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Leucodine blocks mitotic clonal expansion during preadipoctye differentiation through cell cycle arrest

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ABSTRACT

Adipogenesis comprises the signaling program of cell differentiation by which preadipocytes become adipocytes and is a key process that is required in adipocyte hypertrophy in the development of obesity. In this study, we investigated the effect of 11,13 dihydro-[11*R*]-dehydroleucodine (i.e., leucodine), a sesquiterpene lactone obtained from the reduction of dehydroleucodine, on adipogenesis of 3T3-L1 preadipocyte. 11,13 dihydro-[11*R*]-dehydroleucodine blocked the accumulation of lipid droplets stained with Oil Red O and inhibited the increase of triglycerides in the adipocytes (IC_{50}: 71.0 ± 4.1 μM). Expressions of Glut-4, adiponectin and perilipin-2 were blocked in the presence of 11,13 dihydro-[11*R*]-dehydroleucodine. Furthermore, expression of PPARγ and C/EBPα was significantly inhibited by 11,13 dihydro-[11*R*]-dehydroleucodine as well as the expression of C/EBPβ was also strongly inhibited in time- and concentration-dependent manner. This is consistent with the inhibitory effect of 11,13 dihydro-[11*R*]-dehydroleucodine on the early stage of adipogenesis. Thus, the proliferation of preadipocytes were also suppressed by addition of 11,13 dihydro-[11*R*]-dehydroleucodine during the first 48 h after induction. These results indicate that 11,13 dihydro-[11*R*]-dehydroleucodine may selectively affect mitotic clonal expansion to block preadipocyte differentiation. 11,13 dihydro-[11*R*]-dehydroleucodine stopped the cell cycle at the G_0/G_1 phase by diminishing the increase of phosphorylation of Rb, Erk1/2 and Akt while increasing the expression of p27. Collectively, our results indicate that the inhibition of early stage preadipocytes differentiation by 11,13 dihydro-[11*R*]-dehydroleucodine may be associated with cell cycle arrest at the G_0/G_1 phase through upregulation of p27 expression.

1. Introduction

Obesity results from a pathological expansion of adipose tissue mass due to adipocyte hyperplasia and hypertrophy (Spalding et al., 2008; Muir et al., 2016). Aberrant hyperplasia due to preadipocyte proliferation and differentiation into mature adipocytes appears to be the key determinant in this expansion of adipose tissue (Spalding et al., 2008), although hypertrophy due to increased storage of triacylglycerols or endogenous lipogenic pathways also plays a role (Roncari et al., 1981).

The adipose tissue expansion underlying the obesity epidemic can be modeled in vitro by utilizing a 3T3-L1 murine preadipocyte model system (Ruiz-Ojeda et al., 2016). Inducing differentiation in 3T3-L1 preadipocytes through an induction media (IM) cocktail consisting of dexamethasone, isobutylmethylxanthin (IBMX), and insulin over the course of approximately one week results in observable accumulation of triglycerides (Kassotis et al., 2017) This cocktail also results in the expression and/or phosphorylation of numerous proteins that are required for the adipogenic phenotype, including AMPK, Akt1, Erk1/2 (Jeong et al., 2015; Shearin et al., 2016), the transcriptional factor peroxisome proliferator-activated receptor γ (PPARγ), factors from other transcription factor families, including CCAAT/enhancer-binding proteins (C/EBPs) (Tao et al., 2016), signal transducers and activators of transcription (STATs) (Huang et al., 2016), and Kruppel-like factor (KLF) proteins (Jiang et al., 2015).

Essential to the adipocyte differentiation program is the process of mitotic clonal expansion (MCE) (Tang et al., 2003). The acquisition of binding activity by C/EBPβ is temporally coordinated with the initiation of MCE (Lo et al., 2013). C/EBPβ is expressed within 2 to 4 h post-hormonal induction, yet initially lacks DNA binding activity (Lo et al.,

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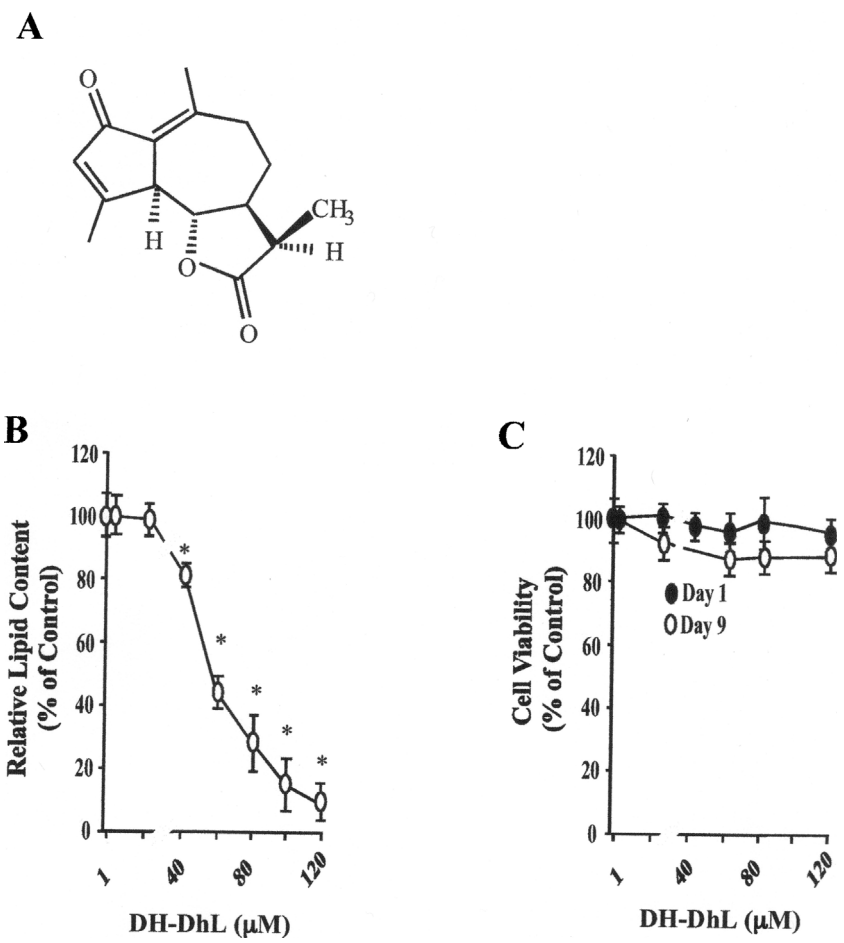

Fig. 1. Inhibitory effect of 11,13-dihydro-[11*R*]-dehydroleucodine on adipocyte differentiation. (A) Chemical structure of 11,13DH-[11*R*] DhL. (B) Preadipocytes were cultured in induction medium containing different concentrations of 11,13DH-[11*R*]DhL as indicated in the figure for 9 days (1–120 μM). Cells were stained with Oil Red O and morphological changes of 3T3-L1 preadipocytes were monitored, and then photographed (×100) after 9 days from the onset of differentiation. The percentages presented are relative to the 11,13DH-[11R]DhL-free control in the presence of induction media (100%). (C) Preadipocytes were cultured in induction medium containing different concentrations of 11,13DH-[11*R*]DhL for both: the course of 1, and a course of 9 days, respectively. Cell viability was determined by the LDH assay. All values are presented as the mean ± SEM of three experiments performed in triplicate. *$P < 0.01$ vs no 11,13DH-[11*R*] DhL-treatment.

2013). Preadipocytes in this early stage express several KLF proteins, and undergo growth arrest (Mori et al., 2005; Oishi et al., 2005; Birsoy et al., 2008; Pei et al., 2011). Beginning at approximately 12 h post induction, and reaching maximal activity at 24 h, C/EBPβ acquires DNA binding activity and the 3T3-L1 preadipocytes begin to undergo two to four rounds of mitosis during the period of MCE which requires activation of the ERK and p38 signaling pathways (Yang et al., 2002; Tang et al., 2003; Aouadi et al., 2007). Progression from G1 to S requires down-regulation of p27 and activation of CDK2 by cyclins E and A (Patel and Lane, 2000; Yang et al., 2002). Cyclin D1 and cyclin E are activated during the G_1 phase of the cell cycle (Choi et al., 2012). Once activated, cyclin D1 assembles with CDK4 or CDK6 and cyclin E binds to CDK2 (Choi et al., 2012). Cyclin A and B are then recruited to CDK2 and CDK1, respectively; associations necessary for the inducement of cell cycle progression through S phase and mitosis (Choi et al., 2012). Activated C/EBPβ triggers the expression of C/EBPα and PPARγ, which are anti-mitotic and eventually terminate the MCE program (Tang et al., 2003). Next, C/EBPβ levels decrease, and co-activation of a number of genes occurs including Ap2, GLUT4, SCD1, PEPCK, and leptin (Tang et al., 2003). In coordination with other genes (Hishida et al., 2007; Johmura et al., 2008; Kawaji et al., 2010; Merkestein et al., 2015), this signaling cascade results in a terminally differentiated adipocyte phenotype characterized by spherical cell morphology and the accumulation of lipid droplets (Fei et al., 2011).

Since adipocyte hyperplasia is a key determinant for the expansion of adipose tissue, it is thought that compounds that inhibit adipocyte differentiation may have therapeutic utility for obesity and its associated disorders (Kim et al., 2011). Previously, it was found that the sesquiterpene lactone dehydroleucodine (DhL), extracted from the aerial parts of *Artemisia douglasiana*, decreased phenotypic (lipid content) and molecular markers (PPARγ and C/EBPα) of adipogenesis, indicating that this compound inhibited differentiation of preadipocytes (Galvis et al., 2011). Enantiomers of 11,13-dihydro-dehydroleucodine (i.e., leucodine and achillin) were found to inhibit 3T3-L1 preadipocytes differentiation (Galvis et al., 2011), but their effects on the various signaling molecules underlying the adipogenic differentiation program were not fully investigated. In this study, we specifically examined the effects of 11,13-dihydro-[11*R*]-dehydroleucodine (11,13DH-[11*R*]-DhL, leucodine), on the timing and expression of molecular markers underlying the adipogenic program, including the process of MCE, in order to further elucidate the action, underlying mechanism, and therapeutic import of dehydro derivatives of dehydroleucodine.

2. Results

2.1. 11,13-dihydro-[11R]-dehydroleucodine inhibits 3T3–L1 adipocyte differentiation

Previous studies have demonstrated that DhL and their epimers inhibited the differentiation of 3T3–L1 preadipocytes into adipocytes (Galvis et al., 2011). In this study, we further examined whether the 11,13DH-[11*R*]DhL epimer (Fig. 1A) modulates early steps of the differentiation of 3T3–L1 preadipocytes into adipocytes. Post-confluent preadipocytes were treated with 0, 10, 20, 40, 80, 120 μM 11,13DH-[11*R*]-DhL every other day for 9 days. 11,13DH-[11*R*]-DhL decreased the accumulation of lipid droplets in a concentration-dependent manner (Fig. 1B). In particular, 160 μM 11,13DH-[11*R*]-DhL completely inhibited the formation of lipid droplets in the adipocytes (data not shown) with a half-maximal inhibitory concentration of 71.0 ± 4.1 μM. Interestingly, cell viability was not affected by