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5-Azacytidine engages an IRE1α-EGFR-ERK1/2 signaling pathway that stabilizes the LDL receptor mRNA

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ABSTRACT

Hepatic low-density lipoprotein receptor (LDLR) is the primary conduit for the clearance of plasma LDL-cholesterol and increasing its expression represents a central goal for treating cardiovascular disease. However, LDLR mRNA is unstable and undergoes rapid turnover mainly due to the three AU-rich elements (ARE) in its proximal 3′-untranslated region (3′-UTR). Herein, our data revealed that 5-azacytidine (5-AzaC), an antimetabolite used in the treatment of myelodysplastic syndrome, stabilizes the LDLR mRNA through a previously unrecognized signaling pathway resulting in a strong increase of its protein level in human hepatocytes in culture. 5-AzaC caused a sustained activation of the inositol-requiring enzyme 1α (IRE1α) kinase domain and c-Jun N-terminal kinase (JNK) independently of endoplasmic reticulum stress. This resulted in activation of the epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinase1/2 (ERK1/2) that, in turn, stabilized LDLR mRNA. Systematic mutation of the AREs (ARE1-3) in the LDLR 3′UTR and expression of each mutant coupled to a luciferase reporter in Huh7 cells demonstrated that ARE1 is required for rapid LDLR mRNA decay and 5-AzaC-induced mRNA stabilization *via* the IRE1α-EGFR-ERK1/2 signaling cascade. The characterization of this pathway will help to reveal potential targets to enhance plasma LDL clearance and novel cholesterol-lowering therapeutic strategies.

1. Introduction

The discovery of the low-density lipoprotein receptor (LDLR) and the subsequent elucidation of its mode of action *in vitro* and *in vivo* have provided a conceptual framework for understanding the mechanisms that control the concentration of the most important cholesterol-carrying lipoproteins in human blood [17]. LDLR is a transmembrane glycoprotein that mediates the clearance of lipoprotein particles composed of apolipoprotein B100 (ApoB100) such as low-density lipoproteins (LDL) or apolipoprotein E such as chylomicron-remnants and intermediate-density lipoproteins (IDL) from the plasma. LDLs are the major cholesterol carriers in the blood and transport approximately 65–70% of plasma cholesterol in humans. Numerous genetic and epidemiological studies have provided conclusive evidence that levels of circulating LDL-cholesterol (LDLc) are strongly and positively correlated with atherosclerosis progression and higher risk of major cardiovascular events [36,50]. Therefore, management of cholesterol levels is essential for the prevention and treatment of cardiovascular disease [5].

In humans, > 70% of the total clearance of plasma LDL occurs through the LDLR in the liver [47]. Cell surface LDLR binds LDL particles through ApoB100, the sole protein constituent of LDL, and the complexes are internalized through clathrin-dependent endocytosis. At the acidic pH of endosomes, the LDL particle dissociates from the receptor and is delivered to lysosomes where it is hydrolyzed into amino acids, fatty acids and free cholesterol. The LDLR can escape lysosomal degradation and is recycled to the cell surface for another cycle. The intracellular LDL-derived cholesterol suppresses the activation of sterol regulatory element-binding proteins (SREBPs) and blunts transcription of LDLR and enzymes involved in cholesterol biosynthesis [10].

A single copy of mutant *LDLR* gene can result in dysfunctional LDLR and cause heterozygous familial hypercholesterolemia (FH), the most common autosomal dominant disorder, which affects about 1 in 200–250 individuals and even more in certain populations [8].

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Mutations in *ApoB100* or in *proprotein convertase subtilisin/kexin type 9* (*PCSK9*), a natural inducer of LDLR degradation, are the two other known causes of FH. FH heterozygotes, which inherit one mutant *LDLR* allele, have a 50% deficiency of LDLR and exhibit a two- to five-fold increase in plasma LDLc from birth. These individuals are at high risk of heart attacks as early as ~30 years of age. In the United States and Western Europe, about 5% of all individuals who have a myocardial infarction before age 60 are FH heterozygotes [8]. The receptor defect impairs the catabolism of LDL, and the resultant elevation in plasma LDLc promotes deposition of cholesterol in coronary arteries and premature atherosclerosis [13]. Homozygous FH, which is very rare and a much more severe form of the disease, affect ~1 in 1 million people worldwide. FH homozygotes often develop skin and tendon xanthomas by age 10, and untreated people rarely live beyond age 30.

The expression of LDLR is regulated at the transcriptional, post-transcriptional and post-translational levels. At the transcriptional level, *LDLR* gene expression is mainly controlled by the transmembrane transcription factor SREBP-2 in response to the accumulation or depletion of intracellular cholesterol [22,29]. In cholesterol-depleted cells, SREBP-2 is transported from the endoplasmic reticulum (ER) to the Golgi complex where it undergoes two proteolytic cleavages. A cytosolic N-terminal fragment, representing the active basic helix-loop-helix transcription factor, is released and enters the nucleus to activate transcription of genes encoding all the enzymes of cholesterol biosynthesis as well as the LDLR. In cholesterol-rich cells, the transport of SREBP-2 to the Golgi complex is blocked and the transcription of the target genes declines, thus preventing cholesterol overload. This mechanism is essential for the action of statin drugs in lowering plasma LDLc levels in individuals at risk for coronary heart disease. Ingested statin drugs are routed primarily to the liver and lower cholesterol production through 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibition. This decrease in liver cholesterol activates SREBP-2 processing as described above and increase the number of LDLR displayed on liver cell membranes [17].

At the post-translational level, PCSK9 and the inducible degrader of LDLR (IDOL) are involved in the lysosomal degradation of LDLR through different mechanisms [40,60]. PCSK9 binds to the LDLR-EGF-A domain at the cell surface and, following endocytosis of the complex, inhibits LDLR endosomal recycling thereby targeting the receptor for lysosomal degradation. IDOL-mediated LDLR degradation is activated by the sterol-sensitive nuclear liver X receptor (LXR). LXR ligands induce the transcription of IDOL, an E3 ubiquitin ligase, which triggers ubiquitination of the cytosolic tail of LDLR and its lysosomal degradation.

The control of mRNA stability is a critical regulatory mechanism of gene expression [6]. However, little is known about the precise mechanism involved in post-transcriptional regulation of LDLR mRNA. Several studies including our own have shown that LDLR mRNA is degraded at a relatively rapid rate with a half-life of approximately 1–2 h in human hepatic HepG2 cells [24,41,57]. It has been previously reported that the plant alkaloid compound berberine can regulate LDLR gene expression through mRNA stabilization in an ERK1/2-dependent manner [24]. We recently showed that the DNA methylation inhibitor 5-Azacytidine (5-AzaC), an anti-cancer drug that has been approved for the treatment of patients with acute myeloid leukemia (AML) and myelodyplastic syndrome (MDS) [14], increases LDLR expression in HepG2 cells [41]. While the expression of several genes involved in lipid metabolism (*e.g.* PCSK9, HMG CoA reductase and SREBP-2) was strongly decreased, we showed that, independently of its DNA demethylating activity, 5-AzaC selectively stabilized LDLR mRNA and greatly increased its protein expression level.

Previous studies showed that stability of rapidly degraded mRNA is controlled through the 3′-untranslated region (3′UTR) that contains AU-rich elements (AREs) [11]. In LDLR, three AREs, named ARE1, 2 and 3, were identified in the proximal 3′UTR based on their similarity to the nonameric motif UUAUUUAUU. Insertion of only one copy of this element into a heterologous fusion mRNA significantly accelerates fusion mRNA turnover and multiple overlapping copies are even more potent at destabilizing the mRNA [56,62]. It was shown that insertion of the ARE1 sequence of LDLR-3′UTR into a β-globin fusion mRNA increased its turnover rate by 3-fold, whereas constructs with all three AREs have a 10-fold higher mRNA turnover rate [56]. Several ARE-binding proteins (ARE-BP) have been shown to regulate the fate of target ARE-containing mRNAs [1,21]. Some ARE-BP, such as the ubiquitously expressed RNA-binding protein HuR, bind and stabilize ARE-containing mRNAs [1]. In contrast, others, including tristetraprolin and KH-type splicing regulatory protein, are decay promoting factors that interact with AREs and recruit the mRNA degradation machinery [15].

In this study, we sought to elucidate the mechanism by which 5-AzaC up-regulates LDLR expression in human hepatocytes. Our results show that 5-AzaC-induced LDLR mRNA stabilization is triggered by the activation of the inositol-requiring enzyme 1α (IRE1α) kinase domain independently of its RNase domain and of ER stress. IRE1α kinase activity recruited a signaling pathway resulting in the activation of the epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinase1/2 (ERK1/2), which induced LDLR mRNA stabilization. Our data demonstrate that LDLR-3′UTR ARE1 is required for 5-AzaC-induced mRNA stabilization *via* the IRE1α-EGFR-ERK1/2 signaling cascade.

2. Results

2.1. 5-AzaC-induced upregulation of hepatic LDLR expression requires ERK1/2 activation

We previously observed that treatment with 5-AzaC lead to increased LDLR mRNA and protein levels in human hepatic cell lines [41]. Therefore, herein we sought to determine the mechanism(s) leading to the increase in LDLR expression. Treatment of HepG2 and Huh7 cell lines with 10 μM 5-AzaC for 24 h increased LDLR protein levels by ~3-fold (Fig. 1A, upper and lower panels), which is correlated with significant stabilization of its mRNA (Fig. 1B). Similar to the alkaloid compound berberine, which stabilizes LDLR mRNA through an ERK-dependent pathway [24], increased LDLR protein levels following treatment with 5-AzaC were concomitant to the activation of ERK1/2 (Fig. 1A, upper and lower panels). Our results also showed that 5-AzaC increases LDLR and p-ERK1/2 expression in a time-dependent manner and that ERK1/2 activation occurred 2 h before the increase in LDLR protein level (Fig. 1C, upper and lower panels). Confocal immunofluorescence microscopy showed that 5-AzaC increased intracellular and cell surface LDLR in both Huh7 and HepG2 cell lines compared with DMEM treated cells (control; Fig. 1D). In comparison with berberine, 5-AzaC induced a greater increase in both LDLR and p-ERK1/2 protein expression; in both cases, pretreatment with the MEK1/2 inhibitor U0126 abolished this increase (Fig. 1E).

2.2. EGFR and JNK signaling pathways are involved in LDLR upregulation

Phorbol 12-myristate 13-acetate (PMA) is a direct activator of protein kinase C and a potent activator of ERK1/2 signaling [54]. However, incubation of Huh7 cells with PMA, which transiently activated ERK1/2, did not increase LDLR protein level (Supplementary Fig. S1A and S1B). In addition, a previous study reported that 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), an activator of AMP kinase, stabilizes LDLR mRNA *via* an ERK-dependent pathway in HepG2 cells [57]. After 24 h of treatment in Huh7 cells, AICAR and 5-AzaC increased ERK1/2 phosphorylation to similar levels; however, AICAR failed to increase LDLR protein expression (Supplementary Fig. S1C). In the same vein, inhibition of AMP kinase activity with Compound C [61] had no effect on the 5-AzaC-induced increase in LDLR protein expression (Supplementary Fig. S1D).

ERK1/2 signaling can be increased when a specific receptor tyrosine