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Method

N-acetylcysteine inhibits lipid accumulation in mouse embryonic adipocytes

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ABSTRACT

Oxidative stress plays critical roles in the pathogenesis of diabetes, hypertension, and atherosclerosis; some authors reported that fat accumulation correlates to systemic oxidative stress in human and mice, but cellular redox environment effect on lipid accumulation is still unclear. In our laboratory we used mouse embryonic fibroblasts (undifferentiated cells: CC), which are capable of differentiating into mature adipocytes (differentiated cells: DC) and accumulate lipids, as obesity model. Here we analyzed the role of the well-known antioxidant and glutathione precursor N-acetylcysteine (NAC) in cellular MAPK modulation and lipid accumulation. We evaluated the effect of NAC on the adipogenic differentiation pathway using different doses: 0.01, 0.1, 1 and 5 mM; no toxic doses in these cells. A dose of 5 mM NAC [DCN-5] provoked a significant decrease in triglyceride accumulation (72 ± 10 [DCN-5] vs 169 ± 15 [DC], $p < 0.01$), as well in Oil Red O stained neutral lipid content (120 ± 2 [DCN-5] vs 139 ± 12 [DC], $p < 0.01$). Molecular mechanisms responsible for adipogenic differentiation involve increase of the expression of phosphoERK½ and phosphoJNK, 5 mM NAC treatment inhibited both pERK½ and pJNK protein levels. We also evaluated the mitotic clonal expansion (MCE) which takes place during adipogenesis and observed an increase in DC at a rate of 1.5 cells number compared to CC at day 2, whereas the highest doses of NAC significantly inhibited MCE. Our results suggest that NAC inhibits lipid accumulation and the MAPK phosphorylation in mouse embryonic fibroblasts during adipogenic differentiation and further contribute to probe the importance of cellular redox environment in adipogenesis.

1. Introduction

Research investigating redox regulation has received a large amount of attention due to the role of oxidative stress in several diseases. Excessive production of reactive oxygen species (ROS) induces oxidative stress in cells, but non-toxic levels of ROS have been described in relation to intracellular signal transduction, thereby regulating fundamental cell behaviors such as proliferation and differentiation [1]. However, ROS have a very short half-life, and their cellular levels are very difficult to reproduce. Therefore, the effect of ROS can be difficult to measure. An alternative strategy to achieve this goal is to evaluate the effects of antioxidants in a systemic study. New adipocytes could develop from precursor such 3T3-L1 fibroblasts or mouse embryonic fibroblasts (MEF). We have previously shown that antioxidant N-acetylcysteine (NAC) inhibits adipogenic differentiation in the 3T3-L1 cell line [2,3]. Here, we explored this antioxidant effects on primary cultures from MEF because 3T3-L1 are committed cells.

The molecular mechanisms that are responsible for the adipogenic differentiation involve regulation of the expression of MAPKs (Mitogen-Activated Protein Kinases) such as phospho-ERK (p ERK½) and phospho-JNK (pJNK). This regulation leads to terminal differentiation and accumulation of triglycerides (Tg) in the adipocytes and as a consequence, the potential to develop obesity [4,5]. As for the role of ERK½ in differentiation process, it is involved in an initial proliferation called mitotic clonal expansion (MCE) that takes place during the two first days of adipogenesis [6]. The signaling pathways that involve JNK are strongly responsive to redox regulation. Thus, an exploration of the molecular regulation of ERK½ and JNK MAPKs during adipogenesis is important for understanding cellular differentiation. Of particular interest is the modulation that occurs during an antioxidant treatment that inhibits the accumulation of triglycerides, which would be the final event in the differentiation of preadipocytes. Questions such as "how does the activation of mitogen-activated protein kinase (MAPK) modules in response to different

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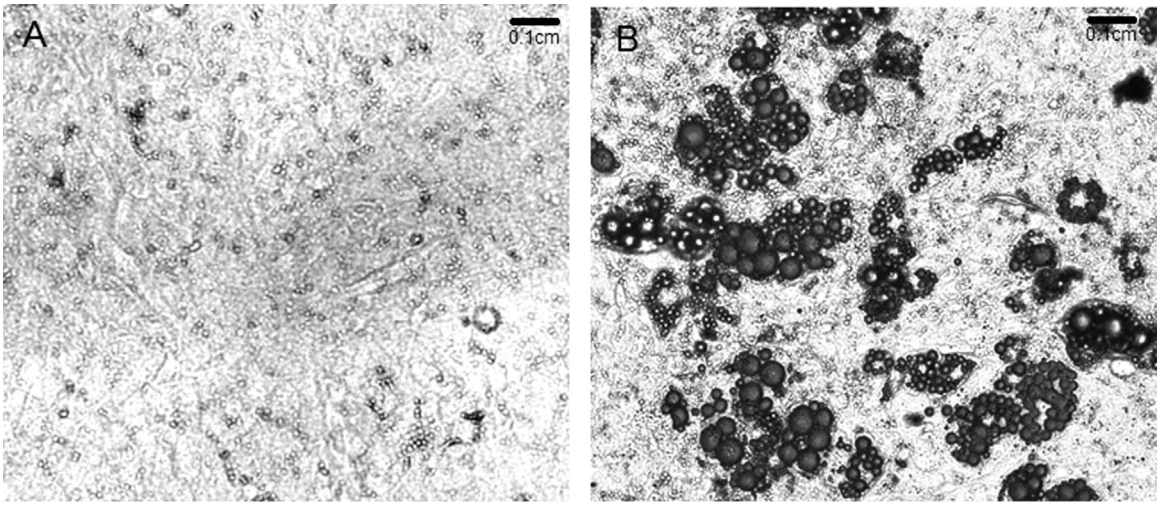

Fig. 1. MEF at day 10 of differentiation. (A) Control cells (CC); (B) MDI-treated cells (DC). Lipid droplets are shown in black. Representative results from one of three experiments with similar results are shown.

extracellular inputs lead to distinct effects in cellular metabolism?" [7] could be answered using this strategy.

The use of NAC as a regulator of the adipogenic process is under discussion [2,3,8,9]. In the present study, our aim is to evaluate the relationship between the accumulation of lipids and MAPK during MEF cellular differentiation through treatment with the antioxidant NAC.

2. Materials and methods

2.1. Isolation of mouse embryonic fibroblasts (MEF)

Mouse embryonic fibroblasts (MEFs) were prepared from CF-1 mouse embryos at day 14 of gestation, by culture of small tissue explants as previously described [10]. Briefly, the embryos were removed from the uterus and washed with PBS. Once the head and red organs were dissected, the embryonic tissue was washed with PBS and finely minced using a sterile razor blade until the tissue could be handled with a pipette. After that, trypsin-EDTA was added and the sample was incubated for 30 min at 37 °C. Trypsin was inactivated by adding Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 25 mM glucose and 10% fetal bovine serum (FBS). Cells were centrifuged at 300 g for 5 min; the pellet was plated in culture bottles with complete media (DMEM plus 25 mM glucose and 10% FBS). The outgrowing primary cell population was passaged by trypsinization at ratio of 1:3 upon confluency and continuously cultured in complete media to favor growth of fibroblastic cells.

2.2. MEF adipocyte differentiation

MEF were first cultured in MDI medium (0.5 mM 3-isobutyl-1-methyl xanthine, 0.1 μM dexamethasone, and 2 μM insulin) for 72 h. They were then transferred to fresh DMEM (25 mM glucose; 10% FBS) supplemented with 2 μM insulin and incubated for three days. The cells were then cultured in fresh complete media for the remainder of the experiment. Day 0 of differentiation was defined as the time at which the cells were first introduced to MDI medium. At day 10, 70–80% of cells dramatically increased their triglyceride (Tg) content, thereby generating refractive droplets that were easily observed by microscopy or Oil Red O staining. MDI-treated MEF were considered differentiating cells (DC), and vehicle-treated MEF were considered control cells (CC).

NAC was added to the MDI medium at day 0 of differentiation and maintained in the medium throughout the remainder of the experiment; these cells were considered NAC-treated differentiating cells (DCN).

2.3. Oil Red O staining

Cell monolayers were washed three times with PBS and then fixed with 4% formaldehyde in PBS, at 4 °C for 30 min. A stock solution of 0.4% Oil Red O (SIGMA) in isopropanol was prepared. To perform the assay, the dye was diluted with water, filtered and added to the fixed cells. Oil Red O staining was developed at room temperature for 30 min. Cells were then washed with water and, the stained lipids droplets in the cells were visualized and photographed. After that, the dye was extracted with isopropanol and its absorbance was determined at 510 nm to quantify the staining lipids.

2.4. Determination of triglyceride and protein levels

Tg accumulation was assessed using a TG color GPO/PAP AA kit (Wiener Laboratory, Rosario, Argentina). Proteins were quantified by the Bradford method using crystalline bovine serum albumin as standard [11].

2.5. MTT assay

To evaluate the toxicity of NAC treatment, MTT (Bromide 3-(4,5-dimethylthiazol-2-yl)-2,5-difeniltetrazol) viability assay was performed [12]. The technique is based on the presence of mitochondrial enzymes in viable cells that reduce the MTT dye and produce a color purple. A stock solution of 5 mg/mL MTT was prepared. To perform the assay, the dye was diluted in complete media to a working concentration of 1 mg/mL. Cells were seeded at 20×10^4 cells/well and 300 μL of MTT were added to each well. After incubation for 1 h, the medium was removed and the cells were treated with 200 μL of ethanol for 10 min. The resulting solution in each well was transferred to an ELISA plate and its absorbance was measured at 550 nm. To further evaluate toxicity, we performed assays in which cell viability was determined under different concentrations of NAC including 0.01 mM, 0.1 mM, 1 mM and 5 mM. The absorbance of control cells (CC) was set to 100% and the results are presented as percentages of CC.