Nicotinamide inhibits adipocyte differentiation of 3T3-L1 cells

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Nicotinamide analogue *Adipocyte differentiation* *3T3-L1 preadipocyte* *ADP-ribosylation* *NAD* *Poly(ADP-ribose) synthetase*

1. INTRODUCTION

3T3-L1 cells are a clone of Swiss/3T3 fibroblasts that spontaneously differentiate into adipocytes with a high frequency when in a resting state [1–4]. The differentiation process normally takes several weeks after the cells have become confluent, but it may be speeded up by treating the cells with the inducers 1-methyl-3-isobutylxanthine (MIX) and dexamethasone (Dex) [5] or others [2,3,6–8].

During differentiation of the adipocytes a number of morphological and physiological changes occur. The 3T3-L1 cells change from spindle-shaped fibroblast cells to larger, spherical cells and accumulate large triglyceride droplets [1–4]. During differentiation almost all the enzymes involved in de novo fatty acid [6–10] and triacylglycerol [11] synthesis increase coordinately and the synthesis of >60 cellular proteins is greatly increased [12,13]. The differentiating cells acquire sensitivity to physiological concentrations of insulin via an increased number of receptors which have a higher binding capacity than preadipocyte insulin receptors [5]. The cells also acquire a catecholamine-sensitive adenylate cyclase system involved in the lipolytic response to catecholamines [14]. Poly(ADP)-ribosylation also changes during adipocyte conversion: the activity of poly(ADP-ribose) synthetase drops sharply upon the induction of differentiation by MIX, Dex and insulin, continues to decrease for several hours, and then, concurrently with the appearance of phenotypic expression, increases to a level higher than that in pre-adipocytes [15]. This transient reduction in poly(ADP)-ribosylation activity has been postulated as an early essential event in differentiation [15] which reflects changes in chromatin structure [16,17]. In a separate study, involvement of poly(ADP-ribose) synthetase in insulin-stimulated cell growth has been suggested [18].

Here, we examined the effect of nicotinamide, which is known to be a strong inhibitor of poly-(ADP-ribose) synthetase [19,20], on the adipocyte differentiation of 3T3-L1 cells. Our results indicated that in fact nicotinamide inhibits differentiation and that this inhibition is correlated with the prevention of inducer-stimulated cell growth. Studies using nicotinamide analogues further support the previous proposition [15] that poly(ADP)-ribosylation is crucial in triggering the adipocyte differentiation program.

2. MATERIALS AND METHODS

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), calf serum (CS), penicillin–streptomycin, sodium bicarbonate, and trypan blue were purchased from Grand Island Biological Co. (New York). Glutamine, nicotinamide and its analogues, 1-methyl-3-isobutylxanthine (MIX), and dexamethasone (Dex) were purchased from Sigma (St Louis). 3T3-L1 cells were purchased from the American Type Culture Collection (Bethesda).

2.1. *Tissue culture*

3T3-L1 cells were routinely grown in DMEM supplemented with 10% CS, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cultures were maintained at 37°C in a 95% air/5% CO_2 atmosphere.

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2.2. *Differentiation*

3T3-L1 cells (1.5×10^5) were plated in 35 mm Petri dishes (Falcon) in 2 ml of either DMEM + 10% CS (DMEMCS10) or DMEM + 10% FCS (DMEMFCS10). Three days after the cells reached confluence the medium was replaced with fresh DMEMFCS10 containing 0.5 mM MIX and 0.25 μM Dex. Two days later the inducer-containing medium was replaced with fresh DMEMFCS10. Medium was changed every 3 days thereafter. The drugs were added at various times as described in section 3.

2.3. *Cell counting*

Cells were washed twice with Dulbecco's phosphate-buffered saline containing 0.02% EDTA and detached by trypsinization at 37°C for 5–10 min. The numbers of live and dead cells were determined by trypan blue staining. Adipocytes were identified by the presence of large lipid droplets and counted. Counting was done on a hemocytometer.

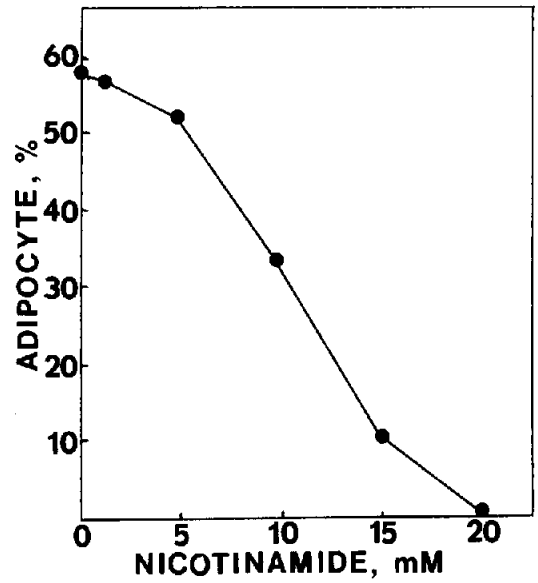

Fig.1. Dose-dependent inhibition of adipocyte differentiation by nicotinamide. Confluent 3T3-L1 cells were treated with MIX and Dex for 2 days (0 d/+2 d) and with nicotinamide for 9 days (0 d/+9 d). Cells were counted on day 9.

3. RESULTS

Since 15 mM nicotinamide significantly inhibited the differentiation of 3T3-L1 cells into adipocytes (not shown), various treatment periods with 15 mM nicotinamide, in relation to the induction period, were tested. All of the treatment periods had some inhibitory effect on differentiation

Table 1

Effects of nicotinamide on adipocyte differentiation and growth of 3T3-L1 cells

Additives	Periods treated[a]	Adipocyte differentiation		Growth	
		% total cells	Relative to B	Cell no. dish ($\times 10^6$)	Relative to A
A. None	–	0.0	–	1.4	1.00
B. Inducers	0 d/+2 d	43.2	1.00	2.6	1.86
C. Nam	−2 d/ 0 d	22.6	0.52	2.0	1.43
D. Nam	−2 d/+2 d	7.4	0.17	1.6	1.14
E. Nam	0 d/+2 d	10.5	0.24	1.7	1.21
F. Nam	+2 d/+7 d	39.6	0.92	0.4	1.71

[a] Inducers (MIX and Dex) were added during the period 0 d/+2 d and then removed, while nicotinamide (Nam) was present at 15 mM during the periods indicated

Adipocyte differentiation and cell number were counted on day 7. Average of 2 determinations

(table 1). The period starting 2 days before the addition of the inducers into growth-arrested 3T3-L1 cells and continuing through the second day of induction (−2 d/ + 2 d) was the most effective. A notable exception was the nicotinamide treatment period that began when the inducers were removed on the second day and continued for 5 days (+2 d/ + 7 d). In this case, nicotinamide no longer inhibited adipocyte differentiation. Table 1 also shows that the inducers (MIX and Dex) stimulate division in the 3T3-L1 cells and that the inhibition of differentiation by nicotinamide is correlated with the prevention of the inducers' mitogenic effect.

Next, the inhibition caused by various concentrations of nicotinamide was investigated (fig.1). The confluent 3T3-L1 cells were treated with MIX and Dex for 2 days (0 d/ + 2 d) and with nicotinamide for 9 days (0 d/ + 9 d) and the cells counted

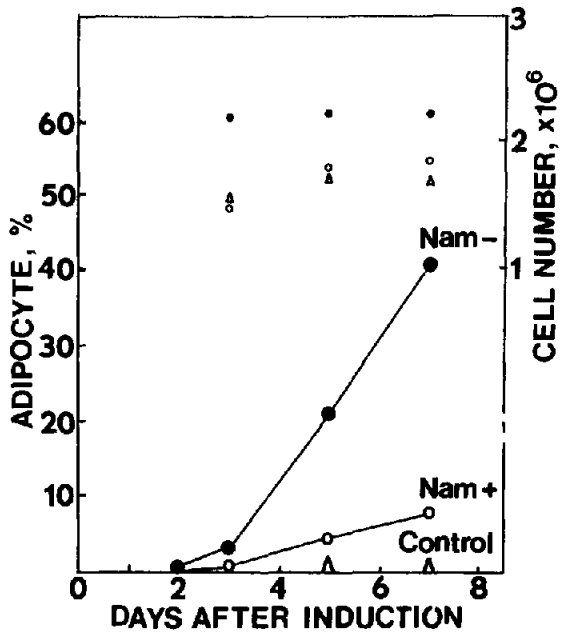

Fig.2. Time-course of adipocyte differentiation and cell growth with and without nicotinamide: (△) control cultures received neither nicotinamide (Nam) nor inducers; (•) Nam (−) cultures were treated with MIX and Dex for 2 days (0 d/ + 2 d); (○) Nam (+) cultures were treated with 15 mM Nam according to the −2 d/ + 2 d treatment schedule described in table 1 and with MIX and Dex for 2 days (0 d/ + 2 d). Cell numbers are indicated with smaller symbols.

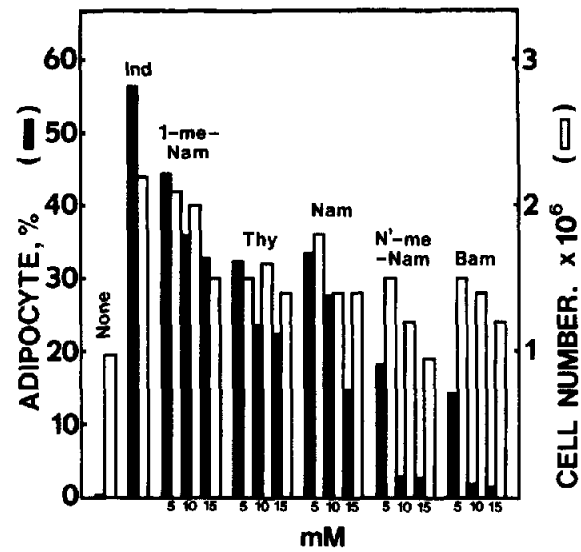

Fig.3. Effects of various nicotinamide analogues on adipocyte differentiation and growth of 3T3-L1 cells. One control culture (None) received neither drugs nor inducers and the other (Ind) received only MIX and Dex for 2 days (0 d/ + 2 d). Other cultures received, in addition to MIX and Dex for 2 days (0 d/ + 2 d), 5 mM, 10 mM and 15 mM (left–right) of 1-methylnicotinamide (1-me-Nam), thymine (Thy), nicotinamide (Nam), *N'*-methylnicotinamide (*N'*-me-Nam), and benzamide (Bam) according to the −2 d/ + 2 d treatment schedule described in table 1. Percent adipocytes and cell number were determined on day 7.

on day 9. Inhibition of differentiation by nicotinamide was dose-dependent with an apparent ED_{50} of 10 mM (fig.1).

Fig.2 shows the time-course of 3T3-L1 cell growth and adipocyte differentiation after inducer treatment in the presence or absence of nicotinamide. As in table 1, nicotinamide clearly inhibited both inducer-stimulated cell growth and differentiation.

Finally, a number of analogues of nicotinamide were tested for the ability to inhibit 3T3-L1 differentiation into adipocytes (fig.3). Of the drugs tested at 3 different concentrations, benzamide was the most potent inhibitor. Following benzamide in potency was *N'*-methylnicotinamide, nicotinamide and thymine. 1-Methylnicotinamide was the least potent inhibitor of differentiation. Again, the inhibition of differentiation was correlated with the prevention of cell growth.

4. DISCUSSION

These data show that nicotinamide and some of its analogues inhibit the differentiation of 3T3-L1 preadipocytes into adipocytes. The mechanism(s) by which the inducers MIX and Dex facilitate adipocyte differentiation is not fully understood [5]. However, it is apparent that growth-arrested 3T3-L1 cells respond to these inducers and double the cell number before entering into adipocyte differentiation [5,6]. This mitogenic response of 3T3-L1 cells to the inducers was suppressed by nicotinamide and some of its analogues. It is known that MIX is an inhibitor of cyclic nucleotide phosphodiesterase and increases the intracellular cAMP level by preventing its degradation [7] and that Dex inhibits the biosynthesis of prostaglandin E1 or its precursor or products [21]. Indirect evidence suggests that addition of prostaglandin E1 to 3T3 cells increases the cAMP level and stimulates DNA synthesis and that this stimulation is synergistically enhanced by the addition of MIX [8]. There is also evidence that addition of dibutyryl cAMP in 3T3-L1 culture enhances the rate of adipocyte differentiation [8]. At present, there is no unified model by which induction of adipocyte differentiation can be understood at the molecular level. Phenomenologically, in the absence of inducing conditions 3T3-L1 cells become arrested in a stage within G1 phase. When inducers are added, they go through one full cell cycle and become arrested at another state, from which they proceed to differentiate into adipocytes. These two states may be designated G_s and G_d, as proposed in [22], for 3T3 T pro-adipocytes, which are similar to 3T3-L1 pre-adipocytes though not identical. Nicotinamide inhibits differentiation apparently by preventing 3T3-L1 cells from leaving the potentiated state and entering the differentiation program.

Nicotinamide is a potent inhibitor of poly(ADP-ribose) synthetase in addition to being a precursor for new NAD^+ biosynthesis [16,17,19,20]. Poly(ADP-ribose) synthetase catalyzes the formation of the ADP-ribose homopolymer from NAD^+ and appears to have a regulatory role in cell proliferation and differentiation [16,17]. As described in section 1, there is a transient decrease of poly(ADP-ribose) synthetase activity and a subsequent increase during the initial period of adipocyte induction [15]. Since nicotinamide inhibited adipocyte differentiation and this inhibition was seen only if the drug was added before or concurrently with the inducers, the recovery of ADP-ribosylation activity from the decreased state during the initial period of induction may be essential for adipocyte differentiation. The strong inhibition of differentiation by benzamide, which is also an inhibitor of poly(ADP-ribose) synthetase [20], further indicates the involvement of this enzyme in regulating differentiation. Its involvement is further suggested by the observation that adipocyte differentiation is inhibited by *N'*-methylnicotinamide which is not an inhibitor of poly(ADP-ribose) synthetase but is an inhibitor of NAD^+ synthesis [20]. In the presence of *N'*-methylnicotinamide, the rise of poly(ADP)-ribosylation may be prevented due to a lack of NAD^+. Although the exact mechanisms remain to be elucidated, this work supports the proposal [15] that adipocyte differentiation involves ADP-ribosylation.

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