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**Culture of human adipose tissue explants leads to profound alteration of adipocyte gene expression**

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**Abstract:**

Primary culture of adipose tissue has often been used to investigate pharmacological or nutritional regulation of adipocyte gene expression. Possible alteration of adipocyte gene expression by primary culture by itself has not been explored in details. In order to address this point, explants were prepared from human subcutaneous adipose tissue issued from plastic surgery, and maintained for 0 to 48 h in DMEM supplemented with 10% serum. At different time points adipocytes were isolated from the explants by collagenase digestion and mRNA expression and lipolysis were studied. Culture was associated with an accumulation of Tumor Necrosis Factor- $\alpha$  (TNF $\alpha$ ) in the culture medium, an increase in anaerobic glycolysis, and an increase in the basal lipolysis. In parallel, a rapid and dramatic decrease in the level of mRNA encoding for several adipocyte-specific proteins such as Adipocyte Lipid-Binding Protein, Hormone-Sensitive Lipase, Lipoprotein Lipase, Peroxisome Proliferation Activating Receptor- $\gamma$ 2, was observed in isolated adipocytes. These down-regulations were reminiscent of a dedifferentiation process. In parallel, primary culture was associated with an increase in adipocyte  $\beta$ -actin, TNF $\alpha$ , Glucose transporter-1 and Hypoxia Induced Factor-1 $\alpha$  mRNAs. Treatment of explants with agents that increase cAMP (isobutylmethylxanthine and forskolin), prevented against TNF $\alpha$  production and expression, as well as against culture-induced alterations of adipocyte gene expression. These data show that primary culture of human adipose tissue explants dramatically alters adipocyte gene expression.

## Introduction

Clarification of the mechanisms of long-term adaptations of *ex vivo* mature adipocytes to hormonal, and nutritional factors requires culture methods that preserves the function and gene expression of the adipocytes over a time course of hours to days. Freshly isolated mature adipocytes can be used to investigate short-term (within 1 or 2 hours) regulation of adipocyte metabolism (lipolysis, lipogenesis, glucose transport) or secretion, but are not appropriate to study long-term regulations because of their limited viability over long time course of culture (several hours to days). Primary culture of adipose tissue explants represents an interesting alternative to improve adipocyte viability because of the partial preservation of its tissue environment. Several reports have indeed demonstrated that this type of culture allows preserving adipocyte metabolism over several days (1-3) without major alterations.

Based on these observations, an increasing number of laboratories are now using the culture of adipose tissue explants to study the influence of various pharmacological or nutritional factors on adipocyte gene expression (4-12). However, in these different studies, little attention has been paid to the consequences of the primary culture by itself on adipocyte gene expression. Primary culture of adipose tissue is indeed preceded by several steps (surgery, transport, dissection) constituting potential aggression (thermic shock, hypoxia) which could alter normal adipocyte gene expression. In addition, several reports have shown that adipose tissue explants in culture produce relatively high amount of Tumor Necrosis Factor- $\alpha$  (TNF $\alpha$ ), (13-15) a cytokine known to inhibit and/or reverse adipocyte differentiation (16-18).

In the present study we analyzed the influence of primary culture of human adipose tissue on the expression of several genes specifically in adipocytes. For that, adipocytes were isolated by collagenase digestion from human subcutaneous adipose tissue explants that have

been maintained in primary culture for various period of time (0 to 48h). We observed that primary culture was associated with a profound alteration of several adipocyte genes reminiscent of a dedifferentiation process. This effect was largely prevented by treatment of explants with agents that increase cAMP.

## **Materials and Methods**

### **Culture of explants**

Human subcutaneous adipose tissue was obtained from healthy, drug free non-obese (BMI  $23.6 \pm 1.1 \text{ kg/m}^2$ , age  $41.3 \pm 5.2$ , n=9) women, undergoing abdominal dermolipectomy for plastic surgery. After rapid transportation in the laboratory adipose tissue was carefully dissected out from skin and vessels, and cut in small pieces (average weight 20-50 mg) under sterile conditions. Explants were placed at  $37^\circ\text{C}$  for 0 to 48h in a humidified atmosphere containing 7%  $\text{CO}_2$  in 100mm diameter dishes (7 g in 12 ml) in DMEM supplemented with 10% fetal calf serum. The medium was changed after 24h culture. Culture aliquots of the culture media were collected to measure the rate of disappearance of glucose (4.5 g/l at time 0), the rate of production of lactate, and the accumulation of  $\text{TNF}\alpha$ . Glucose was measured using a colorimetric assay (Merck). Lactate was measured using the Lactate Reagent (Sigma Diagnostic).  $\text{TNF}\alpha$  was measured using EIA (Immunotech, Beckman Coulter)

### **RNA extraction from isolated of adipocytes**

At different time points, adipose tissue explants were digested (7g in 10 ml) in DMEM containing 0.5 mg/ml type II collagenase (Sigma) and 1% bovine serum albumin (Sigma) for 30 to 40 min at  $37^\circ\text{C}$  under constant shaking. At the end of the incubation period the reaction was stopped by dilution in DMEM, filtered on a silk screen in order to retain undigested explants and isolated adipocytes were separated from the stroma-vascular fraction by floatation. The floating packed cells were washed twice with DMEM. Total RNA was extracted from isolated adipocytes using Rneasy Kit (Qiagen) according to the instructions of the company except that the fat cake was removed by centrifugation before loading the purifying columns.

### **Determination of adipocyte size**

An aliquot of isolated adipocytes was photographed under microscope using a digital camera connected to an image analyzer (Visiolab). Mean fat cell diameter was determined by measuring the diameter of at least 100 cells.

### **Lipolysis of isolated adipocytes**

For lipolysis experiments, the process of isolation of adipocytes was identical than for RNA extraction except that DMEM was replaced by Krebs Ringer bicarbonate buffer supplemented with 6 mM glucose and 3.5% bovine serum albumin (this buffer was called KRBA). Isolated adipocytes were brought to a suitable dilution (2000–3000 cells/assay) in KRBA for lipolysis assays and incubated with pharmacological agents in a final volume of 100  $\mu$ l and for 90 min at 37°C. At the end of the incubation, 20 to 50  $\mu$ l aliquots of the infranatant were taken for glycerol determination (19).

### **Northern blot analysis**

<sup>32</sup>P-labelled probes were obtained by Nick-translation of cDNA fragments purified from the coding region of: rat ALBP, human HSL, human LPL, and human  $\beta$ -actin. Twenty  $\mu$ g of total RNAs were separated by electrophoresis in 1% agarose gel containing 2.2 M formaldehyde. Before transfer, the gel was soaked in an ethidium bromide solution to label ribosomal RNA and verify that the amount of total RNA loaded on the gel was equivalent from one sample to another. RNAs were then transferred onto a nylon membrane (Schleicher and Schuell, Dassel, Germany) and UV-crosslinked. The blot was incubated overnight at 68°C in hybridization buffer containing 0.5 M Na<sub>2</sub>HPO<sub>4</sub>-12H<sub>2</sub>O, 1 mM EDTA, 7% SDS, 1%

BSA, <sup>32</sup>P-labelled cDNA probes pH 7. The blot was finally washed at a final stringency of 0,5X SSC; 0,1% SDS at 65°C and autoradiographed.



## RT-PCR analysis

One  $\mu\text{g}$  RNA was reverse transcribed for 60 min at  $37^\circ\text{C}$  using Superscript II RNase H<sup>-</sup> Reverse Transcriptase (RT) (Gibco BRL life technologies) in 20  $\mu\text{l}$  volume containing 4  $\mu\text{l}$  of 5X first stand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM  $\text{MgCl}_2$ ), 1  $\mu\text{l}$  DTT 0.1 M, 1  $\mu\text{l}$  dNTP (10 mM), 1  $\mu\text{l}$  oligo(dT)<sub>12-18</sub> (0.5  $\mu\text{g}/\mu\text{l}$ ) and 100 units RT. A minus RT reaction was performed in parallel to ensure the absence of genomic DNA contamination, which was always the case in the present study.

RT+ and RT- reaction were subjected to Polymerase Chain Reaction (PCR). PCR was carried out in a final volume of 50  $\mu\text{l}$  containing 3  $\mu\text{l}$  of cDNA, 1  $\mu\text{l}$  dNTP (10mM), 5  $\mu\text{l}$  10x PCR buffer (10 mM Tris-HCl, pH 9, 50 mM KCl and 0,1% Triton X-100), 3  $\mu\text{l}$   $\text{MgCl}_2$  (25 mM), 1.5  $\mu\text{l}$  sense and 1.5  $\mu\text{l}$  antisense specific oligonucleotide primers (10 $\mu\text{M}$ ) and 1.25 unit of Taq DNA polymerase (Promega).

Conditions for the PCR reaction consisted in an initial denaturation step at  $94^\circ\text{C}$  for 2 min, followed by n (depending on target gene) cycles composed of 1 min at  $94^\circ\text{C}$ , 1 min at the annealing temperature of T $^\circ\text{C}$  (depending on target gene) and a final extension at  $72^\circ\text{C}$  for 6 min.

Annealing temperature (T) and number (n) of PCR cycles (optimized for each couple of primer): PPAR $\gamma$ 2:  $60^\circ\text{C}$ , 25 cycles; TNF $\alpha$ :  $57^\circ\text{C}$ , 25 cycles; Glut-1:  $60^\circ\text{C}$ , 33 cycles; HIF-1 $\alpha$ :  $60^\circ\text{C}$ , 27 cycles. PCR products were separated on 1.5% agarose gel and amplification products were visualized with ethidium bromide. Primer sequences and expected size of PCR product:

PPAR $\gamma$ 2 (583 bp):	sens (GCGATTCCTTCACTGATAC)
	antisens (GCATTATGAGACATCCCCAC)
TNF $\alpha$ (362 bp):	sens (CGAGTGACAAGCCTGTAGC)
	antisens (CCTTCTCCAGCTGGAAGAC)



## Results

Subcutaneous adipose tissue was originating from female patients undergoing plastic surgery. Adipose tissue was rapidly (about 10 min) transported in the laboratory, and dissected out in small explants which were cultured for various period of time (0 to 48h) in serum supplemented DMEM. At different time point, the culture medium was collected (for measurement of glucose, lactate and TNF $\alpha$ ), and adipocytes were isolated from the explants following collagenase digestion. Total RNAs were extracted from isolated adipocytes and potential alterations in mRNA level were analyzed by Northern blot or RT-PCR.

### *Analysis of culture medium composition*

Previous studies have shown that adipocytes in culture are able to secrete TNF $\alpha$  (13-15). TNF $\alpha$  was indeed found in relatively high amount in the culture medium after 48 h culture of adipose tissue explants:  $726.3 \pm 66.2$  pg/ml/g of tissue (n=5). In parallel, a time-dependent increase in glucose utilization (29 mg/l/h after 6 h versus 35 mg/l/h after 24h) by the explants was observed. This was associated with an accumulation of lactate (0.52 g/l after 6 h and 1.96 g/l after 24h) in the culture media. These data confirmed that the culture of adipose tissue explants was associated with an increase in TNF $\alpha$  production. Data also showed an increase in anaerobic glycolysis in explants. This last observation was reminiscent of a hypoxic state.

### *Analysis of adipocyte lipolysis*

Lipolysis is a very important metabolic function of adipose tissue which can be regulated by different hormones and neurotransmitters (20). In order to determine whether culture of adipose tissue explants could influence the integrity of cell function, the lipolytic

activity of adipocytes isolated from the explants was measured before and after 48h culture. When compared with time 0 of culture, a significant increase (1.8 fold) in basal lipolysis was observed after 48h culture (Figure 1A). In addition, the concentration-dependent activation of lipolysis by the  $\beta$ -adrenergic agonist isoproterenol revealed no significant decrease in maximal effect with no changes in EC50 (0.22 and 0.19  $\mu$ M at time 0 and time 48 h respectively) (Figure 1B). In addition, activation of lipolysis by the adenylylcyclase activator forskolin was not altered (not shown). In parallel, no significant alteration in the mean diameter of isolated adipocytes between 0 ( $88.8 \pm 1.2 \mu\text{m}$  n=5) and 48h ( $88.2 \pm 0.8 \mu\text{M}$  n=5) culture was observed. These data showed that the functional (i.e. lipolytic process) and morphological integrity of isolated adipocytes were not dramatically altered by primary culture of adipose tissue explants.

#### *Analysis of gene expression.*

In order to determine whether culture of adipose tissue explants could influence adipocyte gene expression, the relative level of several mRNAs was analyzed in isolated adipocytes. As shown in Figure 2, primary culture was associated with a dramatic and time-dependent decrease in the amount of several mRNAs encoding for proteins specifically expressed in adipocyte: Adipocyte Lipid Binding Protein (ALBP), Lipoprotein Lipase (LPL), Hormone Sensitive Lipase (HSL) (Figure 2A), and Peroxisome Proliferation Activating Receptor $\gamma$ -2 (PPAR $\gamma$ 2) (Figure 2B). These down-regulations were associated with a dramatic increase in the level of several other mRNAs:  $\beta$ -actin (Figure 2A), TNF $\alpha$ , Glucose transporter-1 (Glut-1) and Hypoxia Induced Factor-1 $\alpha$  (HIF-1 $\alpha$ ) mRNAs (Figure 2B). In the same time no variation in the level of cyclophilin mRNA was observed (Figure 2B). These results showed that primary culture of adipose tissue explants was associated with important alterations in gene expression. Down-regulation of ALBP, HSL, LPL, and PPAR $\gamma$ 2 mRNAs

was reminiscent of an induction of adipocyte dedifferentiation process. Up-regulations of Glut-1 and HIF-1 $\alpha$  mRNAs were reminiscent of a hypoxic state.

### ***Involvement of TNF $\alpha$ in culture-induced adipocyte dedifferentiation***

As described above, the culture of adipose tissue explants was associated with an accumulation of TNF $\alpha$  in the culture medium. This accumulation was associated with a time-dependent increase in adipocyte TNF $\alpha$  mRNA level (Figure 2B). TNF $\alpha$  is a cytokine known to exert a powerful inhibitory effect on adipocyte differentiation (16-18). In order to test the potential involvement of TNF $\alpha$  in culture-induced adipocyte dedifferentiation, adipose tissue explants were cultured in the presence of a phosphodiesterase inhibitor, a family of molecule which, by increasing intracellular cAMP, inhibit TNF $\alpha$  production (21, 22). For the present study we used the phosphodiesterase inhibitor isobutylmethylxanthin (IBMX), which has previously be demonstrated to suppress endogenous production of TNF $\alpha$  in human preadipocytes (23). IBMX (100  $\mu$ M) strongly reduced the accumulation of TNF $\alpha$  in the culture medium after 48 h culture ( $33.1 \pm 13.5$  pg/ml/g of tissue n=5). IBMX also prevented the increase in adipocyte TNF $\alpha$  mRNA level observed after 48h culture (Figure 3A). In parallel, IBMX prevented the reduction in ALBP, HSL (Figure 3B) and PPAR $\gamma$ 2 (Figure 3A) mRNA levels, as well as the increase in  $\beta$ -actin mRNA level (Figure 3B), observed after 48h culture. The protective effect generated by IBMX was also observed with the same extend with agents that increase cAMP such as the adenylyl cyclase activator forskolin (not shown). These data showed the involvement of cAMP in culture-induced adipocyte dedifferentiation.

## Discussion

The present study shows that primary culture of human adipose tissue explants leads to dramatic alterations in adipocyte gene expression reminiscent of a dedifferentiation process.

Evidences for a culture-induced dedifferentiation process is based on several observations. We observed that primary culture of adipose tissue explants is associated with a dramatic down-regulation of several mRNA encoding for several proteins specifically involved in the acquisition of the adipocyte phenotype such as ALBP, LPL, HSL. Moreover, culture was also associated with a dramatic reduction in the level of mRNA encoding for PPAR $\gamma$ 2, an adipocyte-specific transcriptional factor involved in adipocyte differentiation process (24). Finally, culture was associated with an increase in  $\beta$ -actin mRNA, which was previously shown to be inversely correlated with adipocyte differentiation status (25). Although alteration of gene expression reminiscent of dedifferentiation of adipocytes is obvious, it was not associated with major alteration in adipocyte functionality and morphology (cell size). Indeed, no major changes in the lipolytic activity of adipocytes isolated from the explants after 48h were observed. This could be in apparent contradiction with the dramatic reduction in HSL mRNA after the same period of culture. The difference of turn-over between the HSL mRNA and HSL protein, as previously demonstrated (26), could explain the result. One could expect that, after a longer period of culture (more than 48h), HSL protein would also finally be reduced. This would also likely be the case for the other adipocyte proteins (ALPB, LPL and others) and would lead, after several days of culture, to alterations in adipocyte functions. Previous reports have shown that it is indeed possible to induce a phenotypic dedifferentiation of rat or human adipocytes by forcing them to attach on a culture dish (27, 28). This *in vitro* dedifferentiation is a relatively slow process characterized by a noticeable delipidation (occurring after 2-3 days of culture) followed by the proliferation of some of the delipidated cells (occurring 7 to 10 days of culture). Preliminary experiments

in our laboratory indeed show that, after 7 to 10 days of culture, numerous adherent cells that exhibit a fibroblastic morphology with lipid droplets in their cytoplasm, are present underneath each explant (Gesta et al. personal data). These observations are likely characteristic of a dedifferentiation process.

What could be the mechanisms involved in this dedifferentiation process ?

One possibility is the involvement of  $\text{TNF}\alpha$ , a cytokine known to inhibit and reverse adipocyte differentiation in part by inducing a down-regulation of  $\text{PPAR}\gamma 2$  expression (16-18, 29). Our results show a time-dependent increase in adipocyte  $\text{TNF}\alpha$  mRNA level as well as an accumulation of  $\text{TNF}\alpha$  in the culture medium. We also observed that culture was associated with a slight but significant increase in basal adipocyte lipolysis, without modification of the  $\beta$ -adrenergic-dependent lipolysis. Similar alteration of lipolysis was observed in rat adipocytes exposed to  $\text{TNF}\alpha$  (30). Finally, our results show that it is possible to prevent culture-induced adipocyte dedifferentiation by treating explants with IBMX. The inhibitory action of IBMX on  $\text{TNF}\alpha$  production has been reported in other cell types such as monocytes (21, 22) and also in preadipocytes (23) and results from its ability to increase intracellular cAMP, a powerful regulator of  $\text{TNF}\alpha$  transcription (31-33). Whereas our observations suggest the involvement of  $\text{TNF}\alpha$  in the dedifferentiation process induced by culturing human adipose tissue explants, further complementary experiments (such as the use of blocking  $\text{TNF}\alpha$  antibodies) will have to be performed to prove this hypothesis.

Another possible event involved in the dedifferentiation process induced by culturing human adipose tissue explants, is hypoxia. We indeed observed that the culture of adipose tissue explants was associated with an increase in the anaerobic glycolysis of the adipocytes. In parallel, we observed an increase in the level of mRNA encoding for Glut-1, a protein responsible for the increase in basal glucose transport observed in anaerobic glycolysis (34, 35). In parallel, we also observed an increase in the level of mRNA encoding for HIF-1 $\alpha$ , a

transcriptional factor tightly regulated by O<sub>2</sub> concentration (36) and which controls the expression of several hypoxia-sensitive genes including Glut-1 (37). Whereas HIF-1  $\alpha$  mRNA is constitutively expressed in cultured cells, it is markedly increased by hypoxia (38, 39). Therefore, our data strongly suggest that, in our culture conditions, adipocytes isolated from the explants were in a relative hypoxia. Such a conclusion was expected since adipose tissue explants are issued from a surgery which disconnect the tissues from blood flow and from O<sub>2</sub>. Nevertheless, definitive demonstration of the involvement of hypoxia in the dedifferentiation process induced by culturing human adipose tissue explants, will require further experiments. For example, it will be of particular interest to test the influence of increased oxygen concentration in the culture medium on adipocyte gene expression.

Hypoxia has been reported to be associated with an increase in TNF $\alpha$  production in some cell types (40-42). Hypoxia effect on TNF $\alpha$  is mediated by a reduction of cAMP (43, 44) leading in turn to an inhibition of the cAMP Responsive Element Binding protein, a transcriptional factor that negatively regulates TNF $\alpha$  gene expression (45). It is possible that the hypoxia observed when adipose tissue explants are set in culture could be responsible to the increase in adipocyte TNF $\alpha$  expression and secretion.

In parallel to TNF $\alpha$  and hypoxia one cannot exclude the involvement of other factors or events. We observed that, even after a ten-fold reduction in the amount of adipose tissue explants in the same volume of culture medium down-regulation of adipocyte gene expression persisted (Gesta, personal data). This observation does not favor the involvement of a depletion of some medium compounds such as glucose or seric factors in the dedifferentiation process induced by culturing human adipose tissue explants

In conclusion, the results of the present study have to be taken in consideration when the culture of adipose tissue explants is used to study regulation of adipocyte gene expression.



Investigators have to be aware that the simple fact to set adipose tissue explants in culture generates profound alterations in adipocyte gene expression.

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## Legend of the figures

**Figure 1: Influence of the primary culture of human adipose tissue explants on adipocyte lipolysis.** Adipocytes were isolated from adipose tissue explants by collagenase digestion before (time 0) and after 48h culture. Lipolysis was determined by measuring the amount of glycerol released by isolated adipocytes in the absence (A) or in the presence (B) of increasing concentration of isoproterenol. Values are means  $\pm$  SE from 9 separate experiments. \*  $P < 0.05$  when comparing with time 0 of culture (ANOVA).

**Figure 2: Influence of the primary culture of human adipose tissue explants on adipocyte gene expression.** Adipocytes were isolated from adipose tissue explants by collagenase digestion before (time 0) and after 3, 6, 24, and 48h primary culture. RNA were extracted from isolated adipocytes and mRNAs were analyzed by Northern-blot analysis (A) and RT-PCR (B). Representative of 9 separate experiments.

**Figure 3: Influence of IBMX on adipocyte gene expression.** Adipocytes were isolated from adipose tissue explants by collagenase digestion before (time 0) and after 48h primary culture. RNA were extracted from isolated adipocytes and mRNAs were analyzed by RT-PCR (A) and Northern-blot analysis (B). Representative of 5 separate experiments.