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The induction of adipose conversion in 3T3-L1 cells is associated with early phosphorylation of a 60-kDa nuclear protein

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The induction of adipose conversion in 3T3-L1 cells by bezafibrate has been shown to be enhanced by dibutyryl-cAMP. We here report that the induction of adipose conversion in 3T3-L1 cells, by either bezafibrate and dibutyryl cAMP, or isobutylmethyxanthine alone, is associated with a very early phosphorylation of a 60-kDa acidic protein found in the nuclear fraction.

Adipose conversion; Hypolipidemic fibrate; IMBX; cAMP; Protein phosphorylation

1. INTRODUCTION

Hypolipidemic aryloxyalkanoic acids of varying hydrophobic backbones (i.e. clofibric acid, bezafibrate) have recently been reported to induce adipose conversion in cultured 3T3-L1 cells as well as in primary rat epididymal preadipocytes [1,2]. The induction of adipose conversion in 3T3-L1 cells by bezafibrate was found to be sinergistic with but_2-cAMP or agents elevating intracellular cAMP content, such as forskolin or theophylline. The synergistic effect required the simultaneous presence of the fibrate inducer and but_2-cAMP in the culture medium during the induction phase (usually 48 h), after which both bezafibrate and but_2-cAMP could be removed and replaced by an insulin containing medium, allowing for the differentiated state to gradually develop with a concomitant increase in fat content, mRNAs and respective marker enzymes for adipose conversion. Early events of this complex multistep process (e.g. accumulation of mRNAs for glutamine synthetase and malic enzyme) could be detected as early as five hours following the exposure of the cells to bezafibrate, provided that but_2-cAMP was present in the induction medium [3]. In contrast to the above cAMP effectors which were only poorly capable of initiating adipose conversion in the absence of bezafibrate, IBMX appeared to act as a cAMP effector in the presence of added bezafibrate as well as an inducer per se in the absence of added but_2-cAMP, thus reflecting its dual function as an authentic inducer of adipose conversion together with its capacity for generating intracellular cAMP [3].

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The requirement for cAMP has initiated our interest in evaluating cAMP-dependent protein phosphorylation during the very early stages of bezafibrate- and IBMX-induced adipose conversion.

2. MATERIALS AND METHODS

3T3-L1 cells were grown in 35 or 60 mm plates to confluence in DMEM supplemented with 10% FCS as previously described [1] and subsequently incubated for 2 h with $[^{32}P]H_3PO_4$ in a phosphate-free Tyrode solution supplemented with 0.4% fatty acid free bovine serum albumin. Following this equilibration of the intracellular pool of ATP with ^{32}P [4], the ^{32}P-labelled cells were incubated for an additional 2 h with 1 μM dexamethasone and either 300 μM bezafibrate, 50 μM but_2-cAMP, both or with 500 μM IBMX. The medium was then removed, the cells were washed with saline (4 × 1.0 ml), lysed in 100 μl/35 mm plate of a solution composed of 9.3 M urea, 2% Nonidet P-40, 5% ampholines (pH range 3–10) and 5% β-mercaptoethanol and the lysate was subjected to 2-dimensional gel electrophoresis as described by O'Farrel [5], using molecular weight markers from an Amersham 'Rainbow' kit and pH markers from a Pharmacia 'Carbamylate' kit. Following electrophoresis, the gels were fixed in 10% (v/v) acetic acid–25% methanol (v/v) in water, dried and exposed to X-ray film at −70°C.

The intracellular localization of ^{32}P-labelled proteins was evaluated by lysing the labelled induced cells in 1.5 ml/60 mm plate of a solution composed of 10 mM Tris-HCl buffer, pH 7.5, 5 mM $MgCl_2$, 25 mM KCl, 0.1 mM EDTA, 1 mM DTT and 0.5 mM Nonidet P-40, followed by precipitating the nuclear fraction by centrifuging the cell lysate for 5 min at 500 × g, as described by Zechner et al. [6] and Doglio et al. [7]. The nuclei-rich precipitate was dissolved in 100 μl of a solution composed of 9.3 M urea, 2% Nonidet P-40, 5% ampholines (pH range 3–10) and 5% mercaptoethanol, whereas the supernatant was first dialyzed for 2 h at 4°C against 1000 volumes of H_2O, lyophilized and then dissolved in 100 μl of the same solution. All fractionation steps were carried out at 0–4°C. Both the nuclear and the supernatant fractions were then subjected to 2-dimensional gel electrophoresis as described above.

3. RESULTS

Incubation of non-differentiated 3T3-L1 cells la-

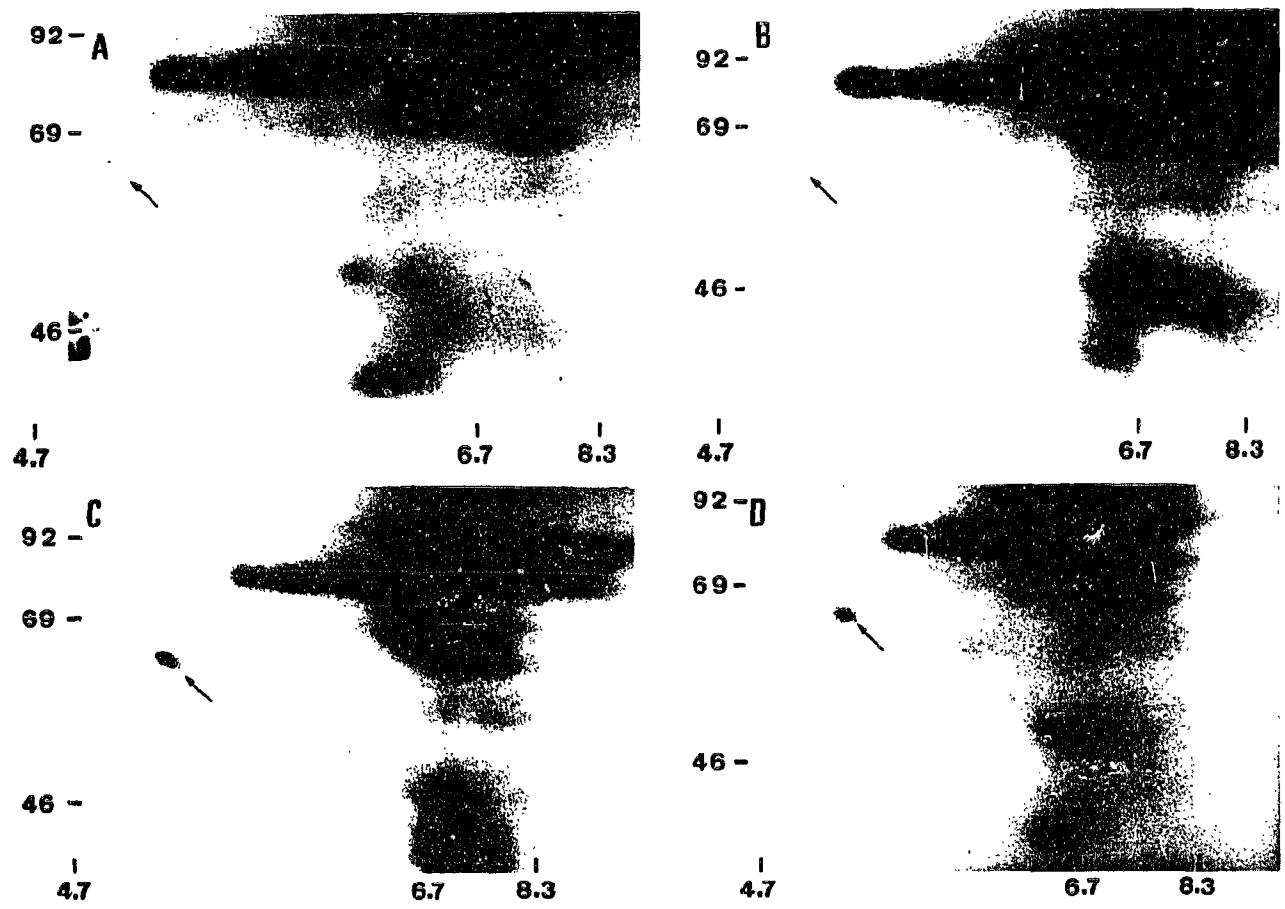

Fig. 1. Protein phosphorylation induced by bezafibrate, but$_2$-cAMP and IBMX: confluent 3T3-L1 cells were incubated at 37°C for 2 h in phosphate-free Tyrode buffer supplemented with 0.4% albumin and 0.2 mCi of [^{32}P]H_3PO_4. 50 μM but$_2$-cAMP (A), 300 μM bezafibrate (B), 300 μM bezafibrate + 50 μM but$_2$-cAMP (C) or 500 μM IBMX (D) were then added to the culture medium together with 1 μM dexamethasone and the culture was further incubated for additional 2 h. Phosphorylated proteins were analyzed by subjecting the respective cells' lysates to 2-dimensional gel electrophoresis and autoradiography as described in section 2. The ordinate specifies mol. mass in kDa and the abcissa specifies pH. The arrows point to the 60-kDa ^{32}P-labelled protein.

belled in their adenine nucleotides pool in the presence of either a combination of bezafibrate and but$_2$-cAMP or IBMX alone resulted in a distinct ^{32}P-labelled spot of 60-kDa having an acidic PI (Fig. 1). The 60-kDa spot was almost absent in controls exposed to either bezafibrate or but$_2$-cAMP alone. Cycloheximide added to the medium together with bezafibrate and but$_2$-cAMP, or with IBMX did not interfere with labelling of the 60-kDa spot (not shown). The 60-kDa spot was the only labelled spot which could be consistently detected in the presence of bezafibrate and but$_2$-cAMP or IBMX, while being absent in the respective controls.

The 60-kDa ^{32}P-labelled spot was found to be associated with the nuclear fraction of 3T3-L1 cells induced for adipose conversion by either bezafibrate and but$_2$-cAMP or IBMX alone, while being essentially absent in the postnuclear supernatant fraction (Fig. 2). The absence of the 60-kDa ^{32}P-labelled spot in the postnuclear fraction could not be accounted for by its processing through dialysis and lyophilization as verified by subjecting unfractionated cells to a similar experimental procedure.

4. DISCUSSION

Induction of adipose conversion by either bezafibrate and but$_2$-cAMP or IBMX alone thus appears to be associated with a very early phosphorylation of a cellular protein found in the nuclear fraction. This phosphorylated protein was hardly detected in cells induced by bezafibrate alone or but$_2$-cAMP alone in correlation with the capacity of the concerned effectors as inducers of adipose conversion [3]. The correlation observed between the capacity of the concerned effectors as inducers of adipose conversion and their capacities in initiating the phosphorylation of the 60-kDa protein may indicate that the phosphorylation observed is intrinsic to the inductive sequel of adipose conversion. Protein phosphorylation may thus account for the fact that the inducer and the cAMp effector may

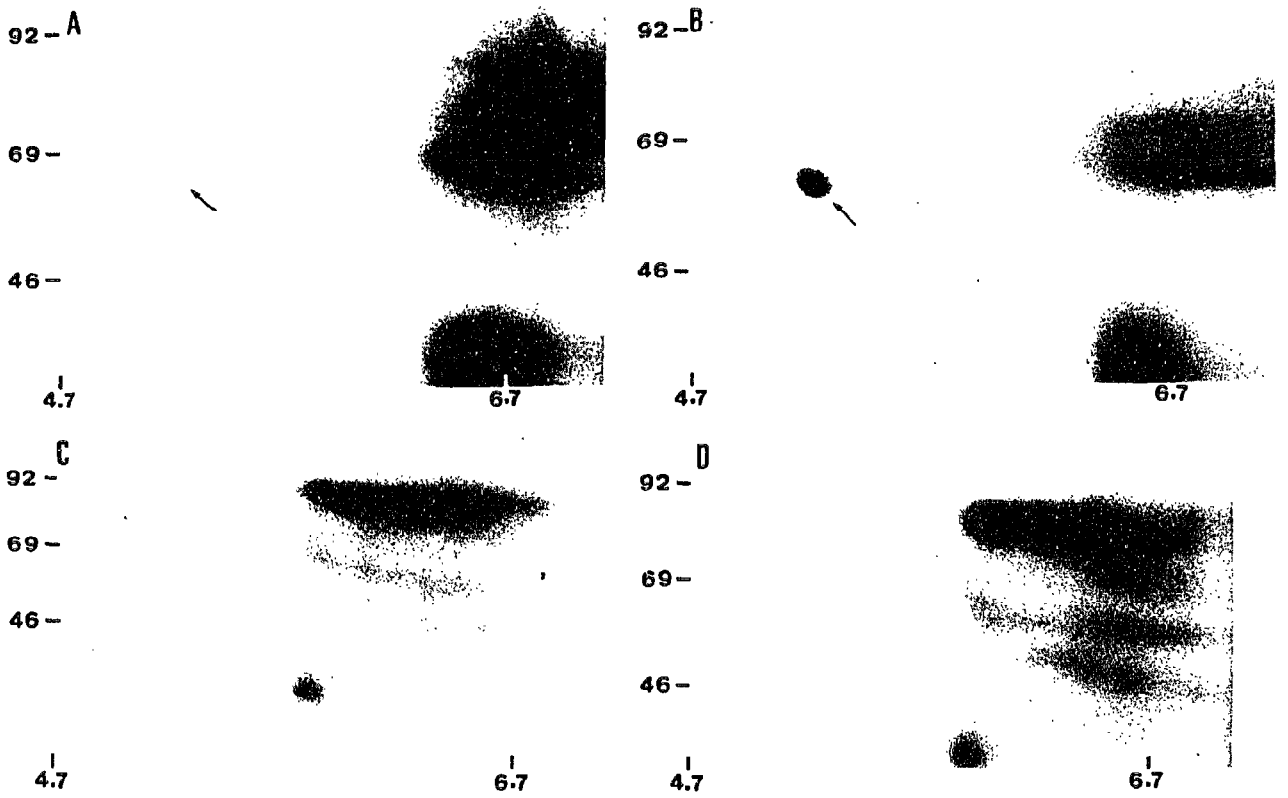

Fig. 2. Cellular localization of the 60-kDa protein: confluent 3T3-L1 cells were incubated as described in Fig. 1, and then fractionated into nuclear (A,B) and supernatant fractions (C,D) as described in section 2. Each fraction was analyzed by 2-dimensional gel electrophoresis and autoradiography. B,D: cells incubated with 300 μM bezafibrate, 50 μM but$_2$-cAMP and 1 μM dexamethasone. A,C: incubated with 50 μM but$_2$-cAMP and 1 μM dexamethasone but in the absence of added bezafibrate. The ordinate specifies mol. mass in kDa and the abcissa specifies pH. The arrows point to the 60-kDa ^{32}P-labelled protein.

both be removed following commitment while the cells are gradually expressing their adipose phenotype throughout the conversion phase [1,3]. It is noteworthy that phosphorylation of the 60-kDa protein in the presence of bezafibrate and but$_2$-cAMP could be detected as early as 15 min following their introduction into the induction medium (not shown), thus making this step the earliest known event of the inductive sequel. The observed capacity of IBMX alone but not of but$_2$-cAMP in initiating the phosphorylation of the 60-kDa protein may corroborate its dual role in adipose conversion, namely as an authentic inducer and as a generator of intracellular cAMP [3].

The identity of the phosphorylated 60-kDa protein still has to be investigated. Its nuclear localization following 2 h of incubating the cells in the presence of the bezafibrate inducer and but$_2$-cAMP does not necessarily point to its original cellular localization. Indeed, the 60-kDa protein could actually be a cytosolic one moving into the nucleus following its cytosolic phosphorylation or alternatively subsequent to its interaction with the bezafibrate or IBMX inducers. The function of this protein presents an intriguing open question. It is tempting to assume that it may act as a transcriptional factor of genes related to adipose conversion and that its transcriptional activity may be modulated by cAMP-dependent phosphorylation similarly to other recently reported transcription factors (reviewed in [8]).

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