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Research paper

CCAAT/enhancer-binding protein-β participates in oxidized LDL-enhanced proliferation in 3T3-L1 cells

Carmela Santangelo*, Rosaria Varì, Beatrice Scazzocchio, Carmelina Filesi, Massimo D'Archivio, Claudio Giovannini, Roberta Masella

Department of Veterinary Public Health and Food Safety, Italian National Institute of Health, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy

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ABSTRACT

Increased circulating oxidized LDL (oxLDL) have been found in obese subjects. Obesity is characterized by an excess of fat mass resulting from an increase in adipocyte number and size. The generation of new adipocytes is a tightly controlled process where multiple factors acting in a signaling cascade follow a precise temporal expression pattern; oxLDL appear to have a role in the impairment of this process. The purpose of this study was to examine the effects of oxLDL on the mechanisms involved in the proliferative stage of the differentiation process in 3T3-L1 cells. After hormonal induction, 3T3-L1 cells undergo approximately two rounds of mitotic clonal expansion (MCE), a process required for adipogenesis. CCAAT/enhancer-binding protein β (C/EBPβ) is immediately expressed after induction, and plays a crucial role in MCE, but its expression must decrease to allow preadipocytes to mature into adipocytes. We found that, in the presence of stimuli to differentiate, oxLDL induced a higher proliferation rate in this cell line, associated with a sustained up-regulation of C/EBPβ, which remained activated inside the nucleus for several days. RNAi-mediated knockdown of C/EBPβ 24 h after oxLDL treatment counteracted the increase in proliferation rate. Both C/EBPβ expression and proliferation processes appear to be influenced by cAMP/protein kinase A (PKA) and extracellular signal-regulated kinases1/2 (ERK1/2) pathways. OxLDL treatment led to increased levels of cAMP, and to a strong, prolonged phosphorylation of ERK1/2 and C/EBPβ. The addition of cAMP and PKA inhibitors, SQ22536 and H-89, respectively, reduced proliferation only in oxLDL-treated cells, whereas the addition of ERK1/2 inhibitor U0126 blocked proliferation in both control and oxLDL-treated cells. C/EBPβ nuclear expression and DNA-binding activity were reduced by U0126, under all tested conditions. These findings show that the altered expression pattern of C/EBPβ is involved in the increase in the number of proliferating cells induced by oxLDL, in hormone-stimulated 3T3-L1 cells.

1. Introduction

Growing evidence indicates that high levels of circulating oxidized low-density lipoproteins (oxLDL) are associated with obesity and the increased incidence of the metabolic syndrome [1–5]. Obesity is characterized by the expansion of body fat mass, a risk factor for various diseases, including coronary heart disease, hypertension, type 2 diabetes mellitus and some types of cancer [6,7]. One of the main functions of adipocytes is to store surplus calories as triglycerides for subsequent retrieval during periods of need. Chronic nutrient overload associated with a reduction in caloric expenditure results in an increased amount of intracellular lipids, greater adipocyte size (hypertrophy), and a higher number of new adipocytes (hyperplasia) from precursor cells. These processes account for adipose tissue expansion and obesity [8,9]. Although the basic number of adipocytes sets in around adolescence in both humans and rodents, there is a continuous turnover of adipocytes in adult humans that enables adipose tissue to generate new adipocytes at any age [10,11]. The observation that obese individuals show a greater number of adipocytes added per year as compared with lean individuals reinforces the concept that adipocyte hyperplasia is an important factor in the development of obesity [11]. Several studies show that oxLDL values correlate positively with degrees of obesity, although the behaviour and metabolism of oxLDL *in vivo* is still poorly understood [2,12,13].

Abbreviations: DMIX, differentiation mixture; oxLDL, oxidized LDL; ctrl, control; C/EBPβ, CCAAT/enhancer-binding protein β; ERK1/2, extracellular signal-regulated kinases1/2; PKA, protein kinase A; siRNA, synthetic short interfering RNA.

* Corresponding author. Tel./fax: +39 0649902763.
E-mail address: c.santan@iss.it (C. Santangelo).

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Adipocyte differentiation is a complex process tightly controlled by multiple factors acting in a signaling cascade that culminates in the activation of a large number of adipocyte genes responsible for the mature fat-cell phenotype [14,15]. Most of the knowledge concerning molecular mechanisms and signal transduction pathways involved in adipocyte differentiation originates from investigations of the 3T3-L1 preadipocyte cell line [16]. After the addition of a differentiation mixture, growth-arrested 3T3-L1 preadipocytes synchronously re-enter the cell cycle, undergo approximately two rounds of mitosis, namely mitotic clonal expansion (MCE), then exit the cell cycle and enter the terminal stages of differentiation [17]. Early expressed in the adipocyte differentiation program, the promitotic transcription factor CCAAT enhancer-binding protein β (C/EBPβ) is essential for MCE, which appears to be a prerequisite for adipogenesis [18]. The intronless C/EBPβ gene is a gene known to generate a single mRNA that can produce two forms: liver-enriched activating protein (LAP, 34 and 38 kDa) and liver inhibiting protein (LIP, 20 kDa). LAP is a transcriptional activator while LIP may act as an inhibitor of C/EBPβ transcritional activity [19]. Upon hormonal induction, C/EBPβ is rapidly expressed (≤2 h), moves to the nucleus where its level remains steady for about 36–48 h, then begins to decline, allowing cells to exit the cell cycle [20,21]. Although C/EBPβ is expressed very early, it lacks DNA-binding activity; the acquisition of this activity requires the sequential phosphorylation of C/EBPβ/LAP first on Thr(188) by mitogen-activated protein kinase (MAPK-ERK) and then on Ser(184) or Thr(179) by glycerol synthase kinase-3β (GSK3β) [22]. Moreover, several lines of evidence indicate that the transcriptional activity of C/EBPβ may be influenced by post-translational modifications occurring also in the DNA-bound C/EBPβ complex, including acetylation [23] and sumoylation [24]. C/EBPβ is also involved in the proliferation of different cell types [25,26], and participates in tumor invasiveness and progression [27,28].

The increase in cAMP concentration has been associated with crucial events in the early program of differentiation, including the induction of C/EBPβ [29]. Cyclic AMP appears to be a remarkable regulator that either activates or inhibits cell proliferation depending on the stimulus and cell type [30]. A recent report indicates that the transient rise in cAMP and its principal effector protein kinase A (PKA) is necessary for MCE in 3T3-L1 cells [31]. Extracellular signal-regulated kinases1/2 (ERK1/2) belong in the group of serine/threonine kinases that are rapidly activated in response to growth factor stimulation. They integrate multiple signals from various upstream second messengers, and regulate the expression of transcription factors leading to cellular proliferation or differentiation [32]. The function of ERK in adipogenesis is multiple [9]; its activation, however, appears to be essential in the early proliferative (MCE) step, including C/EBPβ phosphorylation, required for DNA-binding activity [22]. Several studies indicate that the proliferative activity of oxLDL involves ERK1/2 activation in both macrophages [33] and vascular smooth muscle cells [34]. We have shown elsewhere that oxLDL entered 3T3-L1 cells and delayed preadipocyte differentiation by altering the physiological sequence of transcription factors and signalling pathways [35]. The mechanisms underlying the early stage of the differentiation programme remain to be elucidated. These findings prompted us to examine the effects of oxLDL on the mechanisms involved in the proliferative phase of the differentiation process in the 3T3-L1 cell line. In the present study, we demonstrated that the addition of oxLDL to hormone-stimulated 3T3-L1 cells, results in *i*) the enhancement of cell proliferation rate associated with a *ii*) dramatic and long-lasting nuclear increase of C/EBPβ expression and phosphorylation and *iii*) an increased level of cAMP and ERK1/2 activation. OxLDL-induced proliferation was significantly inhibited by the silencing of C/EBPβ and by PKA, adenylate cyclase, and ERK1/2 kinase inhibitors.

2. Materials and methods

2.1. Reagents

DMEM was purchased from Hyclone (Cramlington, UK); fetal calf serum (FCS), glutamine, and antibiotics were from Flow Laboratories (Irvine, UK). Insulin, isobuthylmetylxanthine, dexamethasone were supplied by Sigma (St. Louis, MO, USA). Electrophoresis reagents were from Bio-Rad (Hercules, CA, USA). Anti-C/EBPβ, anti-lamin B, anti-GAPDH, and horseradish peroxidase-conjugate, anti-mouse, and anti-rabbit antibodies were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). The electrophoresis reagent was from Bio-Rad (Hercules, CA, USA). Antibodies against p44/p42 MAPK (ERK1/2), phospho-p44/p42 MAPK (Thr-202/Tyr-204; phospho-ERK1/2) and p-Thr-188-C/EBPβ were obtained from Cell Signaling Technology (Beverly, MA, USA). H-89 and U0126 were supplied by Calbiochem (Darmstadt, Germany) and SQ22536 was from Santa Cruz (Santa Cruz, CA, USA). Trizol reagent, Lipofectamine RNAiMAX and Opti-MEM I medium were from Invitrogen-Life Technologies (USA), DNAse-I from Promega (Mannheim, Germany). siGENOME SMARTpool C/EBPβ siRNA and siCONTROL Nontargeting siRNA were obtained from Darmacon (Lafayette, CO, USA). TaqMan MGB gene expression assay for C/EBPβ and endogenous control TBP were furnished by Applied Biosystems (Foster City, CA, USA). All the other chemicals were of the highest analytical grade commercially available.

2.2. Plasma LDL isolation and oxidation

LDL (1.019–1.063 g/ml) were isolated by density gradient ultracentrifugation in vertical rotor from fresh pooled plasma of healthy volunteers provided by the Centro Trasfusionale of University of Rome "La Sapienza," as described elsewhere [35].

The protein content was measured by Lowry's methods using BSA as standard [36]. Native LDL (nLDL) were oxidized with 20 μM $CuSO_4$ for 18 h at 37 °C. Oxidation was discontinued by 1 mM EDTA. The degree of LDL oxidation was assessed by determining thiobarbituric acid-reactive substance content [37], which was 45 ± 7 nmol malondialdehyde equivalent/mg LDL protein (*vs.* 4 ± 0.3 nmol in nLDL).

2.3. Cell cultures

Murine 3T3-L1 preadipocyte cells (American Cell Culture Collection) were cultured in DMEM supplemented with 10% FCS, 0.2 mmol/l glutamine, and 10 U/ml antibiotics. Two days after confluence (day 0), 3T3-L1 cells were treated with the differentiation mixture (DMIX) containing 1 μM insulin, 0.25 μM dexamethazone and 0.5 mM isobuthylmetylxantine for 48 h. The medium was changed and 1 μM insulin was added for 48 h.

2.4. 3T3-L1 cells treatment

OxLDL were sterilized with a 0.22 μm membrane (Millipore Corporation, Bedford, MA), and incubated with the cells. After testing different oxLDL concentrations (25–200 mg protein/l), 50 mg protein/l was chosen because of the complete absence of cytotoxicity. Under all the experimental conditions described below, 3T3-L1 cells untreated or treated with nLDL were used as controls. DMIX plus oxLDL (50 mg protein/l), or nLDL (50 mg protein/l) were added to the cultured cells on day 0, and kept in the medium throughout the experiment. In a set of experiments, specific inhibitors were added to the cells at concentrations that effectively inhibited targeted pathways without any signs of cytotoxicity (see figure legends for details). Specifically, 10 μM H-89