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Biologia molecolare. — *Modulation of gene expression in differentiating 3T3-L1 preadipocytes.* Nota di MICHELA FESTA, GAETANO RICCIARDELLI, CONCETTA PIETROPAOLO, ALFREDO COLONNA e ALFREDO RUFFO, presentata (*) dal Socio A. Ruffo.

ABSTRACT. — 3T3-L1 cells can be induced to differentiate by exposing cell culture to a medium containing 3-isobutyl-1-methyl-xanthine together with insulin and dexamethasone. We have previously observed that oxalomalate (OMA), which inhibits aconitase thus blocking the citric cycle, also favours differentiation to adipocytes leading to fatty acids accumulation and increased synthesis of specific proteins. We compared the effects of 3-isobutyl-1-methyl-xanthine-insulin-dexamethasone and OMA on the steady-state levels of mRNA of c/EBP, *H*-ferritin and aconitase/IRE-BP. OMA does not affect the steady-state mRNA amount of c/EBP, *H*-ferritin and aconitase/IRE-BP, but the 3-isobutyl-1-methyl-xanthine-insulin-dexamethasone mixture is able to induce a five-fold increase of mRNA levels for aconitase/IRE-BP. This is the first finding correlating iron metabolism to adipocyte differentiation. Further studies will clarify a possible role for the aconitase inhibitor OMA.

KEY WORDS: Oxalomalate; Aconitase/iron regulatory factor; Adipocytes differentiation; Ferritin; Iron metabolism.

RIASSUNTO. — *Modulazione dell'espressione genica nel corso della differenziazione dei preadipociti 3T3-L1.* Le cellule 3T3-L1 possono essere indotte a differenziarsi dall'aggiunta al mezzo di coltura di 3-isobutil-1-metil-xantina-insulina-desametasone. Noi abbiamo precedentemente osservato che anche l'ossalomalato (OMA), noto inibitore della aconitasi, può indurre la differenziazione di 3T3-L1 con accumulo di acidi grassi e aumento della sintesi di proteine specifiche. Nel presente lavoro sono stati paragonati i livelli di mRNA per c/EBP, *H*-ferritina e aconitasi/IRE-BP in 3T3-L1 differenziate in seguito a trattamento con mezzi contenenti 3-isobutil-1-metil-xantina-insulina-desametasone ovvero OMA. L'OMA non altera i livelli degli mRNA analizzati, ma l'aggiunta di 3-isobutil-1-metil-xantina-insulina-desametasone al mezzo di coltura fa aumentare di circa cinque volte il livello di mRNA per aconitasi/IRE-BP. Questi sono i primi dati che mostrano una correlazione tra il metabolismo del ferro e la differenziazione degli adipociti. Ricerche in corso chiariranno se l'OMA svolga un ruolo in tale correlazione.

INTRODUCTION

Studies on the expression of genes involved in the lipid metabolism (Birkenmeier *et al.*, 1989; Cristy *et al.*, 1989) have shown that the exposure of 3T3-L1 preadipocytes to 3-isobutyl-1-methyl-xanthine together with insulin and dexamethasone (DXM medium) promote the differentiation of 3T3-L1 cells into adipocytes and, moreover, induce the expression of c/EBP (enhancer binding protein) family of transcription regulators, which are able to modulate the expression of genes involved with lipid metabolism. Since oxalomalate (OMA), a well known inhibitor of mitochondrial and extramitochondrial aconitase (Adinolfi *et al.*, 1971), has been shown to inhibit growth and to stimulate the differentiation of 3T3-L1 preadipocytes, leading to fatty acids accumulation and increased protein synthesis (Festa *et al.*, 1988; Ruffo, 1988, 1993), it seemed reasonable to investigate whether the OMA effects on 3T3-L1 differentiation to adipocytes would result from a modulation of the expression of specific genes.

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Moreover, in the past few years a new function of cytoplasmic aconitase has been revealed (Rouault *et al.*, 1992; Melefors and Hentze, 1993; Rouault and Klausner, 1996), following the demonstration of structural identity between the cytoplasmic aconitase and the iron responsive element-binding protein (IRE-BP), also described as iron regulatory factor (IRF). Since the aconitase/IRE-BP regulates iron metabolism by binding to the «iron responsive element» in the ferritin mRNA (Klausner *et al.*, 1993), it is possible to suppose that the binding of OMA to aconitase/IRE-BP may be involved in the expression of genes responsive to iron and ultimately modulate cell differentiation.

We report data comparing the effects of OMA and differentiation medium (3-isobutyl-1-methyl-xanthine-insulin-dexamethasone) on the expression of c/EBP, *H*-ferritin, and aconitase/IRE-BP in differentiating 3T3-L1 adipocytes.

Materials and methods

a) Cell culture and differentiation. 3T3-L1 fibroblasts, obtained from ECACC (U.K.), were maintained in Dulbecco modified MEM supplemented with 10% foetal bovine serum (FBS), 1 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin, at 37 °C in a humid atmosphere containing 5% CO_2. Differentiation of confluent 3T3-L1 cells was induced by exposing cultures to a solution containing 0.5 mM 3-isobutyl-1-methyl-xanthine, 0.25 μM dexamethasone (final concentration) and 1 μg/ml insulin (differentiation medium here indicated as DXM (Birkenmeier *et al.*, 1989)). 48 h later, the medium was removed and fresh medium containing 1 μg/ml insulin was added, until observing adipocytes transformation. Then RNA analysis was performed (Sambrook *et al.*, 1989). To study the OMA effects on adipocytes differentiation, a growing culture (2×10^5 cells/60 mm plate) of 3T3-L1 was exposed to 5 mM freshly prepared OMA for 74 h (Rastrelli and Festa, 1994). Then, medium was removed and fresh medium containing 5 mM OMA was added. After 96 h, RNA analysis was performed (Sambrook *et al.*, 1989).

b) RNA preparation and analysis. Total RNA was prepared following the procedure described by Chirgwin (in Sambrook *et al.*, 1989) and poly A^+ RNA was purified by affinity chromatography on oligo-dT-cellulose (Sambrook *et al.*, 1989). Total RNA and poly A^+ RNA were fractionated on 1.5% agarose-formamide gel, blotted onto Hi-bond-NTC membrane (Amersham) and hybridized with the appropriate probe using standard protocols (Sambrook *et al.*, 1989).

c) RT-PCR amplification and cloning of cDNA for mouse aconitase/IRE-BP. On the basis of the published sequence of mouse aconitase/IRE-BP (Philpott *et al.*, 1991, EMBL accession number X61147) two oligonucleotide primers were synthesized, *a*: 5′-CAATGAGGATCCTATCTGCTACTGAATTTAGG-3′ and *b*: 5′-CAATGAGCATGCGATGCCTCCATTGTGGAAGTAC-3′, that were used for the amplifi-

cation of a 642 bp segment of cDNA coding for IRE-BP, from a cDNA synthesized by reverse trascriptase, using as a template NIH3T3 cells total RNA (Sambrook *et al.*, 1989). The specific IRE-BP sequence primers were flanked by Bam H1 (*underlined* in a primer *a*) and Sph1 (*underlined* in primer *b*) sites to allow the cloning of the amplified sequence in a pGEM-4Z vector.

Results and discussion

The enhancer binding protein (c/EBP) defines a family of transcription factors that have been shown to regulate the expression of several proteins involved in lipid transport and metabolism (Cristy *et al.*, 1989). In order to test whether c/EBP expression is modified in response to OMA, we analysed the specific mRNA level in 3T3-L1 cells exposed to differentiation medium (DXM) or OMA-containing medium (OMA). Figure 1 shows a Northern analysis of RNA extracted from 3T3-L1 cells exposed to the differentiation medium (DXM) or to the OMA-containing medium (OMA), using as a probe a cDNA fragment coding for c/EBP. The β-actin probe was used to standardize the amount of RNA in each lane. The expression of c/EBP was clearly enhanced by exposing the cells to the differentiation medium DXM (Birkenmeier *et al.*, 1989), while OMA did not seem to have any effect.

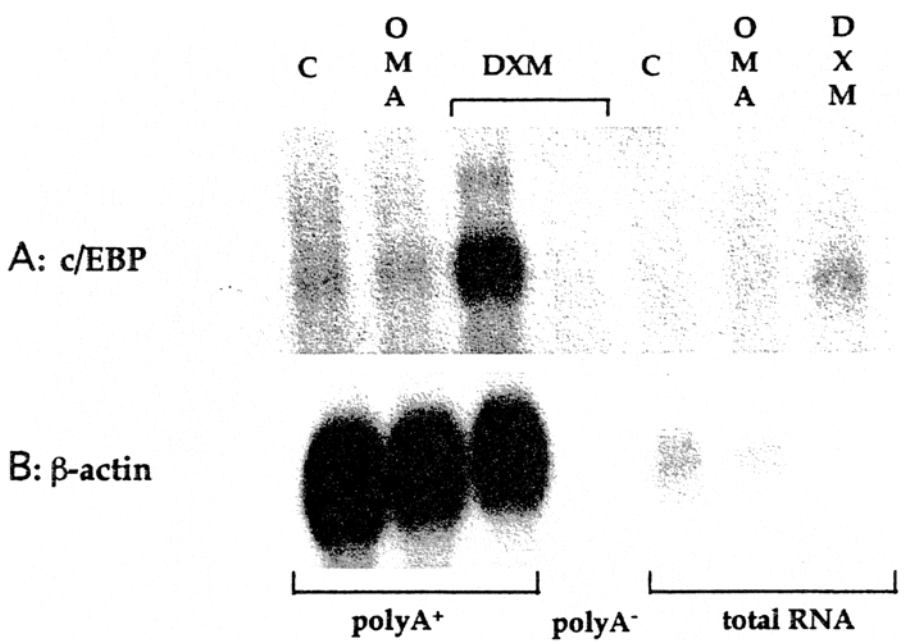

Fig. 1. – Northern blot analysis of RNA from 3T3-L1 cells exposed to 3-isobutyl-1-methyl-xanthine-insulin-dexamethasone (DXM) or oxalomalate (OMA) containing medium. Probes indicated were prepared and labeled as described in the Materials and methods.

Gene expression is not only controlled at transcription level, but several post-transcriptional events contribute to its modulation. The expression of key proteins in the maintenance of iron homeostasis is one of the best characterized model of post-transcriptional gene regulation and involves the binding of a cytoplasmic protein to mature mRNA species. This cytoplasmic protein, that recognizes specific mRNA structures called iron responsive elements (IRE, Melefors and Hentze, 1993), has been identified as the cytosolic counterpart of a citric acid cycle enzyme, aconitase. Aconitase is currently viewed as a bifunctional regulator switching between RNA binding and enzymatic activity.

Since the well known role of OMA as inhibitor of mitochondrial and extramitochondrial aconitase (Adinolfi *et al.*, 1971) and the recently acquired notion that cytoplasmic aconitase is able to sense the intracellular iron levels and regulate translation of proteins involved in iron metabolism (Melefors and Hentze, 1993), we investigated the possible role of OMA in modulating the expression of aconitase/IRE-BP and ferritin. Figure 2 shows the results of a Northern analysis of RNA extracted from cells exposed to the differentiation medium (DXM) or to OMA-containing medium. We used as probe a mouse IRE-BP cDNA fragment (see Materials and methods) and a *H*-ferritin cDNA probe, kindly provided by Prof. Francesco Costanzo (DBBM, Università «Federico II», Naples, Italy). A β-actin probe was used to standardize the amount of

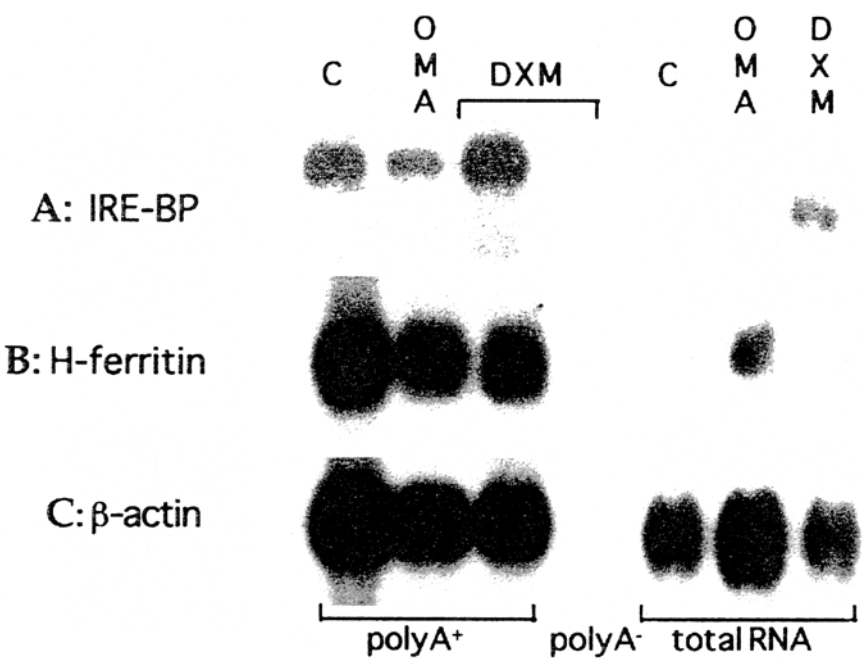

Fig. 2. – Northern blot analysis of RNA from 3T3-L1 cells exposed to 3-isobutyl-1-methyl-xanthine-insulin-dexamethasone (DXM) or oxalomalate (OMA) containing medium. Probes indicated were prepared and labeled as described in the Materials and methods.

RNA in each lane. In the experimental conditions used (confluent cells exposed to the differentiation medium DXM and 96 h exposure to the OMA), OMA did not affect the IRE-BP or *H*-ferritin mRNA level. Interestingly, the DXM differentiation medium, which as OMA, did not alter the mRNA levels for *H*-ferritin, induced a 5-fold increase of the IRE-BP mRNA amount. It has been shown that the expression of ferritin is regulated mostly at translation and the IRE-BP plays an important role in the response of ferritin to the intracellular iron (Klausner *et al.*, 1993). Our results indicate that aconitase/IRE-BP is also involved in the adipocyte differentiation and discloses the issue of the regulation of iron metabolism in mature adipocytes. On the other hand, OMA is a good candidate as a physiological inhibitor of aconitase, since it can be produced *in vivo* by condensation of glyoxylate and oxaloacetate (Ruffo, 1988). It is possible, then, to predict an interplay between mechanisms regulating the citric cycle and mechanisms controlling the expression of specific proteins, including the OMA target, aconitase/IRE-BP.

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