wy 14,643, el bezafibrato y el GW501516 fueron obtenidos de Sigma-Aldrich (St. Louis, Missouri, USA). La rosiglitazona (BRL 49653) fue amablemente cedida por el Dr. L. Casteilla (Toulouse, France) y el activador específico RXR (AGN 194204) también fue cedido por el Departamento de Química de Allergan Inc. (Allergan Pharmaceuticals, Irvine, California, USA). El [α-^{32}P]dCTP se obtuvo de Amersham Biosciences. El *p*-[(E)-2-(5,6,7,8,-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB) fue cedido por Hoffman-LaRoche (Basel, Switzerland) y el Am80 fue cedido por el Dr. K. Shudo (Universidad de Tokio). Los medios de cultivo, el suero fetal bovino (FBS) y las soluciones de antibióticos y antimicóticos fueron obtenidos de Gibco (Carlsbad, California, USA) y el suero delipidado fue obtenido de Sigma-Aldrich.

Los cultivos primarios de adipocitos marrones de ratón se obtuvieron a partir de células precursoras de tejido adiposo marrón de ratón (6). El método se basa en el aislamiento selectivo de las células precursoras de la fracción del estroma vascular de los depósitos de TAM interescapular, cervical y axilar de ratones Swiss de 3 semanas de edad. Los preadipocitos se plaquean una densidad de 5000 células/cm^2 en placas petri de 6cm y se dejan crecer en Dulbecco's modified Eagle's medium-Ham's F-12 (DMEM:F12, 1:1 v/v) suplementado con 10% suero fetal bovino (FBS), 1% antibióticos (penicilina, estreptomicina), 100μM ascorbato, 20nM insulina y 2nM T_3 (medio de diferenciación). Se dejan proliferar durante 4 días y se les vuelve a cambiar el medio de diferenciación, con el que se consigue que a día 8 de cultivo la mayoría de las células presenten la morfología característica de un adipocito marrón diferenciado. Si a día 4 se les cambia el medio a medio deplecionado, que incluye DMEM:F12 suplementado con 1% antibióticos y 10%

suero delipidado, a día 8 se obtienen células totalmente indiferenciadas. Para los estudios de los efectos de la insulina y la rosiglitazona, estos agentes fueron añadidos al medio deplecionado a día 4, a las concentraciones de 20nM y 10μM respectivamente.

El RNA total de las células se obtuvo utilizando el kit RNeasy Mini Kit de Qiagen. Para los análisis Nothern Blot, se cargaron y corrieron 10μg de RNA total en geles al 1.5% de agarosa con formaldehído y se transfirieron a membranas de nylon (N^+, Boehringer Mannheim, Alemania). La presencia de cantidades equivalentes de RNA ribosómico en las muestras se comprobó mediante la visualización ultravioleta por tinción con bromuro de etidio. Las prehibridaciones y hibridaciones de las membranas con la sondas específicas, se realizaron a 55^0C en una solución que contiene 0.25M de Na2HPO4 (pH 7.2), 1mM EDTA, 20% SDS y 0.5% de reactivo de bloqueo (Boehringer Mannheim, Alemania). Las membranas se hibridaron con las sondas del cDNA de rata de UCP-1 (9) y del cDNA de ratón de PGC-1α (2) marcadas radioactivamente con [α^{-32}P]dCTP utilizando el kit Rediprime ™ II Random Prime labelling System de Amersham Biosciences. Las autoradiografias fueron cuantificadas mediante análisis densitométrico (Phoretics 1D software, Phoretics International LTD, Newcastle, UK).

El análisis estadístico se realizó utilizando el test *t* de Student.

Para los análisis de microscopia electrónica de transmisión, las células diferenciadas o indiferenciadas se recogieron en PBS (phosphate-buffered saline) y se centrifugaron a 500g durante 5 minutos. Se fijaron en una solución fosfato 0.1M, 2.5% glutaraldehido y 2% paraformaldehido y posteriormente se volvieron a fijar en una solución fosfato 1% tetróxido de osmio y 0.8% FeCNK. Después de la deshidratación en series de acetona, las muestras fueron embebidas en resina Spurr. Las secciones ultrafinas se tiñeron con acetato de uranilo y citrato de plomo y se examinaron con el microscopio electrónico de transmisión Hitachi H600AB a 75kV.

RESULTADOS

Expresión del gen PGC-1α durante la diferenciación de adipocitos marrones en cultivo primario

Se han determinado los cambios en la expresión de PGC-1α durante el proceso de diferenciación de preadipocitos a adipocitos marrones (**Figura 1**). El perfil de expresión de PGC-1α se asemeja bastante al del marcador típico de la célula adiposa marrón UCP-1. Se observan unos niveles bajos del mRNA de PGC-1α durante los primeros días de cultivo, cuando los preadipocitos se encuentran en estado proliferativo y unos niveles máximos a día 7-8 de cultivo, cuando el 80-90% de las células del cultivo se encuentran totalmente diferenciadas y presentan unos niveles máximos de expresión de UCP-1.

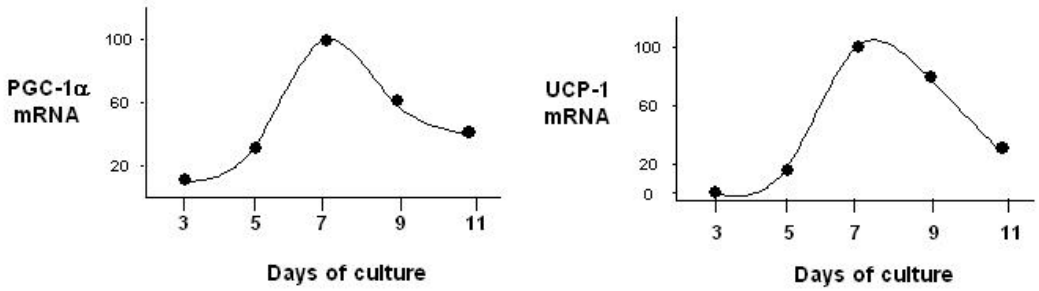


Figura 1. Perfiles de expresión de los genes PGC-1α y UCP-1 durante la diferenciación de adipocitos marrones murinos en cultivo primario. Adipocitos marrones en cultivo primario fueron inducidos a diferenciarse y a día 3, 5, 7, 9 y 11 se recogieron las células y se analizaron los niveles de los mRNA de PGC-1α y UCP-1 mediante Northern Blot. Cada punto es la media de dos-tres experimentos para cada día de cultivo y están expresados como porcentajes respecto al valor máximo de expresión, marcado como 100.

La inducción de la expresión de PGC-1α en adipocitos marrones maduros por la noradrenalina (NA) se realiza vía la activación de receptores β_3-adrenérgicos

Para analizar la respuesta de PGC-1α a un estímulo termogénico como la noradrenalina, adipocitos marrones diferenciados en cultivo primario se trataron con noradrenalina durante 5 horas a la concentración de 0.5μM. La NA induce la expresión del gen PGC-1α de manera muy considerable, así como también incrementa los niveles del mRNA de UCP-1 (**Figura 2**). Para determinar las vías a

través de las cuales la NA está involucrada en este efecto, adipocitos marrones maduros fueron también expuestos a la acción del agonista β-adrenérgico isoproterenol y al activador específico β_3-adrenérgico CL316243. Ambos compuestos son capaces de inducir la expresión de PGC-1α y UCP-1, de manera similar a como lo hace la NA. Seguidamente, se analizó la capacidad del propanolol (inhibidor β-adrenérgico) o del prazosín (inhibidor α-adrenérgico) para modificar la respuesta de la NA sobre PGC-1α y se observó que el propanolol es capaz de reducir significativamente la inducción de PGC-1α y UCP-1 por la NA, mientras que el prazosín no consigue bloquear esta inducción. Estos resultados indican que la vía β_3-adrenérgica sería la responsable de la inducción de PGC-1α por la NA.

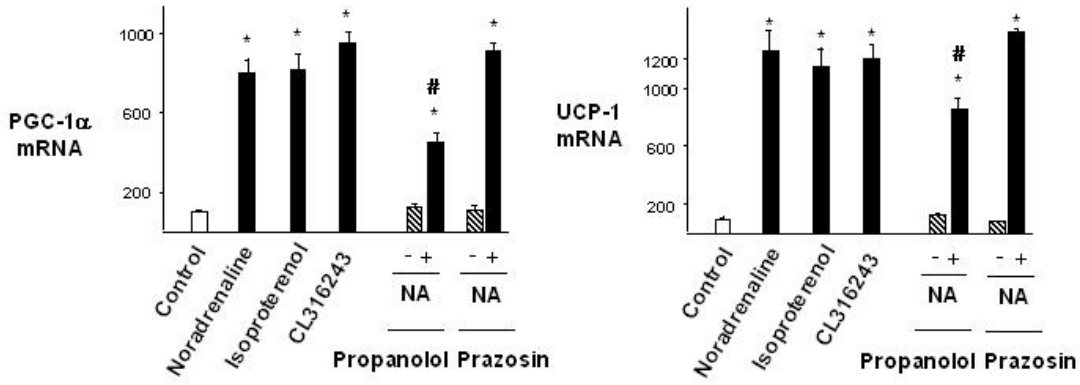


Figura 2. Estudio de la vía adrenérgica por la cual la NA está induciendo la expresión de PGC-1α. Adipocitos marrones diferenciados fueron tratados con 0.5μM NA durante 5 horas o 1μM isoproterenol o 1μM CL316243 durante 24 horas y seguidamente fueron recogidos para extraer RNA total y analizar la expresión de PGC-1α y UCP-1. Los tratamientos con 10μM propanolol o 10μM prazosín en presencia o ausencia de NA también se realizaron durante 24 horas. Cada barra representa la media de 2-3 experimentos ± SEM. Las diferencias estadísticamente significativas ($p<0.05$) de las inducciones por la NA o los agonistas adrenérgicos respecto a los controles se indican mediante *. El símbolo # indica un bloqueo significativo ($p<0.05$) del propanolol sobre la acción de la NA respecto a la inducción de ésta sola.

La inducción de la expresión de PGC-1α por la noradrenalina es dependiente de transcripción génica e independiente de la síntesis proteica

Con el propósito de analizar la implicación de la transcripción génica y de la dependencia de la síntesis proteica en la inducción de PGC-1α por la NA, las células fueron expuestas al inhibidor de la transcripción actinomicina D o al inhibidor de la síntesis proteica cicloheximida, en presencia o ausencia de NA. La **figura 3** muestra

los perfiles de inducción de PGC-1α en presencia de NA con o sin actinomicina D (figura **3A**) y los perfiles de inducción de PGC-1α con o sin cicloheximida (figura **3B**). Se ha observado que la inducción por NA de PGC-1α se pierde completamente en presencia de actinomicina D, demostrando que el efecto mediado por la NA sobre los niveles del mRNA de PGC-1α es dependiente del proceso de transcripción génica. Por el contrario, la cicloheximida no consigue bloquear la inducción de PGC-1α por la NA, con lo que este efecto es independiente de síntesis proteica.

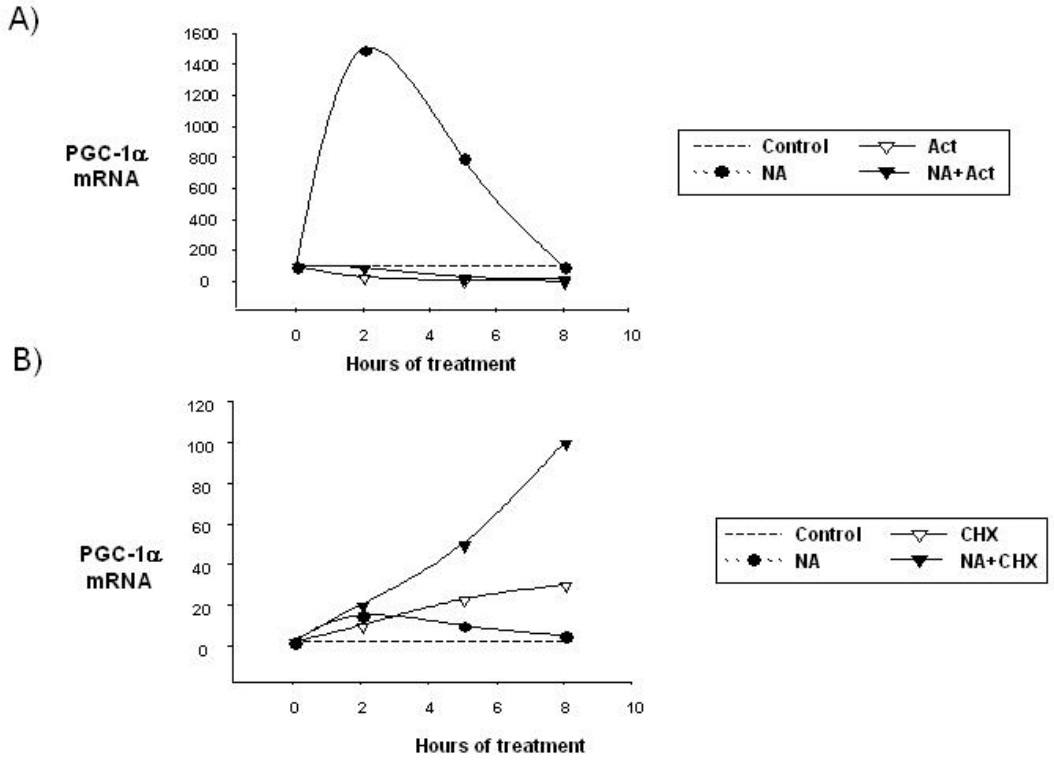


Figura 3. Efectos de la inhibición de la transcripción y de la síntesis proteica sobre la inducción de PGC-1α por la NA. Adipocitos marrones diferenciados fueron tratados con 1 μg/ml actinomicina D (Act) (figura 3A) o 5 μg/ml cicloheximida (CHX) (figura 3B) en presencia o ausencia de 0.5μM NA. A las horas indicadas se recogieron las células y se extrajo el RNA total para analizar la expresión de PGC-1α. Cada punto es la media de dos-tres experimentos para cada tiempo y están expresados como porcentajes respecto al valor máximo de expresión, marcado como 100.

La expresión de PGC-1α y su capacidad de respuesta a la noradrenalina depende del estado de diferenciación del adipocito marrón. Efecto de la insulina y la rosiglitazona

Para analizar en qué medida la expresión de PGC-1α en adipocitos marrones y su inducción por NA depende del estado de diferenciación, hemos utilizado un

modelo celular en el que los preadipocitos se cultivan en presencia de un medio con suero delipidado (*depleted medium*) que no permite a las células diferenciarse. Con este modelo se consigue que los preadipocitos proliferen hasta llegar a confluencia y al final del cultivo las células aparecen como fibroblástos distribuidos en forma de tapiz y sin ninguna acumulación lipídica (ver figura 4, artículo "*Regulation of gene expresión for mitochondrial transcription factors B1 and B2 in brown adipocytes. Opposite effects of differentiation and noradrenaline"*). Juntamente con éstas características morfológicas, las células presentan una biogénesis mitocondrial alterada, a juzgar por la menor cantidad de mitocondrias presente en estas células y la estructura de las crestas mitocondriales poco desarrolladas.

La **figura 4** muestra que los niveles de expresión de PGC-1α en las células indiferenciadas se encuentran significativamente disminuidos respecto a los adipocitos marrones diferenciados, así como también los niveles de UCP-1, que son casi indetectables. En estas células indiferenciadas la respuesta a la NA de PGC-1α, así como la de UCP-1 se mantienen, aunque en menor grado que en las células diferenciadas.

Seguidamente, se analizaron los niveles del mRNA de PGC-1α y sus variaciones en respuesta a la NA cuando al medio deplecionado se le añadió insulina o el agonista PPARγ rosiglitazona, agentes que inducen el proceso de diferenciación de éstas células. La adición de insulina reestablece parcialmente los niveles de expresión de PGC-1α, pero no los de UCP-1 y en estas condiciones la NA es capaz de inducir la expresión de PGC-1α a unos niveles similares a las células diferenciadas. Por otro lado, la presencia de rosiglitazona reestablece completamente los niveles basales de PGC-1α y se consigue una inducción por la NA muy superior al de las células diferenciadas.

Si se analiza la capacidad de respuesta de los preadipocitos (a día 4 de cultivo) a la NA, los resultados muestran que las células en este estado no responden a la inducción de PGC-1α por la NA, mientras que sí aumentan los niveles de UCP-1 bajo este estímulo.

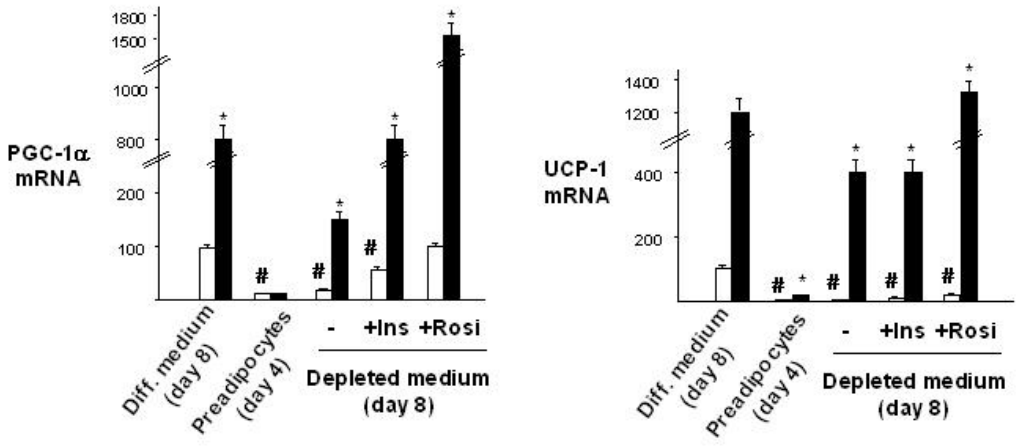


Figura 4. Análisis de la expresión de PGC-1α y su inducción por la NA dependiendo del estado del estado de diferenciación del adipocito marrón. Preadipocitos marrones fueron cultivados en medio de diferenciación o en medio deplecionado sin suplementar o suplementado con insulina o con rosiglitazona, durante 8 días (barras blancas). A día 8 de cultivo fueron tratados o no con 0.5μM NA (barras negras) y a las 5 horas se recogieron las células y por Northern Blot se analizaron los niveles de los mRNA de PGC-1α y UCP-1. Para el estudio en preadipocitos, éstos fueron tratados a día 4 durante 5 horas con 0.5μM NA. Cada barra representa la media de 2-3 experimentos ± SEM. Las diferencias estadísticamente significativas ($p<0.05$) de las inducciones por la NA respecto a los controles respectivos se indican mediante *. El símbolo # indica un bloqueo significativo ($p<0.05$) de la expresión del gen PGC-1α o UCP-1 respecto a los niveles de expresión de adipocitos marrones diferenciados.

El gen PGC-1α también se induce por agonistas PPAR y agonistas RXR

Después de analizar la inducción de la expresión de PGC-1α por la noradrenalina, se llevaron a cabo tratamientos de adipocitos marrones maduros con diferentes compuestos (compuestos de los que se conoce su actividad sobre la expresión del gen UCP-1), durante 24 horas, para analizar su efecto a nivel de la expresión de PGC-1α (**Figura 5**). La figura **5A** muestra una tabla que incluye los compuestos utilizados y sus características principales.

A)

NA- Noradrenaline (0.5μM)	**9cisRA**- 9*cis* retinoic acid (1μM)
Rosi- Rosiglitazone (BRL49653) PPARγ Agonist (10μM)	**AGN**- AGN194204 RXR specific activator (1μM)
Wy 14,643 PPARα Agonist (10μM)	**atRA**- all-*trans* retinoic acid (1μM)
Beza- Bezafibrate PPARα/δ Agonist (100μM)	**TTNPB** RAR specific activator (10μM)
GW- GW501516 PPARδ Agonist (1μM)	**Am80** RAR specific activator (10μM)

Figura 5A. Tabla de los compuestos utilizados para estudiar la posible regulación de la expresión génica de PGC-1α.

El tratamiento de adipocitos marrones maduros con el agonista PPARγ rosiglitazona induce significativamente la expresión de PGC-1α, así como también lo hacen el ácido 9*cis* retinoico (9cisRA) y el activador específico del receptor RXR AGN194204. El bezafibrato, agonista PPARα/δ, también incrementa los niveles del mRNA de PGC-1α de manera importante y el Wy 14,643 y el GW501516, agonistas PPARα y PPARδ, respectivamente, aunque en menor medida también inducen la expresión de PGC-1α. Estos resultados sugieren que el gen PGC-1α estaría regulado a nivel transcripcional a través de posibles elementos de respuesta a PPARs.

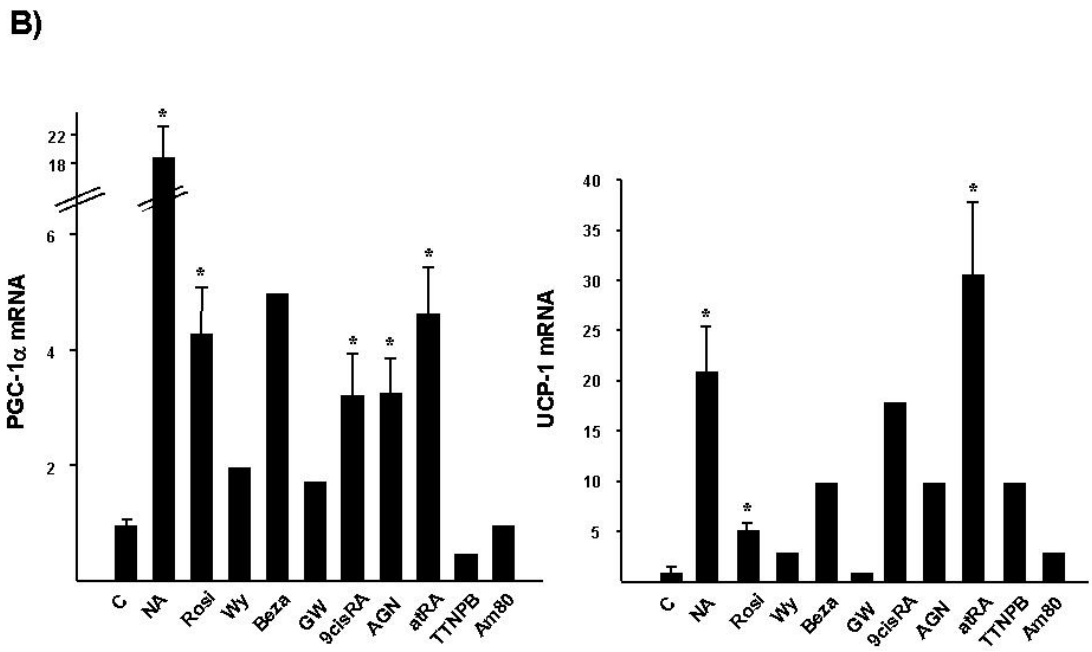


Figura 5B. Estudio de la inducción de la expresión de PGC-1α por diferentes agonistas PPAR, RAR y RXR. Adipocitos marrones diferenciados fueron tratados con los compuestos de la figura 5A, durante 24 horas (excepto para la NA que fueron 5 horas). Seguidamente, se recogieron las células y se extrajo el RNA total para analizar la expresión de PGC-1α y UCP-1 mediante Northern Blot. Las diferencias estadísticamente significativas ($p<0.05$) de las inducciones por estos compuestos respecto al control se indican mediante *, en aquellas situaciones experimentales que se testaron en más de 2 cultivos independientes (las barras representan la media ± SEM).

El aumento en los niveles del mRNA de PGC-1α inducido por el ácido all-*trans* retinoico llevó a pensar en otra posible vía para la inducción de PGC-1α dependiente de RAR, pero esta hipótesis se descartó cuando se observó que el tratamiento con los activadores específicos RAR TTNPB y Am80 no inducía la expresión del PGC-1α. El incremento en los niveles del mRNA de PGC-1α que se

observa en los adipocitos marrones tratados con all-*trans* RA puede ser debido a que en el interior de la célula el all-*trans* RA puede isomerizar a 9*cis*RA y éste ejercer su acción activando los receptores RXR.

La inducción de PGC-1α por la rosiglitazona y la vía RXR es dependiente de PPARγ

Para profundizar en el mecanismo de acción de la rosiglitazona y los activadores de RXR, adipocitos marrones diferenciados fueron tratados con rosiglitazona, 9cisRA y el activador específico RXR AGN194204 durante 24 horas y con NA durante 5 horas, en presencia o ausencia del antagonista PPARγ GW9662, y se analizaron los niveles del mRNA de PGC-1α y UCP-1. En la **figura 6** podemos observar como las inducciones de PGC-1α por la rosiglitazona, el 9cisRA y el activador específico RXR se ven bloqueadas cuando estos compuestos se encuentran en presencia del antagonista PPARγ, mientras que éste no es capaz de bloquear la inducción de PGC-1α por la NA. Estos resultados sugieren que la inducción de PGC-1α por la vía RXR depende de la heterodimerización de RXR con PPARγ y es independiente de la vía de inducción por la NA.

La gráfica para UCP-1 muestra como el antagonista PPARγ sólo bloquea la inducción de UCP-1 por la rosiglitazona mientras que no tiene ningún efecto sobre las inducciones por el 9cisRA, el agonista RXR y la NA. Estos resultados están en concordancia con los datos de Alvarez *et al*., donde se describe que la regulación del gen UCP-1 tiene un importante componente dependiente de RAR y RXR y totalmente independiente de la vía PPAR (10).

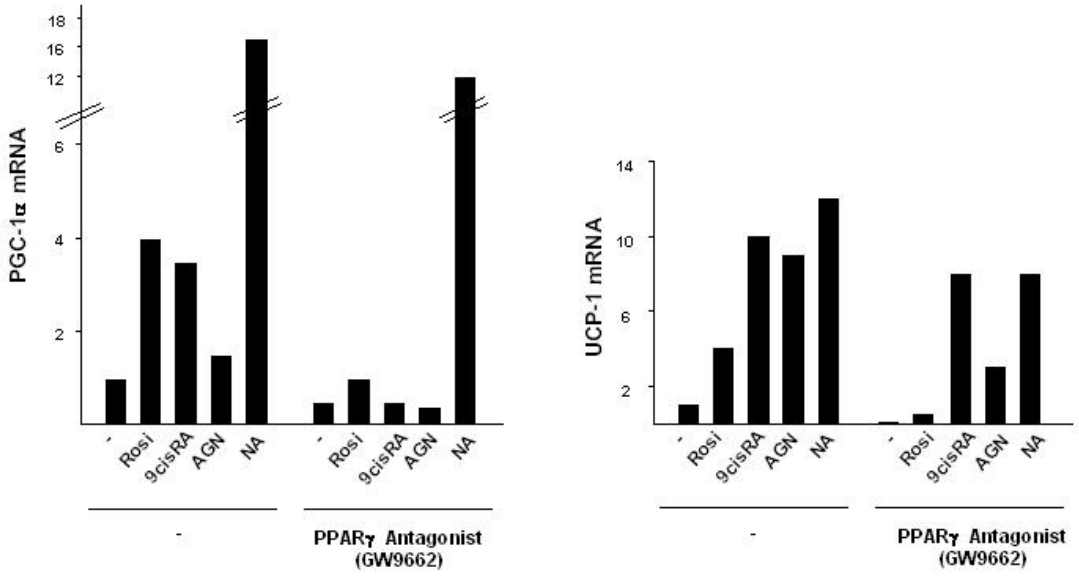


Figura 6. Efecto de la presencia del antagonista PPARγ sobre la inducción de PGC-1α por la rosiglitazona, el 9cisRA y el AGN194204. Adipocitos marrones diferenciados fueron tratados a día 8 de cultivo y durante 24 horas con 10μM rosiglitazona, 1μM 9cisRA o 1μM AGN194204 en presencia o ausencia del antagonista PPARγ GW9662 (30μM). Los tratamientos con 0.5μM NA se realizaron durante 5 horas. El RNA total obtenido de las células fue utilizado para analizar los niveles de los mRNA de PGC-1α y UCP-1 mediante Northern Blot.

La rosiglitazona y el 9cisRA no necesitan de receptores α ni β-adrenérgicos para inducir la expresión de PGC-1α

Para determinar si los receptores α y β-adrenérgicos están jugando algún papel en la activación de la expresión de PGC-1α por la rosiglitazona y el 9cisRA, adipocitos marrones maduros fueron tratados con rosiglitazona y 9cisRA durante 24 horas, en presencia o ausencia del inhibidor α-adrenérgico prazosín y el inhibidor β-adrenérgico propanolol. Los resultados se muestran en la **figura 7**. Las inducciones de PGC-1α por la rosiglitazona y el 9cisRA no se ven alteradas en presencia de los inhibidores adrenérgicos, pero sí se ve parcialmente bloqueada la inducción de PGC-1α por la NA. En el caso de UCP-1, la presencia del propanolol y el prazosín tampoco altera las inducciones de la rosiglitazona y el 9cisRA sobre UCP-1, pero sí bloquea la inducción por la NA.

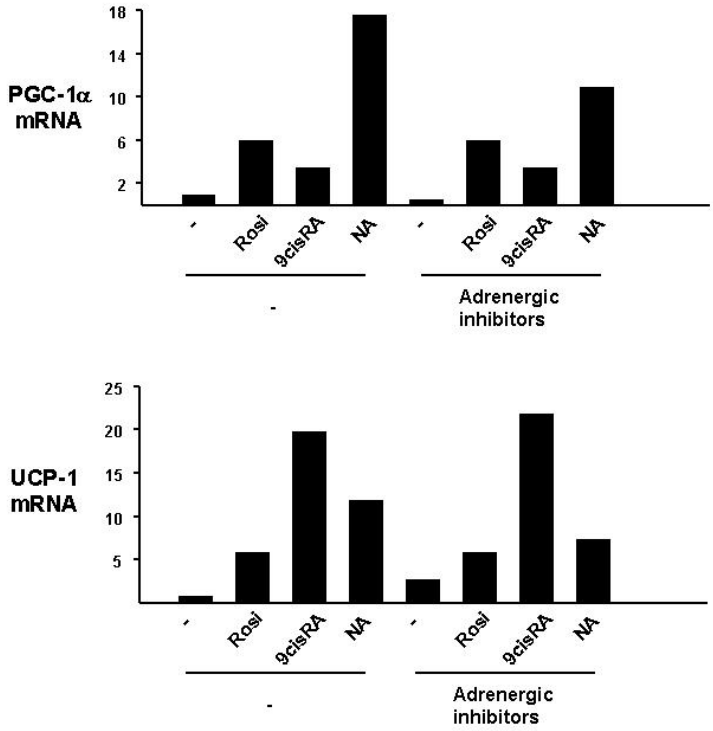


Figura 7. Análisis del efecto de los inhibidores adrenérgicos propanolol y prazosín sobre la inducción de PGC-1α por la rosiglitazona y el 9cisRA. A día 8 de cultivo, adipocitos marrones maduros se trataron con 10μM rosiglitazona o 1μM 9cisRA, durante 24 horas o con 0.5μM NA durante 5 horas, en presencia o ausencia de los inhibidores α y β-adrenérgicos prazosín (10μM) y propanolol (10μM). Los niveles de PGC-1α y UCP-1 de estas células se determinaron a nivel de Northern Blot.

Estudio de la inducción de PGC-1α por rosiglitazona a lo largo del tiempo (*time course*) y a concentraciones crecientes (*dose response*)

Con el objetivo de determinar el perfil de inducción de PGC-1α por la rosiglitazona a lo largo del tiempo (*time course*) y según las concentraciones de la misma (*dose response*), se llevaron a cabo, por un lado, tratamientos de adipocitos marrones maduros con rosiglitazona a la concentración de 10μM y a las 5, 12, 24 y 48 horas se recogieron los adipocitos y se analizó la expresión de PGC-1α y UCP-1 (**Figura 8A**). Por otro lado, se trataron adipocitos maduros a diferentes concentraciones de rosiglitazona durante 24 horas y también se analizó PGC-1α y UCP-1 (**Figura 8B**).

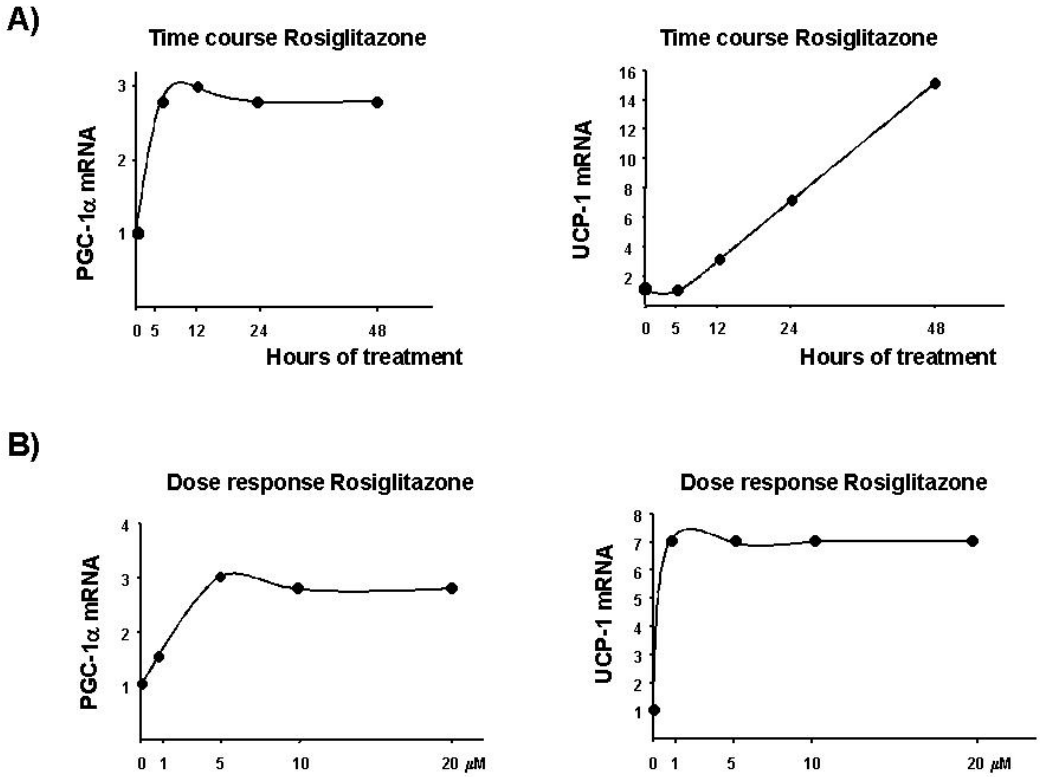


Figura 8. Perfiles *time course* y *dose response* de PGC-1α y UCP-1 de adipocitos diferenciados tratados con rosiglitazona. Los análisis *time course* para PGC-1α y UCP-1 se realizaron tratando adipocitos maduros con rosiglitazona a la concentración de 10μM durante 5, 8, 12 y 24 horas y recogiendo las células a cada tiempo para analizar la expresión de los dos genes mediante Northern Blot. Las curvas *dose response* se consiguieron tratando adipocitos diferenciados con rosiglitazona a las concentraciones de 1, 5, 10 y 20μM y recogiendo las células a las 24 horas del inicio de los tratamientos, para analizar la expresión de PGC-1α y UCP-1 por Northern Blot.

La figura **8A** muestra que la máxima inducción de PGC-1α por la rosiglitazona se consigue aproximadamente a las 12 horas de tratamiento, momento a partir del cual la expresión se mantiene a niveles máximos. Para UCP-1 el incremento en los niveles de UCP-1 es más progresivo, no consiguiéndose llegar a un *plateau* ni a las 48 horas de tratamiento. Los perfiles dosis-respuesta de la figura **8B** nos indican que la concentración más baja a la que se consigue unos niveles máximos de inducción de PGC-1α por la rosiglitazona es 5μM y a partir de esta concentración los niveles se mantiene. En el caso de UCP-1, a la concentración de 1μM de rosiglitazona ya se consiguen los niveles más altos de expresión de UCP-1 inducidos por la rosiglitazona.

La inducción de PGC-1α por la rosiglitazona y la vía RXR no se bloquea cuando se inhibe la síntesis proteica

Con el propósito de analizar si la inducción de PGC-1α por la rosiglitazona, el 9cisRA y el activador RXR depende o no del proceso de síntesis proteica, se trataron adipocitos diferenciados durante 12 horas con dichos compuestos en presencia o ausencia de cicloheximida. Los resultados que se muestran en la **figura 9** indican que la rosiglitazona, el 9cisRA y el AGN en presencia de cicloheximida mantienen su capacidad de inducción de PGC-1α. Para UCP-1 observamos que la cicloheximida bloquea totalmente la inducción por rosiglitazona, pero no así la inducción por 9cisRA o AGN.

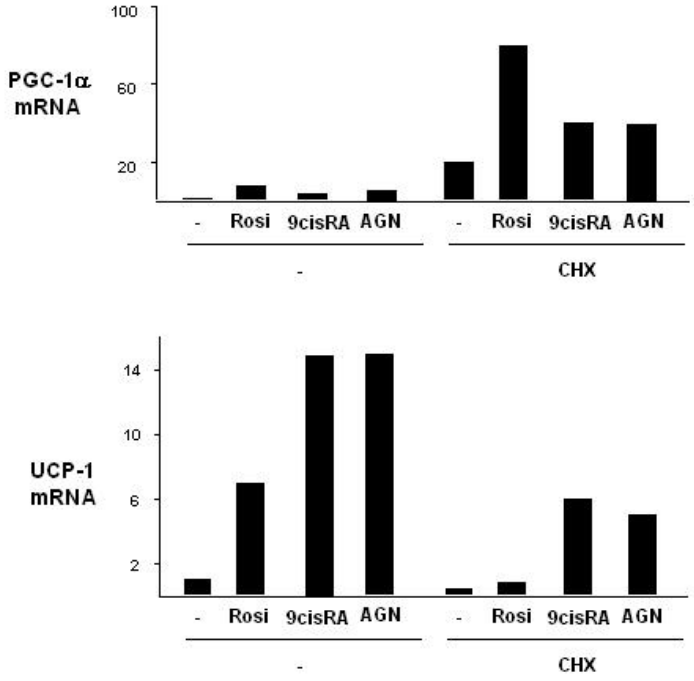


Figura 9. Efecto del inhibidor de la síntesis proteica cicloheximida sobre la inducción de PGC-1α por la rosiglitazona, el 9cisRA y el AGN194204. Adipocitos marrones diferenciados fueron tratados a día 8 de cultivo con 10μM rosiglitazona, 1μM 9cisRA o 1μM AGN194204 en presencia o ausencia de 5μg/ml cicloheximida, durante 24 horas y se extrajo el RNA total para analizar los niveles de expresión de PGC-1α y UCP-1.

Los preadipocitos no responden a la inducción de PGC-1α por la rosiglitazona, el 9cisRA y el activador específico RXR

Se quiso analizar si los preadipocitos en cultivo eran también capaces de responder a la rosiglitazona y a los activadores RXR induciendo PGC-1α y para ello

preadipocitos a día 4 de cultivo fueron tratados con estos compuestos por separado y a las 24 horas se recogieron para analizar la expresión de PGC-1α y UCP-1 (**Figura 10**). Se han representado igualmente en las gráficas los niveles de expresión de PGC-1α y UCP-1 en adipocitos maduros para una mayor comprensión. Los niveles de expresión de PGC-1α de preadipocitos a día 4 de cultivo son alrededor del 30% de los niveles que se encuentran en adipocitos diferenciados. A día 4 de cultivo los niveles del mRNA de UCP-1 son indetectables. Ni el tratamiento con rosiglitazona, 9cisRA ni AGN consiguen inducir la expresión de PGC-1α en preadipocitos. Sin embargo, para UCP-1, observamos que el 9cisRA y el AGN inducen significativamente la expresión de UCP-1, mientras que la rosiglitazona no es capaz de inducir la expresión de este gen.

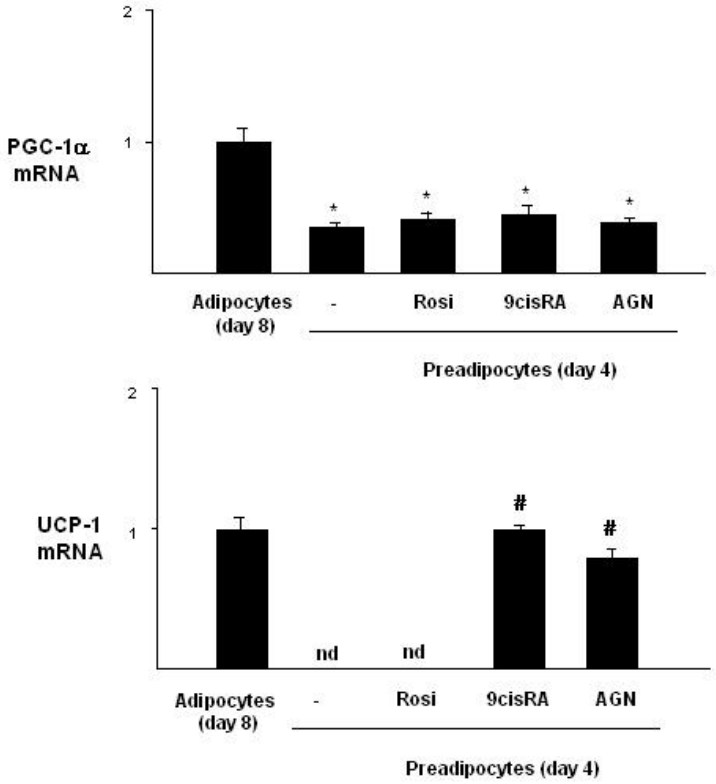


Figura 10. Análisis de la inducción de PGC-1α por rosiglitazona, 9cisRA y AGN194204 en preadipocitos. Preadipocitos a día 4 de cultivo fueron tratados con 10μM rosiglitazona, 1μM 9cisRA o 1μM AGN194204 durante 24 horas y seguidamente, las células fueron recogidas para obtener RNA total y analizar la expresión de PGC-1 y UCP. Cada barra representa la media de 2-3 experimentos ± SEM. Las diferencias estadísticamente significativas ($p<0.05$) entre la expresión de PGC-1α en adipocitos diferenciados a día 8 respecto a los niveles de expresión a día 4 de cultivo se indican mediante *. El símbolo # indica un aumento significativo ($p<0.05$) de los niveles de UCP-1 en preadipocitos tratados, respecto a los niveles de expresión en preadipocitos controles (en este caso indetectables, nd).

DISCUSIÓN

El presente trabajo realizado con el modelo celular de adipocitos marrones murinos en cultivo primario ha permitido estudiar la expresión del gen PGC-1α durante la diferenciación del adipocito marrón, profundizar en la regulación frente a un estímulo termogénico y describir posibles nuevas vías de regulación de su expresión.

PGC-1α, durante la diferenciación del adipocito marrón en cultivo primario, sigue un patrón de expresión similar al de su marcador específico UCP-1, consiguiendo unos niveles máximos de expresión a día 7-8 de cultivo que es cuando los adipocitos marrones están del todo diferenciados, presentan una elevada respuesta termogénica y una gran capacidad oxidativa gracias al elevado número de mitocondrias altamente estructuradas que se hallan en el adipocito diferenciado.

Los niveles del mRNA de PGC-1α se incrementan de manera muy importante cuando adipocitos marrones diferenciados son tratados en respuesta a un estímulo termogénico, como la NA y esta inducción se realiza a través de la activación de receptores β_3-adrenérgicos. Estos resultados están en concordancia con los datos observados *in vivo* donde la exposición de ratones al frío induce considerablemente la expresión de PGC-1α en el TAM y en el músculo esquelético (2) y que esta respuesta está mediada por la vía β_3-adrenérgica en el TAM (3;4).

Los estudios realizados con los inhibidores de la transcripción génica y de la síntesis proteica nos indican que la inducción de PGC-1α por la NA en adipocitos marrones diferenciados es un proceso que depende de la transcripción, pero no requiere de la síntesis de proteínas, resultados que indican que la NA está actuando a nivel de la transcripción génica, posiblemente a través de un elemento de respuesta a AMPc (CRE) en el promotor del gen, elemento que ha sido descrito por Handschin *et al*., en el zona promotora del gen PGC-1α de ratón (11).

Al analizar la expresión de PGC-1α y su respuesta a la NA dependiendo del estado de diferenciación del adipocito marrón, se ha observado que en células indiferenciadas (cultivadas en presencia de medio delipidado), donde los niveles de PGC-1α están disminuidos respecto a las células diferenciadas, la NA es capaz de incrementar los niveles del mRNA de PGC-1α, aunque en menor grado que en las células control. La presencia de la insulina en el medio delipidado recupera parcialmente la expresión basal de PGC-1α, pero las células en este estado son capaces de responder a la NA de manera similar a las diferenciadas. La rosiglitazona, no sólo recupera los niveles basales del mRNA de PGC-1α, si no que la NA en estas células incrementa la expresión de PGC-1α de manera muy superior

a las células diferenciadas, resultado que también se observan para la inducción de UCP-1 por la NA en células cultivadas en medio delipidado más rosiglitazona. Estos resultados indican que la capacidad de los adipocitos marrones para responder a un estímulo termogénico depende de su grado de diferenciación.

En cuanto a la respuesta a la NA por parte de los preadipocitos marrones, se ha observado que éstos no incrementan sus niveles del mRNA de PGC-1α cuando son tratados con NA durante 5 horas, a diferencia de lo que se observa para UCP-1 que sí que se induce su expresión génica por la NA en preadipocitos. Se ha descrito que el estímulo adrenérgico en preadipocitos en cultivo es una señal positiva sobre la diferenciación, incluyendo la biogénesis mitocondrial (12), donde estaría implicado PGC-1α. Sin embargo, el hecho que la expresión del gen PGC-1α no aumente por efecto de la NA en preadipocitos podría explicarse con el hecho que el tratamiento con NA en estas células está siendo sólo de 5 horas, y no crónico. Cabe destacar, sin embargo, que se ha observado que PGC-1α tampoco induce su expresión por el tratamiento con otros agonistas PPARγ o activadores RXR en el estado de preadipocito.

Después de profundizar en la regulación de la expresión de PGC-1α por la NA, se pretendió estudiar otras vías de activación de la expresión de este gen en adipocitos marrones diferenciados. El tratamiento durante 24 horas con diferentes compuestos que se conoce que están activando la expresión génica de UCP-1, actuando a nivel de su promotor, llevó a observar que los niveles del mRNA de PGC-1α se incrementan cuando las células son tratadas con agonistas PPAR o RXR específicos y no se alteran en presencia de agonistas RAR específicos.

Al analizar la respuesta de PGC-1α a estos compuestos en presencia de un antagonista PPARγ, se observó que la acción del agonista PPARγ rosiglitazona sobre PGC-1α depende de que PPARγ sea funcional y que la vía RXR está dependiendo de la heterodimerización de RXR con PPARγ, lo que sugiere la presencia de elementos de respuesta a PPAR en el promotor de PGC-1α. Estos resultados se diferencian de lo que ocurre para UCP-1, donde la respuesta a activadores RXR no se bloquea totalmente por el antagonista PPARγ. De hecho se ha descrito que el promotor de UCP-1 presenta lugares de respuesta a RAR y RXR independientes de los elementos de respuesta a PPAR (10). La inducción de la expresión del gen PGC-1α que se observa por el tratamiento de adipocitos marrones con el agonista PPARα Wy 14,643, el agonista PPARα/δ bezafibrato y el agonista específico PPARδ GW501516, podría estar mediada por este elemento de respuesta a PPARγ sugerido o también podrían estar implicados otros subtipos como PPARα o PPARδ.

La inducción de PGC-1α por la vía PPARγ o RXR no depende de la activación de receptores β-adrenérgicos, indicando que la vía adrenérgica de activación de la

expresión de PGC-1α es independiente de la inducción del gen PGC-1α por agonistas PPAR o RXR a este nivel.

Los estudios *time course* y *dose response* de la inducción de PGC-1α por la rosiglitazona muestran que la respuesta máxima de la expresión de este gen se consigue a las 5 horas de tratamiento y a partir de una concentración de 5μM ya se obtiene la inducción máxima de PGC-1α. El análisis de la inducción de PGC-1α por la rosgilitazona y la vía RXR en presencia del inhibidor de la síntesis proteica cicloheximida, indica que la acción de estos compuestos no depende de la síntesis de proteínas, sugiriendo que la vía transcripcional estaría jugando un papel importante en la activación de PGC-1α por la rosiglitazona. Estos resultados estarían en concordancia con la rápida inducción que se observa de PGC-1α por la rosiglitazona en los análisis *time course*.

Por último, el análisis de la inducción de la expresión de PGC-1α por la rosiglitazona y la vía RXR en preadipocitos indica que estos compuestos no consiguen activar la expresión PGC-1α, resultados que sugieren que la acción de estos compuestos sobre PGC-1α depende del estado de diferenciación del adipocito, como en el caso de la NA.

El compendio de todos los resultados presentados en este capítulo, sitúa a PGC-1α como un gen marcador de la diferenciación morfológica y mitocondrial de los adipocitos marrones en cultivo. Las nuevas vías implicadas en la inducción de la expresión del gen PGC-1α descritas en este trabajo, aunque requieren niveles de estudio más detallados, como analizar la respuesta del promotor, abren nuevas perspectivas en el estudio de la regulación transcripcional de este gen.

PGC-1α, dado el papel que juega en el control de la homeostasis energética, se ha propuesto como una diana potencial para el tratamiento de la diabetes y obesidad. De hecho los agonistas PPARγ tiazolidindionas, que se usan en la actualidad para el tratamiento de la resistencia a la insulina y los retinoides, que están implicados en múltiples aspectos biológicos como la adiposidad y el control del gasto energético, podrían estar regulando la expresión del gen PGC-1α, con lo que las vías de regulación descritas en este estudio permitirían abordar nuevas estrategias de modulación de su actividad.

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Lithium inhibits brown adipocyte differentiation

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Abbreviations: UCP-1 (uncoupling protein 1), CO (citocrome oxidase), PPAR (peroxisome proliferating activated receptor), PGC-1α (PPAR gamma coactivator 1 alpha)

ABSTRACT

Lithium impairs the appearance of the characteristic morphology of the brown adipocyte and down-regulates the expression of marker genes of brown adipocyte differentiation. These effects are dose-dependent and were more pronounced when exposure of preadipocytes to lithium is initiated at early stages of differentiation. Although lithium reduces the expression of genes common to the white and brown adipocyte phenotype (aP2 or PPARγ), the genes more sensitively impaired in their expression by lithium are those expressed differentially in the brown adipocyte, i.e. UCP1, PGC-1α and PPARα. Brown adipocytes appear as preferential targets of the inhibitory action of lithium on adipocyte differentiation.

INTRODUCTION

Lithium is currently used as an efficient primary therapy for bipolar disorder. However, lithium therapy in humans is associated with several side effects, a major one being increased weight gain. Although behavioural changes associated with lithium treatment can contribute to this effect, several indications have pointed out to a direct action of lithium on metabolism as responsible for increased fat accumulation in treated patients [1,2].

Lithium action occurs through several molecular mechanisms, the most relevant being inositol depletion due to inhibition of inositol monophospthatase, inhibition of other phosphomonoesterases and inhibition of glycogen synthase kinase (GSK-3) [3]. GSK3β is a negative regulator of the Wnt signalling pathway. Wnts are a family of secreted glycoproteins involved in embryonic development and other cellular processes. The Wnt signalling pathway involves the interaction of Wnt with the frizzled receptor in the cell surface and the subsequent inhibition of GSK-3β which, through stabilization of β-catenin, leads to transcriptional activation of target genes. Lithium mimics Wnt activation by inhibiting GSK-3β directly. It was known that lithium could inhibit adipocyte differentiation [4] and it has been recently reported that this occurs through the activation of the Wnt pathway [5]. It is not clear how this effect can be related to disturbances in adipose tissue accumulation in lithium-treated patients. Increased adiposity is usually a secondary effect of drugs promoting adipocyte differentiation, such as the antidiabetic PPARγ activators thiazolidinediones [6], not of inhibitors of adipocyte differentiation.

Whereas white adipocytes are the predominant cell type in adipose tissue depots of adult humans, some brown adipocytes are known to remain interespread in adipose tissue depots of adult humans. In some pathological conditions such as phaeochromocytoma, adults develop depots of brown adipose tissue formed of newly developed brown adipocytes [7]. Moreover, a reduced brown adipose phenotype in adipose tissue of adults is associated with metabolic disturbances such as insulin resistance [8]. Brown adipocytes have an opposite role respect to white adipocyte and instead of storing energy as triacylglycerols, brown adipocytes burn metabolic substrates due to their high content of mitochondria and naturally-uncoupled mitochondrial respiratory chain. The unique presence of the uncoupling protein-1

(UCP1) in brown adipocyte mitochondria is responsible for energy expenditure in this cell type [9].

The specific effects of lithium on the brown adipocyte have not been explored to date, and they can be involved in overall effects of lithium treatment on energy balance. Therefore, we undertook the study of the effects of lithium on brown adipocyte differentiation and gene expression.

METHODS

Brown preadipocytes were isolated from 3-week-old mice and differentiated as described elsewhere [10] Lithium chloride or sodium chloride were added at different times of the differentiation process of the culture (day 1, day 4 or day 7) and cells were studied for morphology and gene expression in the day 8, when control cells had achieved full differentiation. When indicated, cells in the day 7 of culture were exposed to 0.5 μM noradrenaline (NA) for 5 h or to 1μM all-trans retinoic acid (RA) for 24h..
RNA was extracted used an affinity column-based method (Qiagen, Hilden. Germany) and analysed using Northern blot in accordance with previously reported methods [11]. Blots were hybridised using the cDNAs for the fatty acid binding protein aP2 (FABP/aP2) [12], peroxisome proliferator activated receptor γ (PPARγ) [13], cytochrome c oxidase subunit II (COII) [14] and subunit IV (COIV) (ATCC, Rokville, USA), UCP1 [15], PPARγ coactivator-1α (PGC-1α) [16], PPARα [17] and a genomic fragment of mitochondrial DNA corresponding to the 16S rRNA gene [18]. The cDNA probes were labelled using ^{32}P-dCTP by the random oligonucleotide priming method. Equal loading of Northern blots was estimated by ethidium bromide staining of gels. Autoradiographs were quantified by densitometric scanning and statistical analysis was performed using the Student's t test.

RESULTS AND DISCUSSION

Brown adipocytes exposed to 5 mM or 25 mM LiCl when already differentiated (day 7 of culture) did not show any effect on morphology 24h later (see Fig 1) or even after longer times of exposure to lithium (not shown.). In contrast, marked effects were

observed due to lithium when it was added in the day 4 of culture, when brown pre-adipocytes had stopped proliferation and they are beginning to differentiate. Cells that had been exposed to 5 mM lithium showed reduced differentiation in the day 8 of culture, as evidenced by a reduced appearance of cells accumulating lipid droplets. When lithium exposure was at 25 mM, almost no cells accumulate lipids and remained with a fibroblast-like morphology. A full suppression of the acquisition of the differentiated brown adipocyte morphology was observed when either 5mM or 25 mM lithium was added from the first day of culture, and the whole proliferation and differentiation processes occurred in the presence of lithium.

Brown adipocyte differentiation can be considered as the result of the overlapping of distinct programs of induction of gene expression. First, there is an adipogenic program of differentiation common to white and brown adipocytes which is based on the accumulation of triacylglycerol stores. This is associated with the expression of gene markers for lipid accumulation, such as the fatty acid binding protein aP2/FABP, or of master transcription factors for genes of lipid metabolism such as PPARγ. The mRNA expression of these genes in brown adipocyte cells cultures exposed to lithium paralleled the effects on morphology. No effects were found when lithium was added to brown adipocytes in the day 7, when already differentiated (not shown). Exposure to 5 mM lithium from day 4 of culture lowered aP2/FABP mRNA and PPARγ mRNA levels whereas these transcripts became non detectable at 25 mM. 5 mM lithium from day 1 to day 8 led to lower but still detectable levels of aP2/FABP mRNA and PPARγ mRNA whereas 25 mM lithium resulted in non detectable levels of both transcripts.

A second aspect of the acquisition of the pattern of differentiation of brown adipocytes is mitochondrial biogenesis [11]. This can be followed by the expression of genes involved in the respiratory chain/oxidative phosphorylation machinery that are encoded by mitochondrial DNA (COII mRNA or 16S ribosomal RNA, for instance) or by the nuclear genome (COIV mRNA, for instance). Lithium treatment decreased the expression of these gene markers but to lesser extent than the adipogenic markers. There was a dose and time-dependent reduction in the mitochondrial DNA-encoded transcripts COII mRNA and 16S rRNA as well as in COIV mRNA, the reduction being more intense in this last case. There was no treatment with lithium that resulted in undetectable levels of these transcripts.

The differentiation of the brown adipocyte has also specific features which are different from white adipocyte differentiation and are functionally related to the differential thermogenic role of this cell type. This can be shown by the induction of the expression of the mRNAs for UCP1, the mitochondrial uncoupling protein of brown fat, by the expression of PGC-1α, a transcriptional co-activator involved in the acquisition of the several features of the differential brown adipocyte phenotype respect to the white adipocyte [16], and by the enhanced expression of PPARα , a subtype of the PPAR family involved in the coordinate regulation of lipid catabolism genes which is expressed preferentially in brown versus white adipocytes [19]. Lithium inhibited dramatically the expression of these genes. Exposure to lithium suppressed UCP1 gene expression and UCP1 mRNA became undetectable at any concentration or time of exposure to lithium (see Figure 2A) with the exception of exposure for 24h of already differentiated cells that had no effect (not shown). Exposure to 25 mM lithium from day 4 of culture or to any lithium concentration from day 1 on led also to undetectable levels of expression of UCP1, PGC-1α and PPARα transcripts. Only in cells exposed to 5 mM lithium from day 4 of culture PGC-1α and PPARα mRNA were detectable although dramatically diminished.

In order to further explore the action of lithium on the brown adipocyte specific functions, cells in the day 7 of culture that had been exposed to lithium at different times of the differentiation process were treated with NA or RA, known activators of the brown adipocyte-specific gene UCP1 (Fig 3). Cells exposed to either 5 mM or 25 mM lithium from day 1 did not show any induction of UCP1 gene expression due to these hormones, and UCP1 mRNA levels remained undetectable (not shown). When lithium was added from the day 4 of culture, 25 mM was completely non permissive for UCP1 gene expression under the action of NA or RA. Only in cells treated from day 4 with 5 mM lithium, UCP1 mRNA levels were induced in response to NA or RA, but to much lower extent respect to control cells. Finally, cells that were treated with lithium for 24 hours when already differentiated (1 day-treatment), showed not only unaltered UCP1 mRNA levels in basal conditions but also unaltered expression due to the action of NA or RA respect to control cells, either at 5 mM or 25 mM lithium concentration.

It is concluded that lithium has an inhibitory role in all the aspects of brown adipocyte differentiation which are common to white adipocyte differentation (lipid accumulation, gene markers common to white and brown adipocyte). This is consistent with studies using white adipocyte differentiation cells models [4,5] which attribute to

the activation of Wnt signalling due to lithium and inhibitory effect on overall adipogenesis. However, the action of lithium was much more powerful on any gene markers specific to brown fat, and concentrations or time of exposure to lithium capable of maintaining some expression of genes common to white and brown fat, were completely suppressive for the expression of brown adipocyte specific gene markers. This strong inhibitory effects could not be counter-acted by addition of NA or RA, the most powerful activators of the specific brown adipocyte gene UCP1. However, the inhibitory action of lithium was unequivocally associated with differentiation and, when brown adipocytes had been already differentiated, lithium did not interfere in the basal expression of brown adipocyte-specific genes nor in the distinct intracellular pathways mediating responsiveness to NA or RA. This indicates that it is the specific program of brown adipocyte differentiation but not the intracellular pathways of enhancement of UCP1 gene expression present in brown fat cells when already differentiated what is affected by lithium treatment.

Considering that the activation of the Wnt pathway appears to be a major mechanism for the action of lithium on adipose cells, the present findings are consistent with recent reports indicating that Wnt-10b over-expression in adipose tissues of transgenic mice leads to a full suppression of the specific gene markers of brown adipocyte, i.e., UCP1, in anatomical depots of brown adipose tissue whereas white adipose tissue gene expression is maintained. This is accompanied with abnormally low energy expenditure in response to food intake and cold intolerance, indicative of impaired brown adipose tissue function [20].

In summary, these results indicate that, although lithium has suppressive effects on overall adipocyte differentiation, the brown adipocyte is particularly sensitive to this effect respect to the white adipocyte differentiation pattern. A particularly intense inhibition of brown adipocyte respect to white adipocyte gene expression patterns due to lithium could be hypothesised to explain preferential effects of lithium inhibiting energy expenditure and therefore promoting weight gain. The potential differences between white and brown adipocytes in their cellular machinery of responsiveness to Wnt or other pathways potentially involved in lithium effects are not known, and deserve further research to explain the highest sensitivity of the brown adipocyte to lithium effects.

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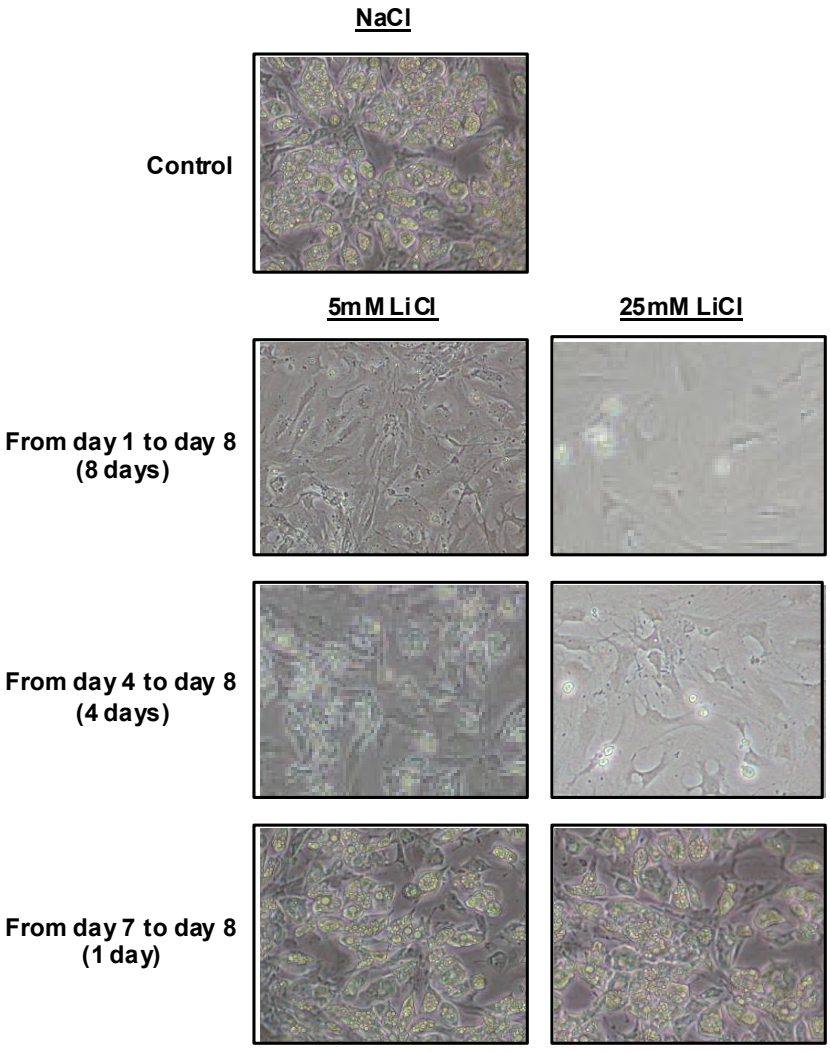
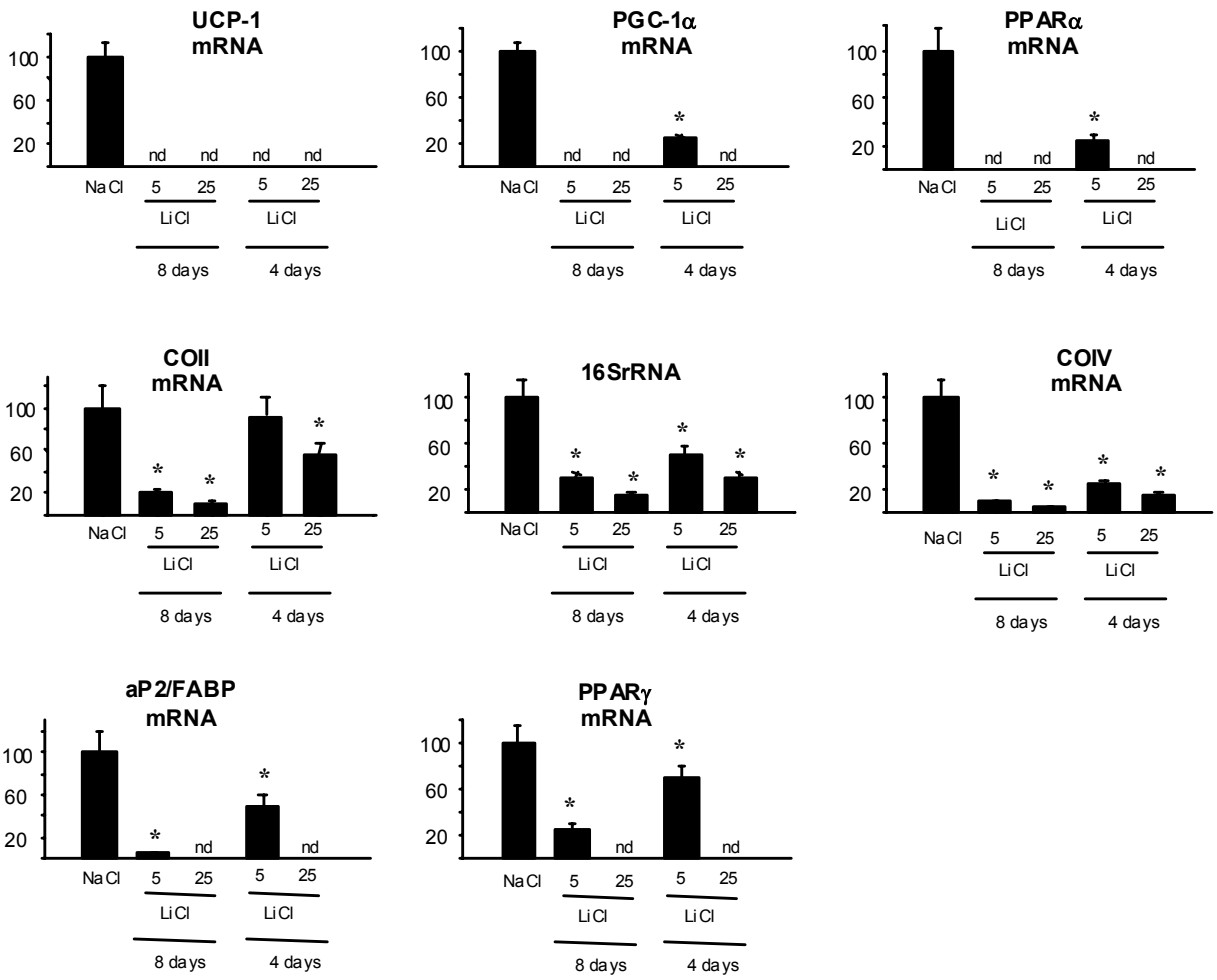


Figure 1

Effects of lithium on brown adipocyte differentiation in primary culture. Brown adipocyte precursor cells were cultured for 8 days in differentiating medium supplemented with sodium chloride (NaCl) from the day 1 of culture (control) of with either 5 mM or 25 mM lithium chloride (LiCl) that was added in the day 1, day 4 or day 7 of culture. Microphotographs were taken at day 8 of culture at X40 magnification.

A)


UCP-1 mRNA
PGC-1α mRNA
PPARα mRNA
COII mRNA
16SrRNA
COIV mRNA
aP2/FABP mRNA
PPARγ mRNA
100
60
20
nd
*
NaCl
5
25
LiCl
8 days
4 days

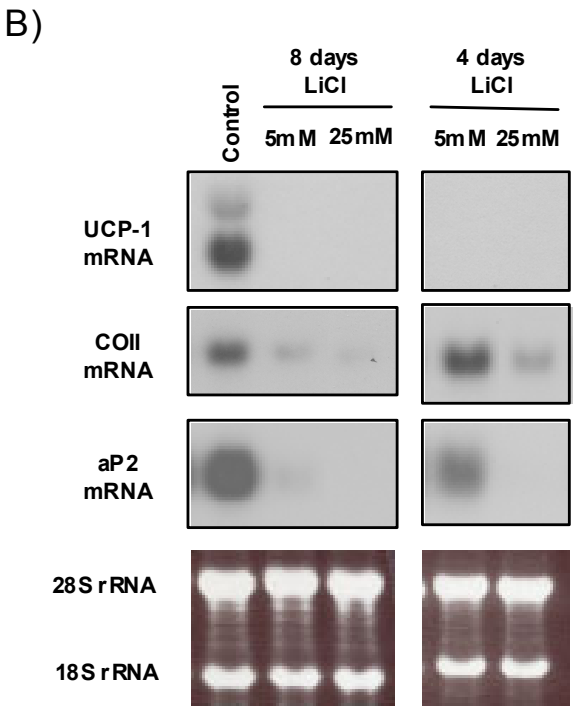


Figure 2.

Effects of lithium on gene expression in brown adipocytes differentiating in culture. Brown adipocyte precursor cells were cultured for 8 days in differentiating medium supplemented with sodium chloride from the day 4 of culture (control) of with either 5 mM or 25 mM lithium chloride that was added in the day 1 (8 days lithium exposure) or in the day 4 (4 days lithium exposure). Relative abundance of gene transcripts was determined by Northern blot analysis of 25 μg RNA. A, bars are means $\pm$ SEM of three independent experiments and are expressed as percentages relative to the mean value in brown adipocytes cultured in differentiating medium plus sodium chloride (NaCl) which was set to 100 (arbitrary units). Statistical differences ($p < 0.05$) from the NaCl-treated group are indicated as *. The cases where there is no detection of the mRNA are indicated by nd (non detectable). B, examples of Northern blot analysis of representative transcripts and ethidium bromide staining of ribosomal RNAs.

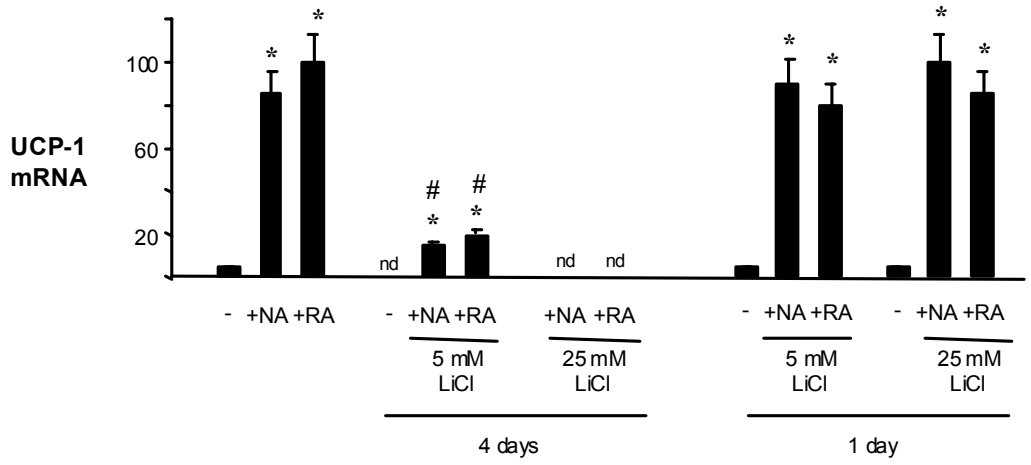


Figure 3.

Effects of noradrenaline and retinoic acid on UCP1 gene expression in brown adipocytes differentiating in culture in the presence of lithium. Brown adipocyte precursor cells were cultured for 8 days in differentiating medium supplemented with sodium chloride from the day 4 of culture (control) of with either 5 mM or 25 mM lithium chloride that was added in the day 4 (4 days lithium exposure) or in the day 1 (1 days lithium exposure). Cells were exposed to 0.5μM noradrenaline for 5 h or 1μM all-trans retinoic acid for 24 h. Relative abundance of UCP1 mRNA was determined by Northern blot analysis of 25 μg RNA. Bars are means ± SEM of three independent experiments and are expressed as percentages relative to the point of maximum value which was set to 100 (arbitrary units). Statistical differences ($p < 0.05$) between the controls and the NA or RA-treated cells for each culture condition are indicated as *, and those between control cells exposed to NA or RA respect to the equivalent treatments in lithium-exposed cells by #. The cases where there is no detection of the mRNA are indicated by nd (non detectable).

Uncoupling protein-1 and mitochondrial biogenesis are targets of reverse transcriptase inhibitor-induced toxicity in brown adipocytes

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Running head: RTIs alter UCP1 and mitochondrial biogenesis

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ABSTRACT

Objective: Human adipose depots contain remnant brown adipocytes interspersed among white adipocytes, and disturbances of brown with respect to white adipocyte biology have been implicated in highly active antiretroviral therapy (HAART)-induced lipomatosis. The aim of this study was to evaluate the effects of reverse transcriptase inhibitors (RTIs) on primary brown adipocytes differentiated in culture.

Design and Methods: We analyzed the effects of RTIs, nucleoside analogs (NRTIs stavudine, zidovudine, didanosine and lamivudine) and non-nucleoside analogs (NNRTIs nevirapine and efavirenz), on differentiation, mitochondrial biogenesis and uncoupling protein-1 (UCP1) gene expression in brown adipocytes.

Results: None of the NRTIs altered brown adipocyte differentiation whereas NNTRIs had opposite effects: efavirenz blocked lipid accumulation but nevirapine induced lipid deposition and expression of adipose markers, promoted mitochondrial biogenesis and increased UCP1 gene expression. Stavudine, zidovudine and didanosine reduced mitochondrial DNA (mtDNA) content. However, mitochondrial genome expression was only impaired in didanosine-treated adipocytes. Stavudine, but not zidovudine, induced expression of the mitochondrial transcription factors and this may explain compensatory mechanisms for the depletion of mtDNA by up-regulating mtDNA transcription. Stavudine caused a specific induction of UCP1 gene expression through direct interaction with a retinoic acid-dependent pathway.

Conclusions: Specific disturbances in brown adipocytes in adipose depots may contribute to HAART-induced lipodystrophy. Mitochondrial depletion does not appear to be the only mechanism explaining adverse effects in brown adipocytes, since there is evidence of compensatory mechanisms that maintain mtDNA expression and because UCP1 gene expression is specifically altered.

Keywords:

reverse transcriptase inhibitor,

lipodystrophy

lipomatosis

mitochondria

uncoupling protein-1

brown adipocyte

retinoic acid

INTRODUCTION

Highly active antiretroviral therapy (HAART) for human immunodeficiency virus type-1 (HIV) combines reverse transcriptase inhibitors (RTIs), which may be either nucleoside analogues (NRTIs) or non-nucleoside analogues (NNRTIs), or RTIs with protease inhibitors (PIs). HAART has led to substantial reductions in morbidity and mortality among patients. However, a serious metabolic syndrome has arisen in treated patients, referred to as the HAART-associated lipodystrophy syndrome [1], with a prevalence of about 40% [2]. The main symptoms are region-specific disturbances of fat distribution: severe loss of peripheral adipose tissue and increased visceral and dorsocervical adipose tissue (abdomen, breast hypertrophy, and cervical fat-pads, known as “buffalo humps”) [3]. Other metabolic disturbances include dyslipidemia, hyperglycemia and insulin resistance [1,3,4].

Alterations in body fat distribution and lipid metabolism among HIV-infected patients on HAART suggest adipocyte dysfunction. Initial reports implicated the PIs in the development of lipodystrophy [4]. Several *in vitro* studies have shown that PIs affect adipocyte differentiation and lipid accumulation in the 3T3 white adipocyte cell lines (for review [5]), and alterations in the transcriptional factor SREBP-1c may be responsible for some of these effects [6,7,8]. It is also widely accepted that RTIs alone can induce the pathogenesis of lipodystrophy [3,9,10,11]. However, *in vitro* experiments on the effects of RTIs are scarce. NRTIs have been reported to interfere to some extent with 3T3 adipocyte differentiation [12,13] and recently, the NNRTI efavirenz has been shown to inhibit the SREBP-1c-dependent lipogenic pathway [14]. Nevertheless, NRTIs can induce mitochondrial toxicity via specific inhibition of DNA polymerase-gamma [15]. Therefore, it has been suggested that depletion of mitochondrial DNA (mtDNA) content through inhibition of mtDNA synthesis in adipocytes contributes to the development of lipodystrophy [16]. Recently, several

studies have shown depletion of mtDNA in subcutaneous fat from NRTI-treated patients with lipodystrophy [17,18]. Furthermore, there is also evidence that NRTIs can affect mitochondrial function through mechanisms not involving inhibition of mtDNA polymerase-gamma, such as mtDNA deletions [19] or direct inhibition of mitochondrial respiration [20].

In mammals, there are two types of adipocyte: white adipocytes (which store metabolic energy as fat) and brown adipocytes (which dissipate metabolic energy as heat due to the presence of the uncoupling protein-1, UCP1) [21]. In humans, brown adipose depots are found in neonates whereas remnant brown adipocytes interspersed among white adipocytes remain in adult adipose depots [22]. The relative amount of brown adipocytes depends on the anatomical site (peripheral *versus* visceral fat depots). Brown adipocytes have a high metabolic rate and contain a large number of mitochondria, potential targets of HAART toxicity. The abundance and activity of brown adipocytes can be monitored by the expression of the UCP1 gene, a qualitative marker of brown respect to white adipose cell [21]. We have recently reported high UCP1 gene expression in lipomas from HAART-treated HIV-patients, thus implicating brown adipocytes in the development of HAART-associated lipomatosis [23]. Whether dysregulation of brown versus white adipocyte biology is more generally implicated in the development of lipodystrophy remains to be analyzed. However, subcutaneous fat from HAART–treated patients shows a mixture of brown and white morphology [24] and Pace *et al.* have recently reported that NRTI treatment is associated with changes in UCP1 gene expression in subcutaneous fat from HIV-patients on HAART [25].

The aim of this study was to evaluate the effects of RTI drugs used in HAART on primary brown adipocytes differentiated in culture. We measured the effects of RTIs on brown adipocyte differentiation, mitochondrial biogenesis and gene expression. Despite a reduction in mtDNA content caused by several NRTIs, gene expression for the mitochondrial oxidative machinery is not essentially impaired except in didanosine-treated cells. In contrast, the NNRTI nevirapine induces brown adipocyte differentiation,

including an increase in UCP1 gene expression. Stavudine specifically induces UCP1 gene expression, and this effect appears to be mediated by a retinoic acid-dependent pathway. Thus, a specific disturbance of brown adipocytes in the adipose depots may contribute to HAART-induced lipodystrophy.

MATERIALS AND METHODS

Cell culture and treatment

Stavudine and didanosine were obtained from Bristol-Myers-Squibb (Princeton, NJ, USA), lamivudine and zidovudine were from GlaxoSmithKline (Greenford, UK). Nevirapine and efavirenz were obtained from Boehringer Ingelheim/Roxane (Ridgefield, CT, USA) and Bristol-Myers-Squibb, respectively. All NRTIs were dissolved in phosphate buffer saline whereas NNRTIs were dissolved in dimethyl sulfoxide (DMSO). All-*trans* retinoic acid, norepinephrine, ascorbic acid, 3,5,3'-triiodothyronine (T_3), insulin, propanolol and prazosin were purchased from Sigma-Aldrich (St. Louis, USA). BRL49653 (rosiglitazone) was kind gift from Dr. L. Casteilla (Toulouse, France). The specific retinoic acid receptor (RAR) antagonist AGN 193109 was kind gift from Dr R. Chandraratna (Allergan Pharmaceuticals, Irvine, USA).

Primary culture of differentiated murine brown adipocytes was performed as described previously [26]. Precursor cells were grown in 4 ml of Dulbeccos's modified Eagle's medium (DMEM)-Ham's F12 medium (1:1, v/v) supplemented with 10% (v/v) FCS, 20 nM insulin, 2 nM T_3 and 100 μM ascorbate. At confluence (day 4 of culture), cells were incubated in the absence or presence of each antiretroviral drug until day 8 of culture (long-term treatments). The short-term experiments (24h or 48h) with the different RTIs, rosiglitazone at 10μM, all-*trans* retinoic acid at 1μM and AGN 193109 at 1μM, were performed on day 8 of culture when 80-90 % of the cells were considered to be differentiated on the basis of lipid accumulation and acquisition of brown adipocyte morphology. Norepinephrine was added at 0.5μM for 5 hours.

Determination of mitochondrial DNA abundance.

Relative mtDNA abundance was assessed as described elsewhere [27]. Total DNA from preadipocytes or from fully differentiated brown adipocytes in primary culture,

treated or not with the various RTIs, was obtained by digesting the cells with proteinase K followed by a phenol/chloroform precipitation. 20μg of total DNA was digested with *Eco*RI endonuclease and analyzed by Southern Blot. Blots were hybridized with the murine mtDNA fragment encoding 16S rRNA [28], thus leading to a 3.0 kb hybridization signal for mtDNA. As a control for equal loading of nuclear DNA, blots were rehybridized with the murine C/EBPβ genomic probe [29], which reveals a 4.5 kb band for the corresponding nuclear gene fragment. The DNA probes were labeled using [α-^{32}P]dCTP by the random oligonucleotide-priming method (Amersham Biosciences).

RNA isolation and Northern blot analysis.

Total RNA was extracted using the RNeasy Mini Kit (Qiagen). For Northern blot analysis, 10 μg of total RNA was denatured, electrophoresed on 1.5% formaldehyde-agarose gels, and transferred to positively-charged nylon membranes (N^+ Boehringer Mannheim). Equivalent amounts of ribosomal RNA in the samples were checked by ethidium bromide UV visualization. Prehybridization and hybridization were performed at 55^{0}C in a 0.25M Na_2HPO_4 (pH=7.2), 1mM EDTA, 20% SDS and 0.5% blocking reagent (Boehringer Mannheim) solution. Blots were hybridized using mtDNA fragments as probes for detection of the mtDNA-encoded cytochrome oxidase subunit II [30] and mitochondrial16S rRNA [28]. The murine cDNAs for the nuclear-encoded cytochrome oxidase subunit IV (ATCC, Rockville, USA), PGC-1α [31], UCP1 [32], UCP2 [33], adipocyte-fatty acid binding protein aP2/FABP [34], mitochondrial transcription factor A [35], and the mitochondrial transcription factors B1 and B2 probes [36] were also used as hybridization probes. The DNA probes were labeled using [α-^{32}P]dCTP by the random oligonucleotide-priming method (Amersham Biosciences).

Preparation of protein extracts and Western blot analysis.

For the protein extracts, fully differentiated brown adipocytes in primary culture treated or not with the RTIs were harvested in a buffer containing 100 mM TrisHCl (pH=8), 250mM NaCl, 1mM EDTA, 1% IGEPAL, 0.5mM PMSF, a cocktail of protease inhibitors (leupeptin, pepstatin, aprotinin at 1 μg/ml and benzamidin 1mM) and 0.5mM DTT. The harvested cells were incubated for 1 hour at 4°C and centrifuged at 13000rpm, for 10 minutes at 4°C. The final supernatant was the protein homogenate. Protein concentration was measured using the BCA Protein Assay Kit (Pierce), using bovine serum albumin as a standard.

For Western blot analysis, samples containing 50 μg of protein extract were mixed with 1/5 volume of a solution containing 50% glycerol, 10% 2-βmercaptoethanol, 0.5% bromophenol blue and 0.5M Tris (pH 6.8), incubated at 90 °C for 5 minutes and electrophoresed on SDS/13% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes (Millipore Corp, Bedford, USA). Blots were probed with a monoclonal antibody for the mtDNA-encoded mouse cytochrome c oxidase subunit I (A-6403, Molecular Probes, Eugene, USA), as well as with the antibodies against the voltage-dependent anion carrier or porin (VDAC) (Calbiochem Anti-Porin 31HL, Darmstadt, Germany), as a marker of mitochondrial protein loading, and against β-actin (Sigma clone AC-15, Saint Louis, USA), as a marker of overall cell protein loading. Immunoreactive material was detected using the enhanced chemiluminiscence method (ECL, Amersham Biosciences).

Transient transfection experiments

The HIB-1B brown adipocyte cell line, kindly provided by Dr. B. Spiegelman, was cultured in DMEM:F12 (1:1) supplemented with 10% heat-inactivated FCS and 4 mg/L biotin. The transient transfection experiments were performed with HIB-1B preadipocytes at 80% of confluence, using the *FuGene6 Transfection Reagent* (Roche Molecular Biochemicals, Mannheim, Germany), following the manufacturer's protocol.

The plasmid (-4551)UCP1-CAT contains the region –4551 to +110 of the rat *ucp1* gene driving the promoterless chloramphenicol acetyltransferase (CAT) gene, and the expression vector pRSV-RARα contains the entire open-reading frame of the human RARα protein [37]. Each transfection contained 1μg of 4.5kbUCP1-CAT, 0.05μg of the hRARα expression vector and 0.1μg of cytomegalovirus β-galactosidase vector, as internal control for variation in transfection efficiency. 24 hours after the transfection process, cells were treated or not with stavudine or nevirapine at 20μM for a further 24 hours. CAT and β-galactosidase activities were measured as described elsewhere [38].

Densitometric analysis. Quantification of autoradiographs and ECL signals was performed by densitometric analysis (Phoretics 1D software, Phoretics International LTD, Newcastle, UK). Only signals in the linear range were analyzed.

Statistical analysis. Where appropriate, statistical analysis was performed by Mann-Whitney U test, and significance is indicated on the figure legends.

RESULTS

Nevirapine induced but efavirenz impaired terminal differentiation of brown adipocytes whereas NRTIs did not interfere with the process

Brown preadipocytes in primary culture proliferate until day 4, after which they differentiate until day 8, as shown by the acquisition of multilocular lipid accumulation and expression of the UCP1 gene [26]. When stavudine, didanosine, zidovudine or lamivudine were added to the medium on day 4 of culture, acquisition of differentiated cell morphology at day 8 was unaltered (Fig. 1, top, and data not shown). The drugs were used at 20 μM, which was in the range of their maximum concentration values and similar to that used in white adipose cell studies [6,12,39,13]. NNRTIs had the opposite effect on brown adipocyte morphological differentiation: nevirapine slightly increased lipid accumulation whereas cells induced to differentiate in the presence of efavirenz did not accumulate lipid droplets (Fig. 1, bottom).

NRTIs, except lamivudine, reduced whereas nevirapine increased mtDNA content

To analyze whether RTIs affect mitochondrial biogenesis, mtDNA content per cell was assessed by quantifying the relative amount of mtDNA with respect to nuclear DNA. Stavudine, didanosine and zidovudine reduced mtDNA in brown adipocytes, whereas lamivudine did not (Fig. 2a). The relative amount of mtDNA almost doubled from day 4 of culture to day 8, indicating that these NRTIs blocked the differentiation-dependent increase in mtDNA abundance (Fig. 2b). In contrast, when brown adipocytes were treated with nevirapine, mtDNA abundance was double that of solvent-control cells (DMSO).

Mitochondrial genome expression was only impaired in didanosine treated cells

To examine whether changes in mtDNA abundance affect mitochondrial biogenesis, gene expression for the mitochondrial-encoded 16S rRNA and subunits I and II of cytochrome c oxidase (CO), a component of the mitochondrial respiratory system, was assessed (Fig. 2c,d). Only didanosine treatment reduced 16S rRNA and COII mRNA, thus suggesting a compensatory up-regulation of mtDNA transcription in stavudine and zidovudine-treated cells (Fig. 2c). In contrast, increased mtDNA abundance in nevirapine-treated cells resulted in a significant increase in mitochondrial transcripts 16S rRNA and COII mRNA. When COI protein levels were analyzed, a parallel decrease in didanosine-treated cells and an increase in nevirapine-treated cells was observed. These changes were specific for the mitochondrial-genome encoded protein and not related to any general effect upon mitochondrial or cell protein content (assessed by nuclear-encoded voltage-dependent anion carrier (VDAC) or β-actin, respectively) (Fig. 2d).

Changes in mitochondrial transcription factors expression as a compensatory mechanism to ensure mtDNA expression

In an attempt to identify how stavudine and zidovudine-treated cells compensate mtDNA depletion, the expression of the transcription factors that regulate mtDNA transcription was evaluated. As depicted in Fig. 3, expression of mitochondrial transcription factor A (TFAM), which is essential for mammalian mtDNA transcription and genome maintenance [40], was decreased in didanosine-treated cells, whereas it remained unaltered when cells were exposed to zidovudine or lamivudine. In contrast, treatment with stavudine increased TFAM mRNA expression. A similar profile of expression in response to NRTI treatment was found for the recently identified transcription factors involved in the initiation and regulation of mitochondrial transcription, the mitochondrial transcription factor B1 (TFB1M) and B2 (TFB2M) [41]. Thus, a compensatory up-regulation of mitochondrial transcription by increased expression of mitochondrial transcription factors can be proposed in cells treated with

stavudine whereas it does not explain unaltered levels of mitochondrial transcripts in zidovudine-treated cells. We next analyzed the expression of the co-activator PGC-1α which has been demonstrated to induce mitochondrial biogenesis in several tissues including brown fat and, in that tissue, to coordinately activate the thermogenic program including UCP1 gene expression [31,38]. Only stavudine induced the expression of PGC-1α mRNA, which was not affected by any other NRTI. The NNRTI nevirapine increased the mRNA expression of TFAM, TFB1M, TFB2M and PGC-1α, in agreement with its global positive effect upon mitochondrial biogenesis. Furthermore, nevirapine increased the expression of the nuclear-encoded subunit IV of cytochrome c oxidase (COIV) mRNA, while the NRTIs did not (Fig. 4a).

Stavudine specifically induced uncoupling protein-1 gene expression

We next analyzed whether the expression of genes that are markers of brown-specific function or adipose metabolism was affected by long-term RTI treatment of brown adipocytes. UCP2 mRNA expression was not affected by any RTI treatment (Fig. 4a). In contrast, stavudine and nevirapine induced UCP1 mRNA expression. Nevirapine also induced expression of the adipogenic marker gene aP2/FABP. The effects of stavudine and nevirapine on differentiated adipocytes on day 8 of culture were examined. Nevirapine had no effect (Fig. 4b, and data not shown), while stavudine caused a two-fold induction of UCP1 mRNA expression after 24 hours (Fig. 4b), which remained after 48 hours (data not shown). The effects of stavudine on differentiated brown adipocytes were specific for UCP1 since neither COII mRNA (Fig 4b) nor COIV mRNA or aP2/FABP mRNA (not shown) levels were altered. The effects of stavudine on UCP1 mRNA expression were dose-dependent, with maximum induction at 20 μM (Fig 4c).

Stavudine induced UCP1 gene expression through a retinoic-acid mediated pathway

We next analyzed whether the effects of stavudine implicate known pathways of regulation of UCP1 gene expression. Noradrenaline directly activates UCP1 gene transcription through a cAMP-response element in the proximal regulatory promoter [42]. Furthermore, noradrenaline highly induces the expression of the co-activator PGC-1α [31], which also contributes to cAMP-mediated up-regulation of UCP1 gene transcription by co-activating nuclear hormone receptors bound to the upstream enhancer of the gene [38]. Among them, all-*trans* retinoic acid receptors (RARs) and 9-*cis*-retinoic acid receptors (RXRs) were responsible for the powerful induction by retinoic acid of UCP1 gene transcription [26,43]. The peroxisome proliferator-activated receptors (PPAR) γ and α also activated transcription of the UCP1 gene promoter [44,38]. Whereas the adrenergic antagonists propanolol and prazosin did not affect stavudine-induction of UCP1 gene expression (data not shown), the specific RAR-antagonist AGN 193109 completely blocked it in both the long-term and the short-term treatment of brown adipocytes (Fig 5a). The inhibitory effects of AGN 193109 were specific for retinoic acid induction of UCP1 mRNA expression without interfering with its induction by noradrenaline or the PPARγ-agonist rosiglitazone (data not shown). Thus, induction of UCP1 gene expression by stavudine involves a retinoic acid-mediated pathway.

To further investigate stavudine regulation of UCP1 gene expression, brown adipocyte derived HIB-1B cells were transiently transfected with a plasmid containing the upstream 4.5 kb of the rat UCP1 gene fused to a CAT reporter gene. Stavudine, but not nevirapine, significantly increased 4,5UCP1-CAT activity only when the expression vector for RAR was transfected (Fig. 5b), further indicating an involvement of an RAR-mediated signaling pathway in UCP1 gene induction by stavudine.

DISCUSSION

NRTI-induced mitochondrial toxicity may explain the lipodystrophy syndrome [16,45,46]. NRTIs inhibit mtDNA polymerase gamma, which is responsible for the replication of mtDNA [15]. Thus, treatment with NRTIs can lead to a depletion of mtDNA and a reduction in the expression of mtDNA-encoded genes. Several studies have reported mtDNA depletion in subcutaneous adipose tissue associated with NRTI therapy [17,47,18], but only very recently, has this been shown to reduce in mitochondrial activity [48]. Although the effect of NRTI has been tested in isolated mitochondria and several cell types [49], only one recent report has implicated adipose cell lines [13]. The present study is the first to examine the effects of NRTIs on mitochondrial biogenesis and gene expression in brown adipocytes.

Brown adipocytes provide a suitable cell model in which to examine whether NRTI-induced mitochondrial toxicity contributes to the pathophysiological mechanisms underlying the lipodystrophy syndrome. Brown adipocytes are characterized by high mitochondrial development and content, and specifically express the mitochondrial uncoupling protein UCP1. In humans, brown adipocytes are interspersed among white adipocytes in adult adipose depots and a reduced brown adipose phenotype in those depots has been associated with insulin resistance [50]. Furthermore, brown adipocytes are involved in the development of HAART-induced lipomatosis [23]. Here we report that RTIs have direct effects on brown adipocytes by interfering with mitochondrial biogenesis, differentiation and specific expression of the brown adipocyte gene marker UCP1.

None of the four NRTI tested (stavudine, zidovudine, didanosine and lamivudine) impaired brown adipocyte differentiation as evaluated by acquisition of morphological features, such as lipid deposition, and adipose gene expression. This is consistent with data from the few studies in the murine white adipose cell lines 3T3-L1 [6,39] and 3T3-F442A [12,13], although stavudine and zidovudine have very recently

been reported to alter terminal lipid deposition in 3T3-F442A cells [13]. In contrast, the NNRTIs studied have the opposite effect on the differentiation of primary brown adipocytes: whereas efavirenz blocked lipid accumulation, nevirapine had a global positive effect. The negative effect of efavirenz on brown adipocyte differentiation was similar to that previously reported in white adipose cell lines and primary cultures of human preadipocytes [14]. In contrast, nevirapine enhanced the differentiation of primary brown adipocytes: it induced lipid deposition and expression of adipose markers, promoted mitochondrial biogenesis and increased the expression of the UCP1 gene. These effects depend on long-term treatment of cells during differentiation and, in contrast to the effects of stavudine (see below), they did not occur in acute treatment, thus further supporting that the effects of nevirapine occur through a positive global effect upon brown adipocyte differentiation. To our knowledge, present study is the first to analyze the effects of nevirapine on adipose cells. Mangiacasale *et al* [51] have recently reported that nevirapine inhibits proliferation and promotes differentiation in several normal and transformed cell types by inhibiting the endogenous nontelomeric reverse transcriptase. Further studies are necessary to determine whether this or other molecular mechanisms contribute to nevirapine induction of brown adipocyte differentiation.

Present results indicate that whereas lamivudine did not alter any mitochondrial-related parameter, the other NRTIs (stavudine, zidovudine and didanosine) reduced the content of mtDNA in long-term treated brown adipocytes. However, only didanosine decreased in the levels of mitochondrially-encoded transcripts 16S rRNA and COII mRNA, thus indicating that transcriptional mechanisms compensate for the depletion of mtDNA in stavudine- and zidovudine-treated cells. NRTI effects are specific to mitochondrial genome expression since expression of the nuclear-encoded genes for mitochondrial proteins such as COIV and UCP2 were unaltered in NRTI-treated cells. Present data constitute one of the first reports on the NRTI effects on mitochondrial biogenesis and gene expression in an *in vitro* adipose cell model. A previous report

indicates that large doses of stavudine and zidovudine also reduce mtDNA concentration in 3T3-L1 adipocytes [52], and a recent report indicates that stavudine and zidovudine, in contrast to didanosine, increase mitochondrial mass but lower the mitochondrial membrane potential in differentiating 3T3-F442A adipocytes [13].

In didanosine-treated cells, the expression of mitochondrial transcription factors TFAM, TFB1M and TFB2M was decreased in agreement with lack of compensation of mitochondrial genome expression. In contrast, stavudine, but not zidovudine, induced expression of mitochondrial transcription factors, thus normalizing mtDNA-encoded transcripts and proteins. Compensatory mechanisms for mtDNA depletion have been adduced to explain the lack of correlation between time on NRTI treatment and the probability of developing adverse effects. Similarly to what happens in patients with mtDNA defects of genetic origin, mitochondrial dysfunction in HAART-treated patients may become evident only after a profound reduction in mtDNA content. In peripheral blood cells from asymptomatic NRTI-treated patients, high levels of mtDNA depletion have been reported to be compatible with the maintenance of mtDNA expression by homeostatic compensatory mechanisms [53]. The intensity of such compensatory mechanisms can differ between individuals and/or between treatments, and identification of the molecular mechanisms involved would be of interest in order to minimize mitochondrial dysfunction. Further studies are required to establish whether mitochondrial transcription factors explain compensation, and if so, to identify potential strategies to promote their expression to compensate mtDNA depletion through up-regulation of mtDNA expression.

An estimated 6% of AIDS patients under HAART therapy develop lipomatosis, often characterized by enlarged adipose depots in the dorso-cervical region [54]. The brown adipocyte cell linage has recently been implicated in HAART-induced lipomatosis due to the expression of the UCP1 gene in the lipomas [23]. Here we report that stavudine, but not other NRTIs, specifically induces UCP1 gene expression in brown adipocyte cultures. The effect is specific since the expression of the

mitochondrial and adipose related genes remains unaltered, and only correlates with increased expression of PGC-1α ,which is a potent transcriptional coactivator of UCP1 gene transcription besides its role in regulating mitochondrial biogenesis. Positive action of stavudine on UCP1 gene expression occurs during long-term treatment of differentiating brown adipocytes but also in short-term treated mature brown adipocytes, indicating a direct effect of stavudine that is independent of differentiation. Among the known transcriptional regulators of the UCP1 gene, we demonstrate that stavudine specifically mimics the action of the retinoic acid pathway. Activation by retinoic acid, which is the active form of vitamin A, of the human and murine UCP1 genes occurs at the transcriptional level and it is mediated by retinoic acid receptors [26,55,43]. Accordingly, present results showed that the positive effect of stavudine on the UCP1 gene promoter requires the retinoic acid receptor. This is also reinforced by the inhibitory effect of AGN 193109, a retinoic acid receptor antagonist, on stavudine induction of UCP1 gene expression. The hypothesis that drugs used in HAART may affect vitamin A metabolism and/or retinoid-mediated signaling pathways has been related to HIV protease inhibitors. In fact, PIs were believed to interfere with retinoid metabolism [56] and indinavir with the retinoic acid pathway [57]. More recently, it has been described that some patients under HAART treatment have alterations in the plasma retinol levels, which has been related to changes in retinoic acid synthesis [58]. In fact, white and brown adipose tissues, together with the liver, constitute the main active depots of vitamin A in the body [59]. Whether stavudine interferes with vitamin A metabolism and/or retinoic acid signaling pathway specifically in brown adipocytes or this is a more wide effect remains to be determined. Another question would be the physiological consequences of increased expression of UCP1 in adipose tissues [46]. Mitochondrial uncoupling in the adipocyte due to the presence of UCP1 can alter the intracellular energetic status of cells which may influence metabolism (i.e. promote lipid oxidation and energy expenditure), the output of endocrine signals (i.e. leptin, adiponectin) and/or intracellular signals for apoptosis in the adipocyte, which may lead

to lipoatrophy. Experimental studies using mice genetically engineered to express UCP1 in white adipose depots, which result in low levels of mitochondrial uncoupling, have shown an altered distribution of subcutaneous versus visceral adipose tissue depots, reminiscent of lipodystrophy [60].

In conclusion, brown adipose mitochondria are direct targets of the NRTIs stavudine, zidovudine and didanosine in an *in vitro* brown adipose model. However, transcriptional mechanisms compensate for the loss of mtDNA content, which results in unaltered levels of mitochondrial expression in stavudine and zidovudine treated cells. At least for stavudine, the increased expression of the mitochondrial transcription factors TFAM, TFB1M and TFB2M may be involved in such compensatory mechanisms. These findings are relevant to the lipodystrophy syndrome, since they explain differences in the development of mitochondrial dysfunction between the NRTIs and their combinations in HAART therapy. Our results may also help to identify molecular targets to minimize the impact of HAART-induced mitochondrial toxicity. Identification of UCP1 as a specific target of stavudine through retinoic-mediated pathways is of importance in HAART-induced lipomatosis. It remains to be determined whether it is involved in the alterations in subcutaneous and visceral fat depots associated with lipodystrophy.

ACKNOWLEDGEMENTS

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Fig. 1

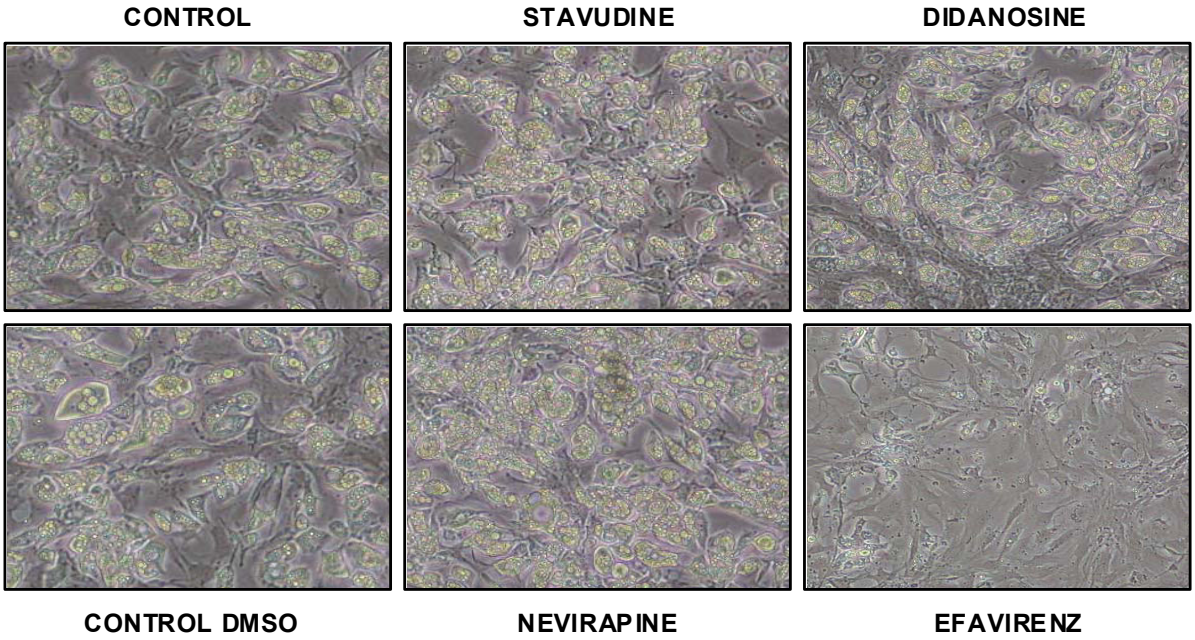

FIGURE 1
Effect of RTIs on morphological differentiation of primary brown adipocyte cultures. Confluent primary brown preadipocytes (day 4) were differentiated in the absence (control and control with solvent DMSO), or presence of various RTIs at a concentration of 20 μM until day 8 of culture. NRTIs stavudine and didanosine were dissolved in phosphate-buffered saline, whereas NNRTIs nevirapine and efavirenz were dissolved in a final concentration of 0.1% DMSO. Control DMSO cells were differentiated in the presence of 0.1% DMSO. Cells were examined by phase contrast microscopy at X40 magnification on day 8 of culture. The images are representative of at least three independent experiments performed in different primary cultures.

Fig. 2

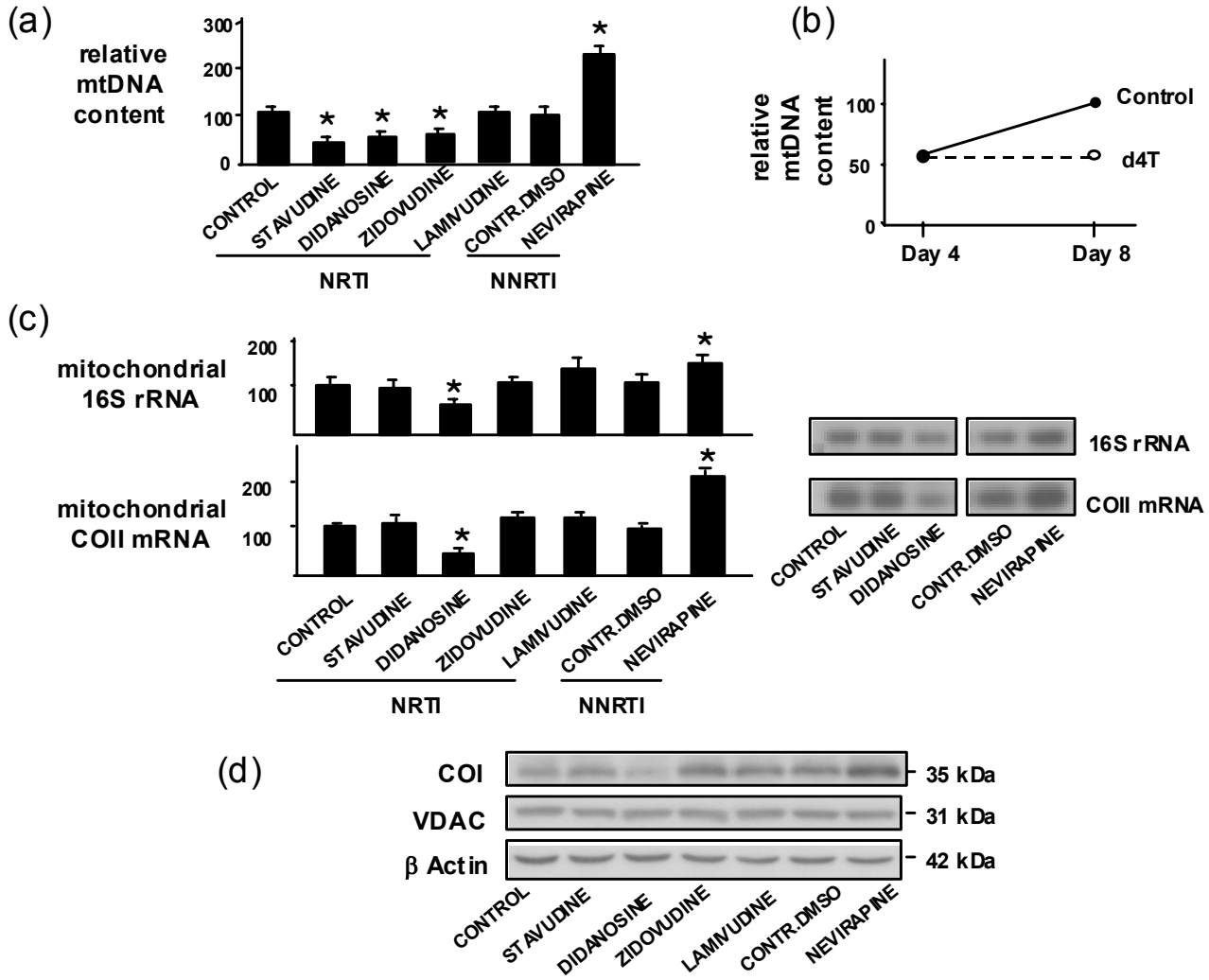

FIGURE 2

Effect of RTIs on mitochondrial biogenesis in brown adipocytes differentiating in culture. Confluent brown preadipocytes (day 4) were differentiated in the absence (control and control 0.1% DMSO), or presence of the indicated NRTIs (stavudine, didanosine, zidovudine and lamivudine) or NNRTI nevirapine at 20 μM until day 8 of culture. Bars are means ± SEM of two–three independent experiments with duplicate plates and are expressed relative to the untreated control cells, set to 100. Statistical significance of comparisons between treated cells and their respective controls are shown by: * $p \leq 0.05$, (a) Relative abundance of mtDNA was assessed by quantifying the relative amount of mtDNA with respect to nuclear DNA by Southern blot analysis, (b) Profile of relative mtDNA content during differentiation. Untreated preadipocytes at day 4 of culture were compared to cells at day 8 differentiated from day 4 in the absence (control) or presence of 20 μM stavudine (d4T). Points are means of two independent experiments and are expressed relative to control cells at day 8, which was set to 100. (c) Mitochondrial genome expression as measured by the relative abundance of the mitochondrial transcripts 16S rRNA and cytochrome oxidase subunit II (COII) mRNA as determined by Northern blot analysis, with representative Northern blots shown on the right, and (d) relative abundance of cytochrome oxidase subunit I (COI) protein as determined by Western blot analysis of whole-cell lysates, and control of mitochondrial protein (voltage-dependent anion carrier porin, VDAC) and total protein loading (β-actin). A representative immunoblot from three independent experiments is shown.

Fig. 3

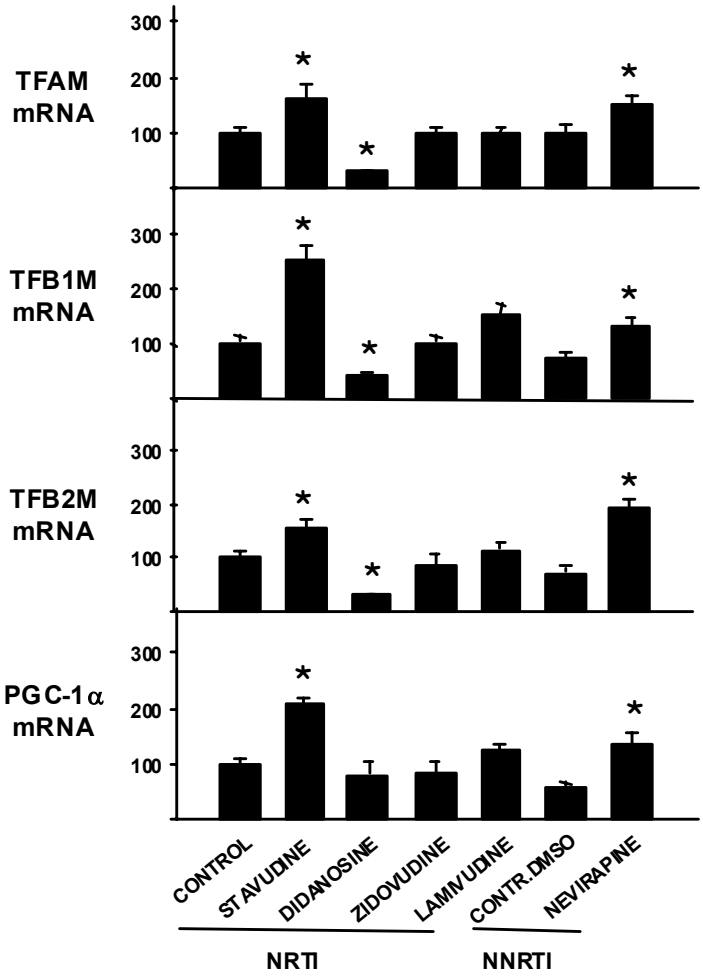

FIGURE 3
Effect of RTIs on gene expression of mitochondrial transcription factors and coactivator PGC-1α. Messenger RNA expression of mitochondrial transcription factors TFAM, TFB1M and TFB2M and of coactivator PGC-1α were analyzed by Northern blot. For details, see the legend to the Fig. 2.

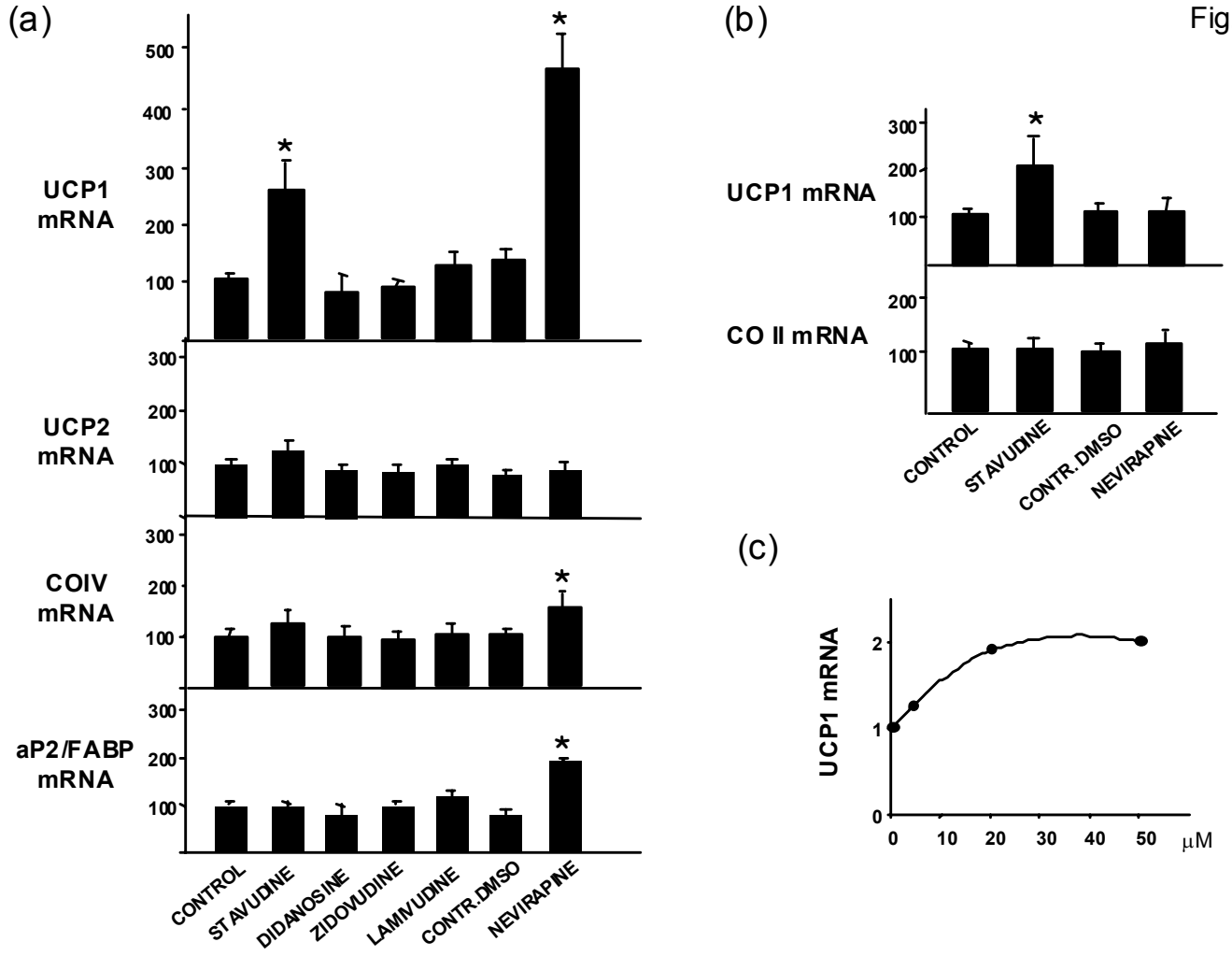

FIGURE 4

Effect of RTIs on the expression of uncoupling proteins, mitochondrial and adipose gene markers. Stavudine specifically induced uncoupling protein-1 gene expression. (a) mRNA expression of uncoupling proteins UCP1 and UCP2, of nuclear-encoded mitochondrial cytochrome oxidase subunit IV (COIV) and of the adipogenic marker adipocyte-fatty acid–binding protein (aP2/FABP) were analyzed by Northern blot. For details, see the legend to the Fig. 2. (b) Differentiated brown adipocytes at day 8 of culture were maintained for 24 hours in the absence (control and control 0.1% DMSO), or presence of either 20 μM stavudine or 20 μM nevirapine in 0.1% DMSO. Bars are means ± SEM of two–three independent experiments with duplicate plates and are expressed as relative to the untreated control cells which was set to 100. Statistical significance of comparisons between treated cells and their respective controls are shown by: * $p \leq 0.05$, (c) Dose-response curve for the effects of the NRTI stavudine on UCP1 gene expression. Differentiated brown adipocytes (day 8) were exposed to the indicated concentrations of stavudine for 24 hours. Points are means from two-three independent experiments with duplicate plates, in which the variation within the experimental groups is less than 15%..

Fig. 5

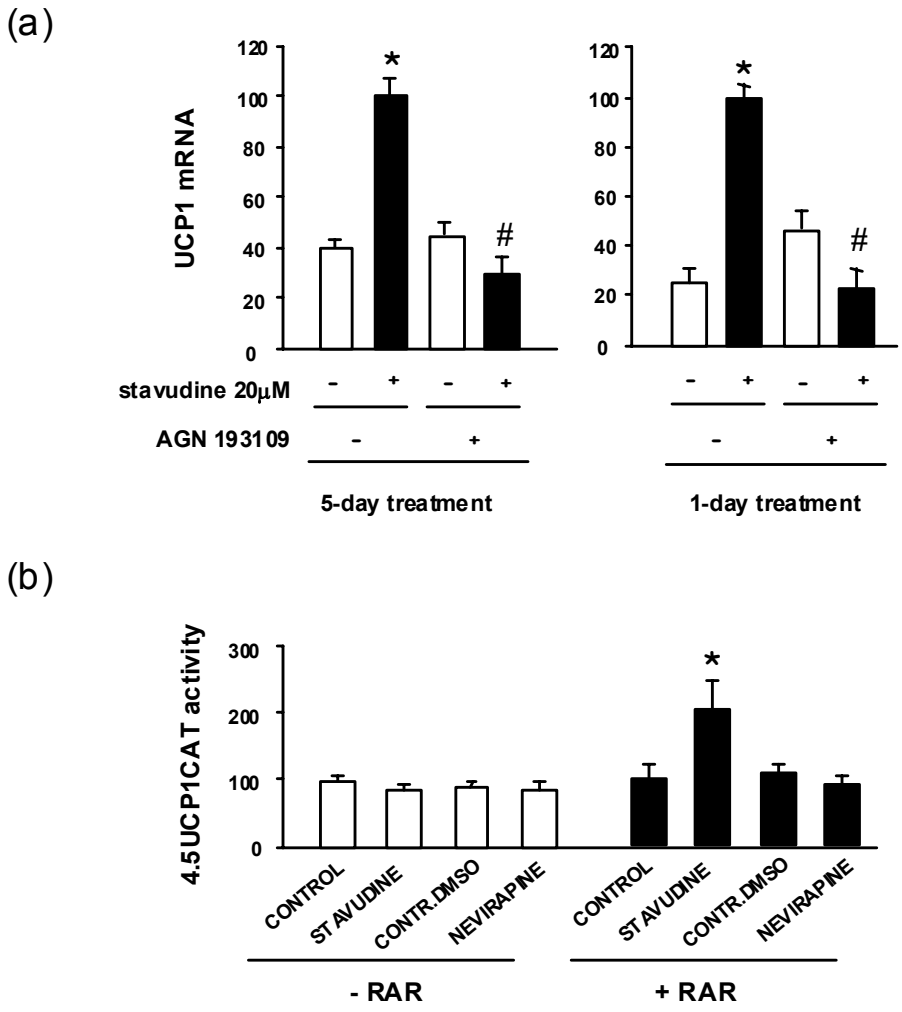

FIGURE 5

Effect of the retinoic acid receptor antagonist AGN 193109 on the induction of uncoupling protein-1 gene expression by stavudine, and effects of stavudine on the transcriptional activity of the UCP1 gene promoter. (a) Confluent brown preadipocytes (day 4) were differentiated in the absence or presence of 20 μM stavudine and/or 1μM AGN 193109 (an specific RAR-antagonist) until day 8 of culture, or (b) differentiated brown adipocytes (day 8) were exposed to 20 μM stavudine and/or 1μM AGN 193109 for 24 hours. UCP1 mRNA expression was analyzed by Northern blot. Bars are means ± SEM of two–three independent experiments with duplicate plates and are expressed relative to the point of maximal expression (stavudine-treated cells) which was set to 100. Statistical significance of comparisons is shown by: * $p \leq 0.05$, for the effect of stavudine, and # $p \leq 0.05$, for the effect of AGN 193109. (c) Transient transfection experiments of HIB-1B brown preadipocytes were performed with 1μg plasmid containing the upstream 4.5 kb of the UCP1 gene promoter fused to a cloramphenicol acetyltransferase (CAT) reporter gene (4.5UCP1CAT). When indicated, 0.05μg of the expression vector for the retinoic acid receptor α (pRSV-RARα) was cotransfected. Cells were exposed to 20 μM stavudine or nevirapine for 24 hours or not (control or 0.1%DMSO solvent control for nevirapine). Results are expressed as CAT activity relative to control which is set to 100, and are means ± SEM of two independent experiments, each performed in duplicate.

DESARROLLO DE UN MODELO DE DIFERENCIACIÓN DE ADIPOCITOS BLANCOS HUMANOS EN CULTIVO PRIMARIO. EFECTO DEL INHIBIDOR DE LA TRANSCRIPTASA REVERSA ANÁLOGO DE NUCLEÓSIDOS (NRTI) DIDANOSINA

INTRODUCCIÓN Y PLANTEAMIENTO EXPERIMENTAL

La lipodistrofia asociada al tratamiento antiretroviral de elevada actividad (HAART), que se ha descrito en pacientes de SIDA en tratamiento, es un síndrome del cual aún se desconoce su etiopatología. Este síndrome se caracteriza por una pérdida de la grasa de los brazos, piernas y cara y una acumulación de ésta en la zona abdominal y nucal, con alteraciones metabólicas asociadas (1-3). Las alteraciones propias del síndrome en la distribución de la grasa corporal, que son región-específicas, y las alteraciones en el metabolismo lipídico son probablemente debidas a una disfunción adipocitaria.

Se han realizado numerosos estudios *in vitro* del efecto de los fármacos antiretrovirales con líneas celulares de adipocitos blancos, como las líneas celulares 3T3-L1 o 3T3-F442A (4). Existen, sin embargo, un menor número de estudios realizados a partir de cultivos primarios de adipocitos blancos humanos que estudien de manera más específica el efecto de dichos fármacos sobre la biología de la célula adiposa humana (5). Los cultivos primarios, aunque implican un menor rendimiento y la imposibilidad de mantener el cultivo a largo plazo, presentan un modelo celular de estudio mucho más fiable a nivel de expresión génica y de comportamiento celular que una línea celular, que presenta características de células tumorales. Además, el hecho que las líneas celulares de adipocitos blancos mencionadas sean de ratón, hace que los resultados obtenidos de estudios con cultivos primarios de adipocitos blancos humanos sean mucho más extrapolables a lo que en realidad está ocurriendo en el tejido adiposo de los pacientes de SIDA con lipodistrofia.

Uno de los objetivos del trabajo que se presenta a continuación es poner a punto la técnica de cultivo primario de adipocitos blancos humanos y caracterizar la funcionalidad de las células adiposas en cultivo primario. Este tipo de cultivo se realiza a partir de células precursoras obtenidas de liposucciones de pacientes sanos que se cultivan en placa y se tratan con un cóctel proadipogénico para conseguir adipocitos blancos diferenciados. Para la caracterización de dichos cultivos, se ha estudiado, a nivel morfológico, la diferenciación del adipocito blanco,

la expresión de uno de los marcadores del estado de diferenciación del adipocito maduro, el gen aP2/FABP (proteína que liga ácidos grasos o *fatty acid binding protein*) y la capacidad de respuesta lipolítica frente a un estímulo adrenérgico.

El otro objetivo de este trabajo es estudiar algunos de los efectos del inhibidor de la transcriptasa reversa análogo de nucleósido didanosina (didanosina) sobre el adipocito blanco humano en cultivo primario. Uno de los mecanismos propuestos como responsable de la lipodistrofia asociada a HAART se basa en el hecho que los NRTIs que se usan actualmente en la terapia HAART inhiben la síntesis de DNA mitocondrial (mtDNA). Una depleción en la cantidad de mtDNA en las células adiposas induciría una disminución de los enzimas codificados por el mtDNA y una disfunción de este orgánulo (6). La didanosina ha sido el fármaco escogido para este estudio ya que los resultados del estudio anterior indicaban que la didanosina producía una depleción de la cantidad de mtDNA que no se compensaba a nivel transcripcional. Basándonos en esta hipótesis mitocondrial y a partir de los estudios realizados con el modelo experimental del adipocito marrón murino en cultivo primario, se ha abordado el estudio con el objetivo de determinar el efecto del didanosina sobre la diferenciación del adipocito blanco, a nivel de la morfología microscópica, estudiar los efectos del didanosina sobre los cambios a nivel de la expresión génica de diferentes marcadores de diferenciación adipocitaria y biogénesis mitocondrial y estimar el potencial efecto de este fármaco como inhibidor de la función oxidativa mitocondrial, mediante la determinación de las concentraciones de lactato en el medio de cultivo.

MATERIALES Y MÉTODOS

El NRTI didanosina (didanosina) se obtuvo de la farmacéutica Bristol-Myers-Squibb y se disolvió en PBS. Los medios de cultivo, el suero fetal bovino (FBS), las soluciones de antibióticos y antimicóticos y el Hepes fueron obtenidos de Gibco (Carlsbad, California, USA). La biotina, el pantotenato cálcico, el 3-isobutyl-l-methylxantina (IBMX), la dexametasona, la insulina humana y el isoproterenol se obtuvieron de Sigma-Aldrich (St. Louis, Missouri, USA). El agonista PPARγ BRL49653 fue amablemente cedido por el Dr. L. Casteilla (Toulouse, France).

Los preadipocitos humanos fueron obtenidos de Advancell (Barcelona, España) y cada vial utilizado incluía precursores de adipocito blanco aislados de tres individuos sanos (*pool* de tres individuos). Se plaquean las células a una densidad de 30000 células/cm^2 en Dulbecco's modified Eagle's medium: Ham's F10 medium

(DMEM/F10) (1:1) suplementado con 10% suero fetal bovino (FBS), 15mM Hepes pH:7.4 y 1% antibióticos y antimicóticos (penicilina, estreptomicina y amfotericina B). Después de plaquear, se dejan las células en el incubador sin moverlas durante un mínimo de 16 horas para facilitar que su adhesión. Se deja que las células proliferen durante 48 horas y cuando éstas han llegado a confluencia, se les cambia el medio a un medio de inducción que incluye: DMEM/F10, 3% FBS, 1% antibióticos/antimicóticos, 15mM Hepes, 33μM biotina, 17μM pantotenato cálcico, 100nM insulina, 1μM dexametasona, 0.25mM IBMX y 1μM BRL49653. Se mantiene el medio de inducción durante 3 días y al cabo de este tiempo se les vuelve a cambiar el medio a un medio igual que el medio de inducción pero sin IBMX ni BRL49653. Cada 2 ó 3 días se les cambia el medio. Al cabo de unos 15 días, el 80% de las células presentan acúmulos de grasa en forma de multivacuolas lipídicas, indicativas de que la célula adiposa blanca está diferenciada.

Los tratamientos con el NRTI didanosina se realizaron a partir del primer día de inducción de la diferenciación y el fármaco se añadió cada vez que se les cambió el medio a las células. Cuando las células estuvieron del todo diferenciadas, se les hicieron las fotos al microscopio óptico, se recuperó el medio de cultivo y se recogieron las células para extracción del RNA total.

El RNA total de las células diferenciadas se obtuvo utilizando el kit RNeasy Mini Kit de Qiagen. Para el análisis Northern Blot, se cargaron y corrieron 5μg de RNA en un gel al 1.5% de agarosa con formaldehído y se transfirieron a membrana de nylon (N^+, Boehringer Mannheim, Alemania). Las prehibridaciones y hibridaciones de la membrana con la sonda específica, se realizaron a 55^0C en una solución que contiene 0.25M de Na2HPO4 (pH 7.2), 1mM EDTA, 20% SDS y 0.5% de reactivo de bloqueo (Boehringer Mannheim, Alemania). La membrana se hibridó con la sonda del cDNA de ratón de la aP2/FABP (7), que se marcó radioactivamente con [α^{-32}P]dCTP utilizando el kit Rediprime ^{TM} II Random Prime labelling System de Amersham Biosciences.

El resto del análisis de la expresión génica se realizó mediante PCR a tiempo real (*real-time PCR)*. Se trató una alícuota de RNA con DNAsaI y posteriormente se retrotranscribió 1 microgramo de RNA utilizando la transcriptasa reversa MultiScribe-RT y hexámeros con secuencia al azar, de acuerdo con las instrucciones del proveedor (Applied Biosystems). Se analizó la cantidad de los mRNAs individuales mediante el sistema TaqMan "Assay-on-demand" (Applied Biosystems) específicamente diseñado para cada uno de los tránscritos a analizar (PGC1α,

NRF1, PPARγ, subunidad IV (COX4) y subunidad II (COXII) de la citocromo oxidasa, citocromo b, TFAM, TFB1M y TFB2M). La reacción se realizó de acuerdo con las instrucciones del proveedor y utilizando un lector "ABI PRISM HT799 *sequence detection system*" (Serveis Científico Tècnics, Universitat de Barcelona).

La determinación de la cantidad de lactato en el medio se llevó a cabo utilizando el reactivo de determinación de lactato Lactate Reagent de Sigma-Aldrich, según las instrucciones del fabricante. Se determinó la cantidad de lactato en el medio de células con y sin el fármaco, así como la cantidad de lactato en el medio que no ha estado en contacto con células, que se restó de los valores anteriores.

La respuesta lipolítica de las células se determinó cuantificando el glicerol secretado al medio en respuesta al tratamiento de éstas con isoproterenol. Se realizaron dos tiempos de tratamiento: Se añadió 1μM isoproterenol a las células, a las 6 horas se recuperaron 200μl de medio y a las 24 horas se recuperó el resto de medio. El reactivo utilizado para la determinación de glicerol es el Free Glycerol Reagent de Sigma-Aldrich y se siguieron las instrucciones del fabricante. A partir de una recta patrón de glicerol, se determinó la cantidad de glicerol de los medios recogidos de las diferentes situaciones experimentales a las 6 y 24 horas.

Los análisis estadísticos se realizaron utilizando el test *t* de Student.

RESULTADOS

Los preadipocitos humanos en cultivo primario bajo un estímulo adipogénico se diferencian a adipocitos blancos que presentan las características morfológicas y funcionales típicas de un adipocito

Los preadipocitos humanos, con una morfología típica fibroblástica, proliferan hasta llegar a confluencia, momento en el cual se les induce a diferenciarse mediante un cóctel de inducción. A día 15 de cultivo, aproximadamente el 80% de las células presentan acumulaciones de lípidos en forma de gotas refringentes en sus citoplasmas. (**Figura 1**).

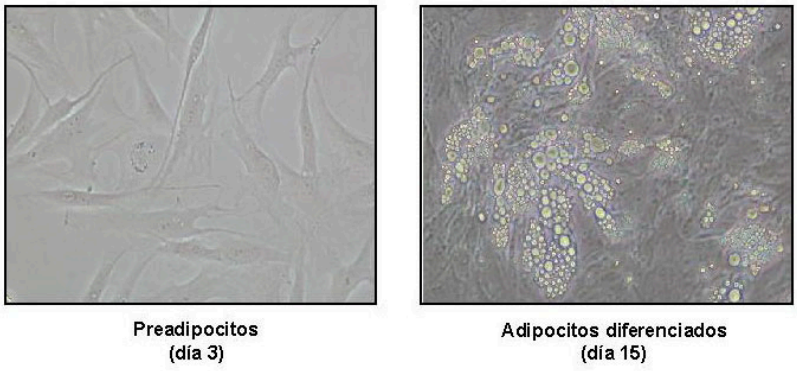

Figura 1. Micrografía (contraste de fases) de preadipocitos humanos diferenciados a adipocitos, en cultivo primario. Precursores de adipocito blanco (fotografía izquierda) fueron plaqueados a la densidad de 30000 células/cm^2 y al llegar a confluencia, se les indujo la diferenciación con un medio de inducción, durante 3 días. Posteriormente, se mantienen las células con un medio de diferenciación y a día 10-15 de cultivo, las células presentan los acúmulos de lípidos característicos del adipocito (fotografía izquierda). Las fotografías están tomadas a 40 aumentos.

El análisis por Northern Blot del mRNA de células diferenciadas muestra que los adipocitos humanos diferenciados en cultivo primario presentan unos niveles considerables de expresión del gen aP2/FABP, marcador del estado de diferenciación del adipocito. (**Figura 2**)

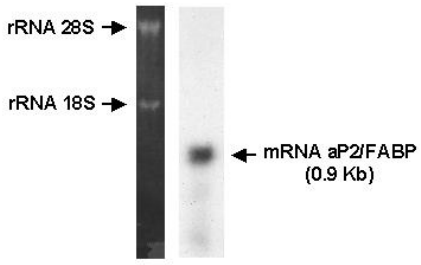

Figura 2. Los adipocitos blancos humanos diferenciados en cultivo primario expresan el gen aP2/FABP. Estudio de la expresión del gen aP2/FABP mediante Northern Blot a partir de 5μg de RNA total de adipocitos humanos diferenciados. Se muestra la señal del mRNA de aP2/FABP de 0.9kb y las señales de los dos RNA ribosómicos 18S y 28S, como referencia.

El ensayo de determinación de la respuesta lipolítica mediante el tratamiento con isoproterenol muestra que, tanto a las 6 horas de tratamiento como a las 24 horas, los adipocitos diferenciados son capaces de responder al estímulo lipolítico liberando glicerol al medio de cultivo. (**Figura 3**)

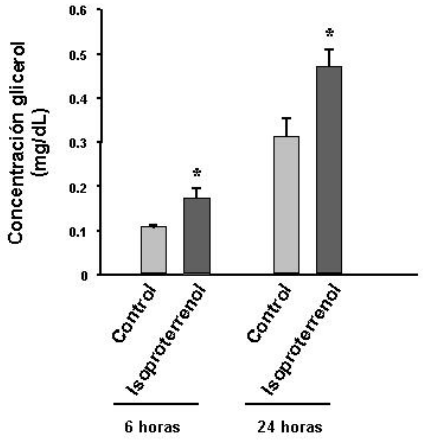

Figura 3. Respuesta lipolítica de adipocitos blancos humanos diferenciados en cultivo primario. Adipocitos blancos diferenciados fueron expuestos al agonista β-adrenérgico isoprotereno durante 6 y 24 horas y se determinó la cantidad de glicerol liberado al medio. Cada histograma es la media ± error estándar de al menos tres placas de cultivo independientes. Las diferencias estadísticamente significativas ($p<0.05$) respecto a los controles se indican mediante *.

El tratamiento de los adipocitos blancos humanos con el NRTI didanosina durante la diferenciación no altera la diferenciación morfológica de las células

El NRTI didanosina se añadió al medio de cultivo de las células en el mismo momento en que se les añadió el medio de inducción y estuvo presente durante todo el proceso de diferenciación (tratamiento crónico). Las fotografías de microscopia óptica muestran que la presencia del fármaco durante el proceso de diferenciación no afecta el grado de diferenciación de éstas. (**Figura 4**)

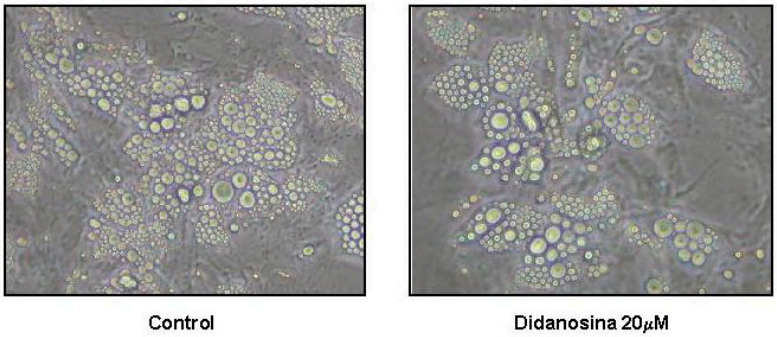

Figura 4. Micrografía (contraste de fases) de adipocitos humanos diferenciados en cultivo primario en condiciones control o cultivados en presencia de didanosina. El NRTI didanosina a la concentración de 20μM fue añadido al medio de inducción y se añadió cada vez que se cambió el medio. Las fotografías están tomadas a 40 aumentos.

El tratamiento de adipocitos blancos humanos con didanosina durante la diferenciación altera la expresión de genes marcadores de biogénesis mitocondrial y diferenciación adipocitaria

Con el objetivo de estudiar el efecto a nivel de expresión génica de la didanosina sobre la diferenciación de adipocitos blancos humanos en cultivo primario, se obtuvo el RNA total de los adipocitos blancos sin tratar y los tratados crónicamente con didanosina y se analizó por *real-time PCR* los niveles del mRNA de los siguientes genes: PPARγ, como marcador del estado de diferenciación del adipocito, PGC-1α, como coactivador regulador de la biogénesis mitocondrial, COIV, COII y Cit b (citocromo b), como marcadores mitocondriales, siendo COIV codificado por el DNA nuclear y COII y Cit b por el DNA mitocondrial, y TFAM, TFB1M y TFB2M, factores de transcripción del mtDNA. Los niveles de los mRNAs de cada gen en células control y células tratadas con didanosina, respecto a la expresión de genes de referencia usados como controles endógenos ("*housekeeping*") se muestran en la **figura 5**.

Los niveles de expresión de los diferentes genes analizados muestran un patrón de expresión muy similar según se refieran al control endógeno rRNA 18S, mRNA ciclofilina o mRNA β2-microglobulina.

Los resultados indican que la expresión de PPARγ se ve disminuida por el tratamiento de las células con didanosina, mientras que PGC-1α presenta una débil tendencia de disminución en las células tratadas. El mRNA del gen COIV también está ligeramente disminuido por el efecto del didanosina, mientras que los marcadores mitocondriogénicos COII y Cit b, que están codificados por el mtDNA, se encuentran claramente disminuidos. Al analizar los niveles de los mRNA de los factores de transcripción TFAM, TFB1M y TFB2M se observa que los dos primeros se encuentran sobreexpresados en las células tratadas, con un aumento muy claro en la expresión de TFB1M y menos marcado en TFAM.

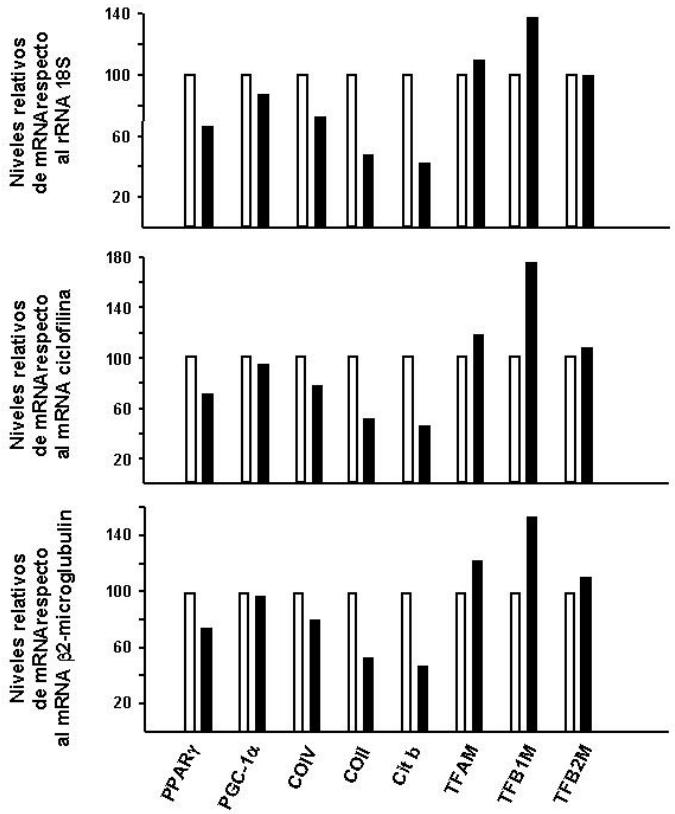

Figura 5. Análisis por *real-time PCR* de los niveles de mRNA de marcadores mitocondriogénicos y de diferenciación adipocitaria de adipocitos blancos humanos tratados o no con didanosina. Cada gráfica muestra los niveles de expresión de cada gen con respecto a cada uno de los controles endógenos, en ensayos realizados con el RNA total procedente de "*pool*" de dos cultivos independientes. A los valores de expresión de cada gen en las células control se les ha designado el valor de 100 y respecto a estos se han calculado los valores de expresión de las células tratada. Las barras blancas representan las células control y las negras las células tratadas con 20μM didanosina.

La presencia de la didanosina durante el proceso de diferenciación de los adipocitos humanos induce la producción de lactato

La determinación de la cantidad de lactato en el medio de cultivo de los adipocitos que han estado en contacto o no con la didanosina durante la diferenciación, muestra que las células tratadas con didanosina presentan unos niveles de lactato en el medio significativamente más altos que los de las células no tratadas. (**Figura 6**)

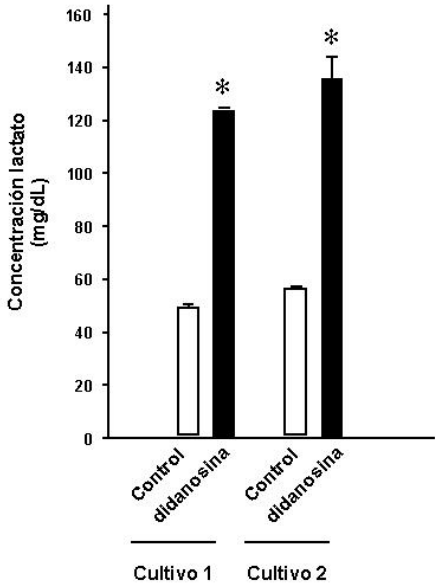

Figura 6. Efecto del tratamiento crónico de adipocitos blancos con el NRTI didanosina sobre la concentración de lactato en el medio de cultivo. Los medios de cultivo de adipocitos blancos humanos diferenciados de dos viales de células independientes y tratados o no con didanosina, se utilizaron para determinar la cantidad de lactato secretado por las células. Las diferencias estadísticamente significativas ($p<0.05$) respecto a los controles se indican mediante *.

DISCUSIÓN

El presente trabajo presenta la puesta a punto del modelo de adipocitos blancos humanos en cultivo primario y su aplicación como modelo para estudiar el efecto de los fármacos antiretrovirales sobre la biología del adipocito blanco humano.

Células precursoras obtenidas de liposucciones de pacientes sanos, cultivadas en placa y diferenciadas con un medio proadipogénico son capaces de dar lugar a células adiposas diferenciadas, que presentan gran cantidad de lípidos en forma de multivacuolas en el citoplasma, que expresan el gen aP2/FABP (proteína citoplasmática que liga ácidos grasos) y que son capaces de responder a un estímulo lipolítico liberando glicerol (procedente de la lipólisis de los triacilglicéridos del interior celular). Así, esta caracterización muestra que este modelo celular permite la obtención de adipocitos blancos en cultivo que presentan las características morfológicas y funcionales típicas de un adipocito blanco, ofreciendo grandes posibilidades de utilización de estas células en múltiples estudios.

Uno de los primeros estudios que se han llevado a cabo utilizando este modelo celular ha sido el de analizar los efectos del NRTI didanosina sobre la diferenciación de adipocitos blancos humanos. Se ha querido analizar específicamente el efecto a nivel mitocondrial que tiene este fármaco sobre el adipocito blanco humano para profundizar más en la etiopatología de la lipodistrofia asociada a HAART, en la que se plantea que los NRTIs podrían estar afectando la funcionalidad mitocondrial de las células adiposas y así contribuir a este síndrome (6).

Dado que la cantidad de adipocitos diferenciados que se obtiene es altamente limitante, este estudio se ha restringido a analizar el efecto de la didanosina, fármaco que en los estudios con el modelo de adipocitos marrones murinos en cultivo primario presentados en el trabajo anterior da lugar a una depleción del mtDNA que a nivel transcripcional no se compensa y a una reducida expresión de los productos génicos codificados por el mtDNA (ver trabajo anterior).

El análisis de la morfología celular a nivel de microscopia óptica de adipocitos blancos tratados con didanosina durante todo el proceso de diferenciación, indica que este fármaco no altera la diferenciación morfológica de estas células.

Para profundizar sobre los efectos de la didanosina sobre los adipocitos blancos humanos, se determinaron los niveles de expresión de varios genes relacionados con la diferenciación adipocitaria y la biogénesis mitocondrial, mediante *real-time PCR*. Dado que la cantidad de RNA total obtenido de estas

células es también muy limitante, la utilización de una técnica tan sensible como la *real-time PCR* ha permitido analizar la expresión de varios genes, ya que se necesita muy poco RNA de partida. Los genes a analizar se escogieron para analizar la diferenciación adipocitaria y la biogénesis mitocondrial y también según los resultados obtenidos en el trabajo del efecto de los inhibidores de la transcriptasa reversa sobre el adipocito marrón presentados anteriormente.

El análisis por *real-time PCR* de la expresión de PPARγ, marcador del estado de diferenciación del adipocito, indica que las células tratadas crónicamente con didanosina presentan unos niveles de expresión más bajos que las células control, dato que sugiere que el NRTI didanosina podría estar alterando el proceso de diferenciación de los adipocitos blancos humanos, aunque a nivel morfológico las células no se vean afectadas.

Los niveles de los mRNAs de los genes COII, Cit b aparecen disminuidos en las células tratadas respecto a las control, indicando que la didanosina podría estar afectando la cantidad de mtDNA de las células y como consecuencia la cantidad de los tránscritos mitocondriales COII y Cit b.

Los resultados para el coactivador PGC-1α y para COIV, que se encuentran también disminuidos en las células tratadas, indicarían una afectación mitocondrial más generalizada que no sólo implicaría al mtDNA, si no también a la expresión de genes nucleares.

Al analizar los niveles de los mRNAs de los factores de transcripción mitocondriales TFAM, TFB1M y TFB2M se observa que éstos se encuentran aumentados, sobretodo el TFB1M, sugiriendo que éstas células estarían intentando compensar la posible depleción del mtDNA producida por el didanosina a nivel de la transcipción del mtDNA, aunque no se refleja en los niveles de transcripción de la COII y el Citb.

El aumento en la producción de lactato por parte de los adipocitos blancos tratados crónicamente con didanosina sugiere, juntamente con la disminución de los tránscritos mitocondriales COII y Cit b, que en estas células se está produciendo una inhibición de la función oxidativa mitocondrial. Cabría no excluir la posibilidad de que la didanosina y los otros NRTIs indujeran toxicidad mitocondrial a través de otras vías (como daños o mutaciones en el mtDNA o interferencia con la actividad respiratoria mitocondrial) (8-10).

De manera global, los resultados presentado en este trabajo indican que la didanosina ejerce un efecto de toxicidad mitocondrial evidenciado por la disminución de la expresión de los tránsitos codificados por el mtDNA, como la COII y el Cit b, y el aumento de la producción de lactato. A diferencia de los adipocitos

marrones (en el caso de la estavudina), el incremento de TFAM y TFB1M no es capaz de normalizar la expresión de los tránscritos codificados por el mtDNA. El modelo de adipocitos blancos humanos en cultivo primario es un modelo idóneo para el estudio de la biología del adipocito blanco y una buena herramienta para analizar el papel de los fármacos antiretrovirales sobre la fisiología del adipocito blanco humano.

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