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MK-2206, an allosteric inhibitor of AKT, stimulates LDLR expression and LDL uptake: A potential hypocholesterolemic agent

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ABSTRACT

Background and aims: Induction of low-density lipoprotein receptor (LDLR) plays a significant role in reduction of plasma LDL-cholesterol (LDL-C) levels. Therefore, strategies that enhance the protein level of LDLR provide an attractive therapeutic target for the treatment of hypercholesterolemia. With this aim in mind, we concentrated our effort on studying the role of AKT kinase in regulation of LDLR levels and proceeded to examine the effect of MK-2206, an allosteric and highly selective AKT inhibitor, on LDLR expression.

Methods: Cultured human hepatoma cells were used to examine the effect of MK-2206 on the proteolytic processing of sterol regulatory element-binding protein-2 (SREBP-2), the expression of LDLR and cellular internalization of LDL. We also examined the effect of MK-2206 on LDLR levels in primary human hepatocytes.

Results: MK-2206 induced the proteolytic processing of SREBP-2, upregulated LDLR expression and stimulated LDL uptake. In contrast to statins, induction of LDLR levels by MK-2206 did not rely on 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) inhibition. As a result, cotreatment of cells with MK-2206 and mevastatin potentiated the impact of mevastatin on LDLR. Importantly, MK-2206 stimulated the expression of LDLR by primary human hepatocytes.

Conclusions: MK-2206 is a novel LDLR-inducing agent that, either alone or in combination with statins, exerts a stimulating effect on cellular LDL uptake.

1. Introduction

A major risk factor for development of cardiovascular disease (CVD) is elevation of plasma levels of low-density lipoprotein cholesterol (LDL-C) [1,2]. The maintenance of plasma cholesterol homeostasis is primarily carried out by the liver through its ability to internalize LDL-C by LDL receptor (LDLR)-mediated endocytosis [3,4]. Thus, an increase in hepatic LDLR expression results in reduction of plasma LDL-C levels.

Expression of LDLR is mainly controlled at the level of transcription by the sterol regulatory element-binding protein-2 (SREBP-2) whose activity is regulated by a negative feedback loop in response to the intracellular cholesterol levels [5]. Precursor SREBP-2 is sequestered in the endoplasmic reticulum (ER) in a mulitprotein complex that includes sterol regulatory element-binding protein cleavage-activating protein (SCAP) and insulin-induced genes (INSIGs). Upon depletion of intracellular cholesterol, SREBP-2, in conjunction with SCAP, is transported to the Golgi apparatus where it undergoes two sequential proteolytic cleavages to release its N-terminal transactivation-competent domain [6]. Once inside the nucleus, this mature form of SREBP-2 binds to its cognate sterol regulatory element-1 (SRE-1) site in the promoter of *LDLR* gene and activates its transcription [7,8].

Results from a number of studies suggest that, in addition to sterols, AKT signaling also regulates the activity of SREBPs by a diverse set of mechanisms, including alteration of SREBPs gene transcription, protein maturation or protein stability. For instance, while activation of the mTORC1 branch of AKT pathway induces both *SREBP-1* gene expression and SREBP-1 processing, inhibition of the GSK-3β arm of AKT signaling prevents degradation of nuclear SREBP-1 [9–11]. While most research have focused on the relationship between AKT and SREBP-1, a few studies have examined the effect of AKT signaling on SREBP-2 activity and the results have been conflicting. For example, whereas activation of SREBP-2 by

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insulin-like growth factor-1 or cholesterol-depletion has been reported to depend on AKT activity [12,13], another study has failed to find a link between AKT activation and SREBP-2 processing [14].

The AKT kinase family comprises three related and differentially expressed isoforms, AKT1, AKT2 and AKT3, that act as molecular hubs to link both extracellular and intracellular stimuli to various cellular processes such as cell proliferation, apoptosis and metabolism [15]. It is therefore not surprising that aberrant AKT activity underlies the pathophysiological properties of a variety of human diseases [15]. Accordingly, multiple AKT inhibitors, including MK-2206, are currently being explored in clinical trials [16]. MK-2206 is an orally active and highly selective allosteric inhibitor of all AKT isoforms that binds in a cavity formed at the interface of the catalytically active kinase domain and the regulatory pleckstrin homology domain, locking the kinase in a closed, inactive conformation [17–19]. MK-2206 has been examined as mono- or combination-therapy in a number of clinical trials [20–23]. The results of these studies show that MK-2206, although modest in its ability to achieve the desired clinical antitumor activity, is well tolerated.

In the course of our research on LDLR regulatory mechanisms, we found that MK-2206 affects the expression of LDLR, prompting us to focus our attention on the relationship between MK-2206 and LDLR. Here, we show that MK-2206 activates SREBP-2 and exerts an LDLR-inducing and LDL- C-lowering effect in a manner that is independent of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) inhibition, endoplasmic reticulum (ER) stress or apoptosis. Furthermore, we show that MK-2206 augments the effect of mevastatin on LDLR levels. Based on these results, we believe that further research is warranted to examine the feasibility and potential of using MK-2206 as a hypocholesterolemic drug.

2. Materials and methods

2.1. Reagents and antibodies

MK-2206 2HCl and mevastatin were from Selleckchem (Houston, Texas). Actinomycin D (Act D), cycloheximide (CHX), 25-hydroxycholesterol (25-HC) and (±)-mevalonolactone (which turns to mevanolate in water) were obtained from Sigma-Aldrich (St. Louis, MO). LDLR antibodies (3839; for Western blot analysis) and (61087; for detection of cell-surface LDLR by flow cytometry) were purchased from BioVision (Milpitas, CA) and Progen (Heidelberg, Germany), respectively. Antibodies against the N-terminus of SREBP-2 (ab30682) and β-actin (ab8227) were purchased from Abcam (Cambridge, UK). Antibodies directed against the C-terminus of SREBP-2 (557037) were obtained from BD Biosciences (San Jose, CA). Anti-GAPDH (Glyceraldehyde 3-phosphate dehydrogenase; G9295) was purchased from Sigma-Aldrich. Antibodies against pAKT (S473; AF887), AKT (MAB2055), pPRAS40 (T246; MAB6890) and PRAS40 (MAB6408) were obtained from R&D Systems (Minneapolis, MN). Anti-β-tubulin (T9154-05G) was purchased from USBiological (Swampscott, MA). Anti-pAKT1 (S473; 9018) and anti-HA (ab18181) were obtained from Cell Signaling (Danvers, MA) and Abcam (Cambridge, UK), respectively.

2.2. Cell culture and treatment

HepG2 and HeLa cells (European Collection of Cell Cultures, Salisbury, UK), human telomerase reverse transcriptase (hTERT)-immortalized human hepatocytes (IHH; provided by Dr. Philippe Collas, University of Oslo, Oslo, Norway), SV40 large T antigen-immortalized mouse hepatocytes (IMH; obtained from Dr. Angela M. Valverde [Instituto de Investigaciones Biomedicas Alberto Sols, Madrid, Spain] were cultured on collagen-coated culture vessel (BD Biosciences, San Jose, CA) in HyClone Minimum Essential Medium (GE Healthcare Life Sciences, Pittsburg, PA) containing 10% fetal bovine serum (FBS; Sigma-Aldrich) and non-essential amino acids (Biowest, Nuaillé, France). Chinese hamster ovary (CHO) cells (American Type Culture Collection, Manassas, VA) were maintained in Ham's F-12 medium (Biowest) supplemented with 10% FBS. UT-2 cells (a gift from Dr. Joseph L. Goldstein, University of Texas Southwestern Medical Center, Dallas, TX) were cultured in the same media as CHO cells and supplemented with 0.2 mM mevalonate. Hepac1c7 cells (provided by Dr. Jørn Andreas Holme, Norwegian Institute of Public Health, Oslo, Norway) were cultured in MEM Alpha medium without Nucleosides (Thermo Fisher Scientific, Waltham, MA) containing 10% FBS. For culture of cells in the absence of exogenous lipoproteins, cells were first grown for 24 h in complete medium containing 10% FBS and then washed twice with phosphate-buffered saline (PBS) before being provided with complete medium supplemented with 5% lipoprotein-deficient serum (LPDS). All media were supplemented with 2 mM L-glutamine Sigma- Aldrich), 50 U/ml penicillin and 50 μg/ml streptomycin (GE Healthcare Life Sciences). Freshly plated human hepatocytes were obtained from QPS Hepatic Biosciences (Research Triangle Park, NC). Upon arrival, Storage Medium was replaced with Fresh Maintenance Medium supplemented with 0.1% dexamethasone (QPS Hepatic Biosciences). After a 24 h acclimatization in a cell culture incubator, cells were exposed to reagents for 14 h before harvesting for Western blot analysis. To inhibit HMGCR activity without affecting prenylation of proteins, cells were incubated with a mixture of mevastatin and 20 μmol/L mevalonate. All cells were grown in monolayer cultures in 5% CO_2 at 37 °C. All drugs were added in dimethyl sulfoxide (DMSO) with a constant DMSO concentration of 0.1% (v/v). To control for possible DMSO effects, control samples were treated with DMSO alone at final concentrations of 0.1%.

2.3. Cell fractionation

HepG2 cells were harvested by trypsinization and pellets were washed in PBS before incubation in hypotonic buffer (10 mM Tris [pH 7.6], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) for 15 min on ice. Cells were then lysed by 25 passages through a 25-gauge needle and centrifuged at 1000 × *g* for 10 min at 4 °C to collect the nuclei. The nuclei pellet was resuspended in 100 μl RIPA buffer (for SREBP-2 detection; 50 mM Tris [pH 7.5], 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5 mM EDTA, 10 mM NaF, 5 mM β-glycerophosphate, 0.1 mM Na_3VO_4 and Complete Protease Inhibitor Cocktail [Sigma- Aldrich]) and the suspension was incubated on ice for 40 min with intermittent vortexing at 2000 rpm and then centrifuged at 20,000 × *g* for 30 min at 4 °C. The recovered supernatant was designated as nuclear extract. The supernatant recovered from the 1000 × *g* centrifugation was spun at 20,000 × *g* for 30 min at 4 °C to pellet membranes. The membrane pellet was then resuspended in 100 μl RIPA and incubated on ice for 40 min with intermittent vortexing at 2000 rpm to extract membrane proteins and then clarified by centrifugation at 20,000 × *g* for 30 min at 4 °C. The supernatant was designated as the membrane fraction.

2.4. Western blot analysis

For detection of SREBP-2, 25 μg of nuclei extracts or membrane fractions were separated on a 10% SDS-PAGE. For detection of other proteins, cells were lysed in Triton X-100 lysis buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 1% Triton X-100, 10 mM EDTA and Complete Protease Inhibitor Cocktail) and separated on a 4–20% SDS-PAGE. After transfer to PVDF membrane (Bio-Rad, Hercules, California), proteins were detected by use of standard immunoblotting

procedures. For plotting of the results, the immunoblots were scanned and the intensity of the target protein was normalized to that of the internal loading control (GAPDH, β-actin or β-tubulin). The obtained values were then plotted relative to vehicle-treated values or the values obtained from cells at time 0, which were set at 1.

2.5. Quantitative real-time PCR

Total RNA was purified using the QIAamp RNA Isolation Kit (Qiagen, Hilden, Germany). cDNA was synthesized with the AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA). Quantitative real-time PCR (qPCR) was performed using Brilliant III Ultra-Fast QPCR Master Mix on Mx3005P QPCR system (Agilent technologies). The assay id of the PrimeTime Predesigned qPCR Assays (Integrated DNA Technologies, Coralville, Iowa) are: *GAPDH* (Hs.PT.39a.22214836); *HMGCR* (Hs.PT.58.41105492); *LDLR* (Hs.PT.58.14599757), proprotein convertase subtilisin/kexin type 9 (*PCSK9*; Hs. PT.58.203171419) and transferrin receptor (*TFRC*; Hs. PT.39a.22214826). The experiments were carried out in duplicate for each data point. The housekeeping gene *GAPDH* was used for normalizing target mRNA expression. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.6. Analysis of cell-surface LDLR and LDL internalization by flow cytometry

For detection of the amount of cell-surface LDLR expression, HepG2 cells were harvested using Non-enzymatic Cell Dissociation Solution (Sigma-Aldrich), washed twice with Staining Buffer (PBS + 1% BSA) and incubated with anti-LDLR (1:20 dilution in Staining Buffer; Progen) at room temperature for 40 min. Cells were then washed three times and incubated with Alexa Fluor 647-conjugated anti-mouse (1:600 dilution in Staining Buffer; Abcam) at room temperature for 30 min in the dark. After antibody incubation, cells were washed twice with Staining Buffer, resuspended in PBS and analyzed on a FACS Canto flow cytometer (BD Biosciences) for quantification of Alexa Fluor 647 fluorescence. To measure LDLR internalization activity, human LDL was isolated and labelled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD; Invitrogen, Carlsbad, CA) as previously described [24]. Cells were incubated with DiD-LDL (10 μg/ml) at 37 °C for 2 h. At the end of the incubation period, cells were harvested and washed three times with PBS containing 0.5% BSA before analysis by flow cytometry.

2.7. Plasmid constructs, transfection and reporter assays

pcDNA3.1-HA-V5/His plasmid was constructed by inserting a synthetic oligonucleotides duplex encoding an HA tag between the *Kpn*I and *Bam*HI sites of pcDNA3.1-V5/His (A) (Thermo Fisher Scientific). pcDNA3.1-HA-AKT1-WT-V5/His was constructed by subcloning the *Bam*HI-*Eco*RI fragment from pcDNA3-T7-AKT1 (a gift from Dr. William Sellers; Addgene plasmid #9003) into the corresponding sites of pcDNA3.1-HA-V5/His. The pcDNA3.1-HA-AKT1-DD-V5/His mutant was generated using the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) to mutate the ACC and TCC codons encoding T308 and S473, respectively, in pcDNA3.1-HA-AKT1- WT-V5/His to GAC to create Asp codons. Both pcDNA3.1-HA-AKT1-WT-V5/His and pcDNA3.1- HA-AKT1-DD-V5/His encode HA-tagged proteins without V5/His tag at their C-termini due to presence of a stop codon located at the end of AKT coding sequences. pLR1563-luc was a gift from Dr. Youngmi Kim Pak (Asian Institute for Life Sciences, University of Ulsan College of Medicine, Seoul, Korea) [25]. To construct pLR1563/mutSRE-1-luc, CC (underlined) in the *LDLR* promoter SRE-1 motif (ATCACCCCAC) in pLR1563-luc was replaced with GG using QuickChange II XL mutagensis kit (Agilent Technologies). pcDNA3.1-2xFLAG-SREBP-2 (Addgene plasmid #26807) was a gift from Dr. Timothy Osborne (Molecular Biology and Biochemistry, University of California, Irvine, CA) [26]. For transfection with expression vectors, cells were transfected with 312 ng DNA/cm^2 of culture vessel growth surface area at a 4.5:1 (for HepG2) or 3.0:1 (for HeLa) FuGENE HD transfection reagent:DNA ratio following the manufacturer's instructions (Promega, Madison, WI). To monitor the effect of transfection process, control cells were transfected with empty vector. For gene knockdown studies, HepG2 cells were reverse transfected during plating with 24 nmol/L gene-specific or non-targeting (*NT*) AllStars negative siRNA (Qiagen) siRNA using Lipofectamine RNAiMAX (Thermo Fisher Scientific) with cells at 70% confluency. Drug treatments were performed at 24 h post-transfection. For measurement of *LDLR* promoter activity, HepG2 cells were transfected with pRL-SV40 vector (Renilla luciferase as internal control; Promega) and pLR1563-luc or pLR1563/mutSRE-1-luc at a 1:10 ratio. Cells were subjected to drug treatment at 24 h post-transfection, and then harvested for analysis of reporter gene activities by Dual-Luciferase Reporter Assay (Promega) following the manufacturer's instructions.

2.8. Measurement of cellular cholesterol synthesis

HepG2 cells were first cultured in complete growth medium containing 5% LPDS for 24 h. The medium was then replaced with fresh medium supplemented with 0.2 μCi/ml [^{3}H]acetate (PerkinElmer, Waltham, MA). After 1 h, cells were treated with reagents and incubated for further 24 h. Cells were then washed twice with PBS and harvested by trypsinization. The cell pellet was mixed in 0.5 ml methanol and 1 ml hexane and incubated for 1 h with agitation at room temperature. The cellular lipid extract in the upper phase was evaporated to dryness, resuspended in 30 μl chloroform and then resolved by thin-layer chromatography (TLC) on silica gel 60-coated glass plates (Z292974, Sigma-Aldrich) using a 1:1 (v/v) mixture of diethyl-ether and hexane as the mobile phase. A solution of 10 μg/ml cholesterol (Sigma-Aldrich) was used as standard. After staining the TLC plate with iodide vapor, the migrated spots were scraped off and then analyzed for the amount of radioactivity by liquid scintillation.

2.9. Statistical analysis

An unpaired, two-tailed Student's *t*-test was used to determine the significance of differences between the means of two independent groups.

3. Results

3.1. MK-2206 increases the expression of LDLR

The role of AKT in activation of SREBP-2 and thus the expression of its target gene, LDLR, remains controversial, as there is conflicting evidence suggesting that AKT activity either has no effect on or is required for activation of SREBP-2[12–14]. We therefore considered it important to investigate the relationship between AKT and the SREBP-2/LDLR axis. As a first step towards this aim, we decided to focus on the effect of AKT inhibition on LDLR expression. For this purpose, we utilized MK-2206, an allosteric and highly selective pan-AKT inhibitor [17], and for a cellular model system for expression of LDLR, we used the human liver-derived cell line HepG2. We first cultured HepG2 cells in the presence of either FBS or LPDS for 24 h (hereafter referred to as sterol-fed and sterol-

starved, respectively). Sterol-starved cells have been shown to contain lower cholesterol levels relative to sterol-fed cells [27,28]. Cells were then exposed to various concentrations of MK-2206, harvested after 14 h and examined for the expression of LDLR protein. Fig. 1A shows that MK-2206 induced LDLR expression in a dose-responsive manner with 5 and 10 μmol/L of MK-2206 exhibiting a maximal effect on LDLR protein levels in sterol-starved and sterol-fed cells, respectively. Above 10 μmol/L, the LDLR-inducing

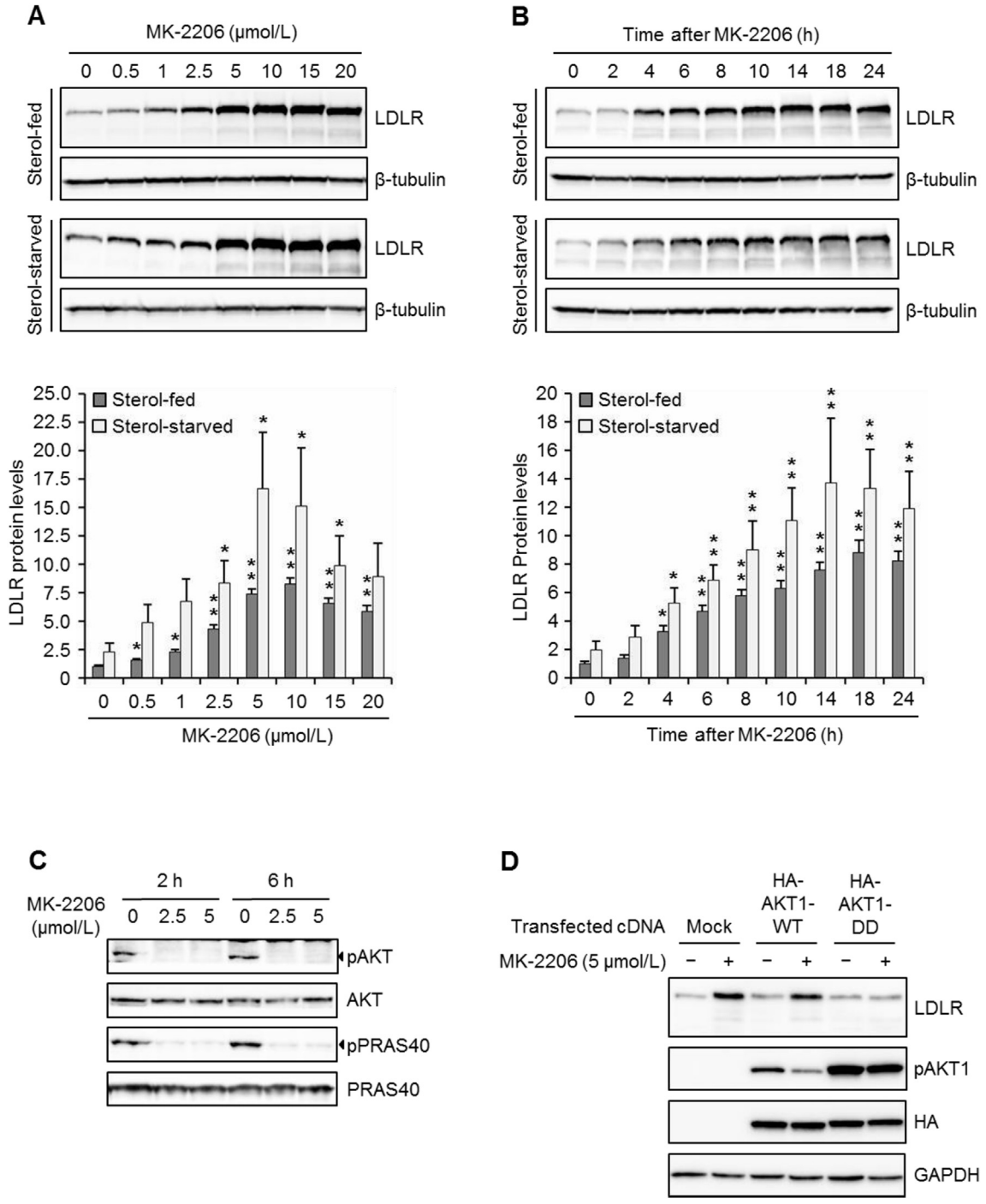

Fig. 1. MK-2206 induces the expression of LDLR regardless of intracellular cholesterol levels.
(A) HepG2 cells were treated with the indicated doses of MK-2206 for 14 h and then examined by Western blotting. Upper panel: representative blot from seven experiments. Lower panel: Western blot data were quantified and plotted relative to the value from vehicle-treated, sterol-fed cells. (B) HepG2 cells were cultured as in A, treated with 5 μmol/L MK-2206 and then harvested at the indicated time points for immunoblot analysis. Upper panel: representative experiment of seven. Lower panel: Western blot data were quantified and plotted relative to the value from vehicle-treated, sterol-fed cells that were harvested at 0 h. Error bars represent SD. One asterisk and two asterisks indicate $p < 0.05$ and $p < 0.01$, respectively, compared with matched vehicle-treated cells (A) or matched cells harvested at 0 h (B). (C) Sterol-fed HepG2 cells were treated with the indicated concentrations of MK-2206 and harvested at the indicated times for immunoblot analysis. One representative blot is shown (n = 3). pAKT, phosphorylated AKT; pPRAS40, phosphorylated PRAS40. (D) Sterol-fed HeLa cells were transfected with either empty vector, pcDNA3.1-HA-AKT1- WT-V5/His or pcDNA3.1-HA-AKT1-DD-V5/His. At 24 h after transfection, cells were exposed to MK-2206 for 14 h before harvesting for Western blot analysis. pAKT, phosphorylated AKT. One representative blot is shown (n = 3).

effect of MK-2206 was mitigated presumably as a result of the cytostatic effect of such high concentrations of MK-2206. Examination of the kinetics of the MK-2206-mediated induction of LDLR showed that 5 μmol/L MK-2206 significantly induced LDLR levels within 4 h (Fig. 1B). LDLR reached its maximum levels in sterol-starved and sterol-fed cells at 14 and 18 h post-treatment, respectively, followed by a slight decline by 24 h. In the remainder of the experiments of this study, cells were incubated with 5 μmol/L MK-2206 for 14 h unless otherwise noted. Importantly, MK-2206 upregulated LDLR levels at the cell surface and induced DiD-LDL uptake in both sterol-fed and sterol-starved cells (Fig. 2A–B). Finally, examination of the effect of MK-2206 on LDL uptake by LDLR-knockdowned cells showed that induction of LDLR is the causative factor in MK- 2206-mediated upregulation of LDL uptake (Supplementary Fig. 1). Collectively, these results suggest that MK-2206 increases LDLR protein levels and activity under both normal and sterol-deficient conditions.

To validate that induction of LDLR by MK-2206 is mediated by inhibition of AKT, we first examined the effect of MK-2206 on phosphorylation of AKT at S473 and its downstream target protein, PRAS40, as indicators of AKT kinase activity [15]. As shown in Fig. 1C, a concentration as low as 2.5 μmol/L of MK-2206 inhibited phosphorylation of AKT and PRAS40 within 2 h, showing that MK-2206 potently inhibits AKT activity in HepG2 cells. Next, we assessed the effect of MK-2206 on LDLR levels in HeLa cells that overexpressed HA-tagged AKT1-T308D/S473D (AKT1-DD) mutant. Substitution of T308 and S473 with aspartic acid generates a phosphomimetic AKT1 that is locked into a constitutively active state, thus rendering the kinase refractory to the action of MK-2206[15]. As expected, cells expressing HA-tagged AKT1-WT displayed decreased phosphorylation of the transgene concomitant with elevated levels of LDLR (Fig. 1D). In contrast, MK-2206 failed to induce the expression of LDLR in cells that expressed the constitutively active mutant of AKT1. This result indicates that MK-2206 increases LDLR levels through inhibition of AKT kinase.

To examine whether the MK-2206-mediated induction of LDLR protein is reflected at the mRNA level, we measured *LDLR* mRNA levels by qPCR and found that MK-2206 induced the expression of *LDLR* mRNA in both sterol-fed and sterol-starved HepG2 cells (Fig. 2C and Supplementary Fig. 2). Furthermore, comparison of the expression profiles of LDLR protein (Fig. 1B) with those of *LDLR* mRNA (Fig. 2C and Supplementary Fig. 2) showed that there was a correlation between both the magnitude and the kinetics of changes in LDLR protein and *LDLR* mRNA levels after treatment of cells with MK-2206. This result, together with the observation that MK-2206 had no significant stabilizing effect on *LDLR* mRNA (Fig. 2D), suggested that MK-2206-mediated induction of LDLR protein was the result of enhanced *LDLR* gene transcription.

The rapid induction of *LDLR* mRNA within 2 h after MK-2206 treatment suggested that it might occur independently of *de novo* protein synthesis. To examine this idea, sterol-fed HepG2 cells were pretreated with the protein synthesis inhibitor cycloheximide (CHX) for 1 h before addition of MK- 2206 and then harvested after 5 h for examination of LDLR protein and *LDLR* mRNA levels. Inhibition of protein synthesis, as judged by the inability of MK-2206 to induce LDLR protein levels in the presence of CHX, had no effect on MK-2206-mediated induction of *LDLR* mRNA (Fig. 2E). This result indicates that MK-2206-induced *LDLR* transcription does not require new protein synthesis.

Finally, examination of the effect of MK-2206 on LDLR in a panel of both hepatic and non-hepatic cell lines from different species showed that MK-2206 exerts its LDLR-inducing effect in a non-cell type-specific manner (Figs. 2F–3B).

3.2. MK-2206 stimulates the expression of LDLR through a mechanism distinct from that of statins

Statins induce the expression of LDLR as a result of their ability to block the endogenous cholesterol production. To examine whether MK-2206 utilizes a mechanism similar to that of statins to upregulate LDLR levels, we first examined the effect of MK-2206 on endogenous cholesterol synthesis. Fig. 3A shows that MK-2206 inhibited cholesterol biosynthesis, albeit with significantly less efficiency when compared to mevastatin or 25-hydroxycholesterol (25-HC). Given the potent LDLR-inducing effect of MK-2206, this observation implied the possibility that MK-2206 might regulate the expression of LDLR in a manner that is independent of the HMGCR activity and cholesterol biosynthesis. To assess the validity of this suggestion, we examined the effect of MK-2206 on the expression of LDLR protein in UT-2 cells, a mutant clone of CHO cells that is deficient in HMGCR and thus, in contrast to CHO cells, should be resistant to the LDLR-inducing effect of HMGCR inhibition [29]. As shown in Fig. 3B, MK-2206 induced the expression of LDLR in both CHO and UT-2 cells. These results shows that MK-2206 affects the expression of LDLR independent of HMGCR activity.

The distinction between the mechanisms by which MK-2206 and statins increase the expression of LDLR suggested that MK-2206 might exert a complementary effect on statin-induced upregulation of LDLR. To test this hypothesis, sterol-starved HepG2 cells were exposed to the individual and combination of MK-2206 and mevastatin and examined for the expression of LDLR by immunoblotting. The results in Fig. 3C clearly showed that combination of MK-2206 and mevastatin markedly increased LDLR levels as compared with treatment with either agent alone.

3.3. MK-2206 stimulates the cleavage of SREBP-2 and increases the LDLR promoter activity

To determine whether the inducing effect of MK-2206 is restricted to *LDLR* or extends to other genes, we examined the effect of MK-2206 on the expression of two sets of genes: 1) *PCSK9* and a number of cholesterogenic genes that are co-regulated with *LDLR*, and 2) *SREBP-1c*, *IDOL*, *ACACA*, *FASN* and *SCD1* whose patterns of expression differ from that of *LDLR*. As shown in Fig. 4A and S3, MK-2206 induced the expression *PCSK9*, *HMGCR*, *SREBP-2* and *HMGCS1* mRNAs, indicating that it affects the expression of genes that are controlled by a regulatory mechanism similar to that of *LDLR*. In contrast, whereas MK-2206 had no effect on *IDOL* mRNA levels, it only modestly induced the expression of *ACACA*, *FASN* and *SCD1* mRNAs with a statistically significant increase observed at 8 h post-treatment (Supplementary Fig. 4). The delayed induction kinetics of *ACACA*, *FASN* and *SCD1* mRNAs (compare Supplementary Fig. 4 with 4A and Supplementary Fig. 3) suggests that the regulation of these genes occurs as a secondary response to MK-2206. The induction of *HMGCR* mRNA by MK-2206 (Fig. 4A) suggests that MK- 2206-treated cells exhibit an increased cholesterol *de novo* synthesis, a conjecture that is contrasted by the observation that MK-2206 inhibits cholesterol biosynthesis (Fig. 3A). To resolve this apparent inconsistency, we examined the effect of MK-2206 on HMGCR protein expression. As shown in Supplementary Fig. 5, MK-2206 reduced HMGCR levels in an MG-132-sensitive manner. This result provides a potential explanation for the inhibitory effect of MK-2206 on *de novo* cholesterol synthesis.

To verify our aforementioned suggestion that MK-2206 stimulates the transcription of *LDLR* gene, sterol-fed HepG2 cells that were transfected with the human *LDLR* promoter reporter construct, pLR1563-luc, were treated with MK-2206 and then analyzed for luciferase activity. Consistent with its ability to increase the expression of *LDLR* mRNA, MK-2206 strongly induced

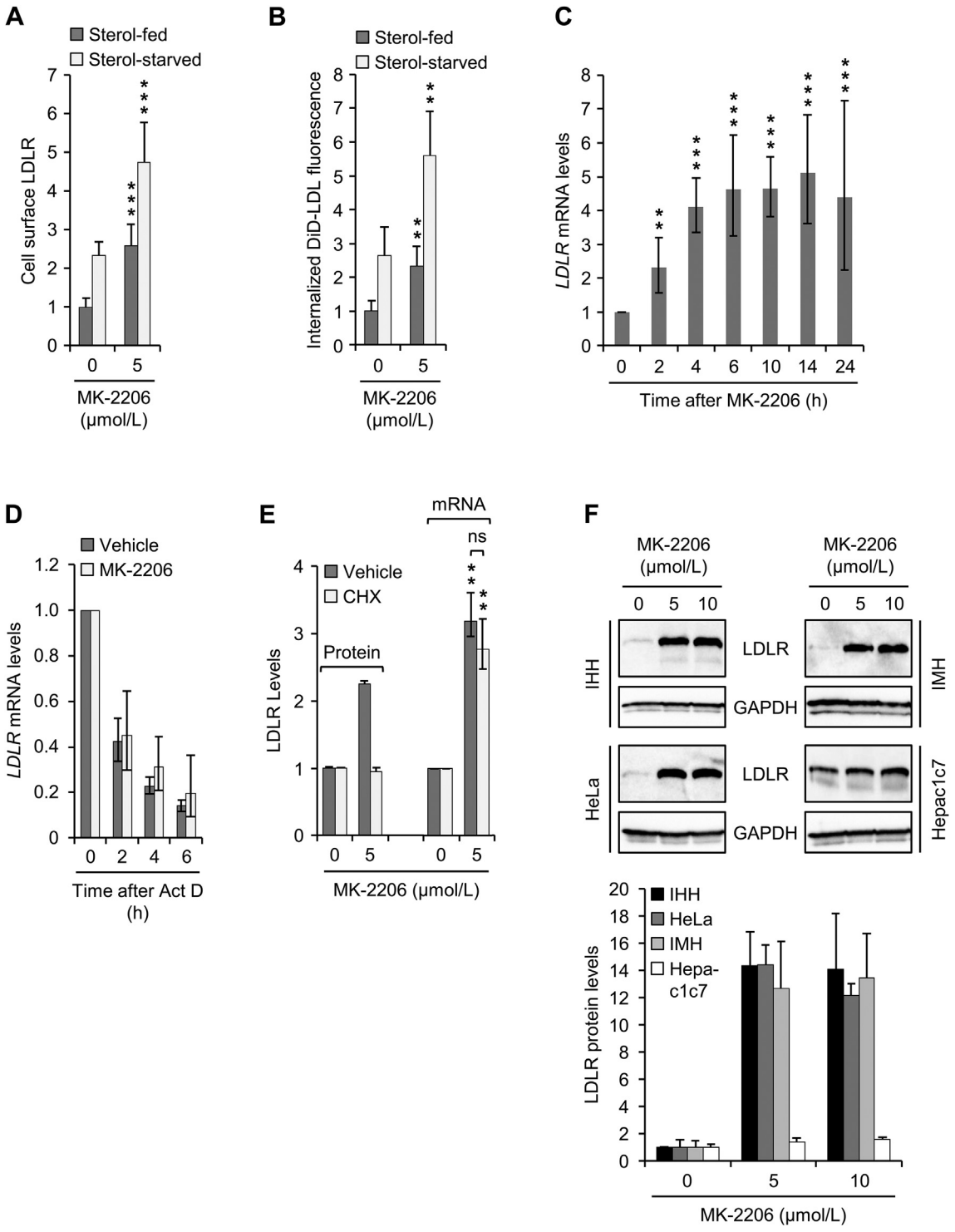

Fig. 2. Induction of LDLR activity by MK-2206.
(A) HepG2 cells were exposed to MK-2206 and then harvested after 14 h for flow cytometric analysis of mean fluorescence intensity as an indicator of cell-surface LDLR expression. Results were then plotted relative to vehicle-treated, sterol-fed cells (n = 6). (B) HepG2 cells were cultured and treated as in A. Cells were then exposed to DiD-LDL (10 μg/ml) for the last 2 h of treatment before harvesting for flow cytometric analysis of the mean fluorescent intensity of internalized DiD-LDL. Results were then plotted relative to vehicle-treated, sterol-fed controls (n = 5). (C) Sterol-fed HepG2 cells were treated with or without 5 μmol/L MK- 2206 before harvesting at the indicated times for determination of *LDLR* mRNA levels by qPCR. *LDLR mRNA* levels were then plotted relative to the value obtained at time 0 (n = 5). (D) Sterol-fed HepG2 cells were treated with vehicle or 5 μmol/L MK-2206 for 12 h before treatment with actinomycin D (Act D; 5 μg/ml). Cells were harvested at the indicated time points after Act D treatment for determination of *LDLR* mRNA levels by qPCR. *LDLR* mRNA levels were plotted relative to respective vehicle-treated controls (n = 4). (E) Sterol-fed HepG2 cells were preincubated with 10 μg/ml cycloheximide (CHX) for 1 h before treatment with vehicle or MK-2206 for 5 h. After harvesting the cells, each sample was split in two. Lysates prepared from one half of each sample were analyzed by immunoblotting. The immunoblots data were plotted relative to matched vehicle-treated controls (n = 3). The other halves of samples were processed for determination of *LDLR* mRNA levels by qPCR. *LDLR* mRNA levels were plotted relative to those obtained from matched vehicle-treated cells. ns, not significant. (F) Upper panel: sterol-fed immortalized human hepatocytes (IHH), HeLa, immortalized mouse hepatocytes (IMH) and Hepac1c7 cells were treated with vehicle or the indicated concentrations of MK-2206 for 14 h before analysis by immunoblotting. One representative experiment of three is shown. Lower panel: Western blot data were quantified and plotted relative to the value from vehicle-treated cells. mRNA data are displayed with error bars representing the 95% confidence interval. Otherwise, error bars represent SD. Two and three asterisks indicate $p < 0.01$ and $p < 0.001$, respectively, compared with matched vehicle-treated cells (A, B and E) or matched cells harvested at 0 h (C).

the *LDLR* promoter activity (Fig. 4B). Importantly, mutational inactivation of the sterol regulatory element-1 (SRE-1) [30] in LDLR promoter led to not only a reduction in basal luciferase expression but also loss of MK-2206- induced transactivation activity (Fig. 4B). Thus, MK-2206 stimulates *LDLR* promoter activity in a manner that is dependent on functional SRE-1. This conclusion is further supported by the observation that MK-2206 induces the expression of other SRE-regulated genes (Fig. 4A and S3). Because SRE-1 is the binding site for SREBP-2[8], this result implied that MK-2206 is dependent on SREBP-2 to induce the expression of *LDLR*. To examine this notion, we silenced SREBP-2 expression in HepG2 cells with *SREBP-2*-specific siRNAs and examined them for the expression of *LDLR* mRNA in response to MK-2206 treatment. Knockdown of *SREBP-2* expression (Supplementary Fig. 6) significantly diminished the *LDLR*-inducing effect of MK-2206 (Fig. 4C). This result suggests that SREBP-2 mediates the effect of MK-2206 on *LDLR* gene expression.

Proteolytic cleavage of SREBP-2 in the Golgi apparatus produces two products: the transactivation-competent N-terminal fragment (hereafter "NTF-SREBP-2") that upon entering the nucleus activates the transcription of its target genes, and the C-terminal fragment (hereafter "CTF- SREBP-2") that remains associated with the Golgi [31]. The observation that MK-2206 requires the activity of SREBP-2 to induce the expression of *LDLR* predicted the possibility that MK-2206 may stimulate the proteolytic cleavage of SREBP-2 and thereby increase the level of NTF-SREBP-2. We evaluated this prediction by immunoblotting the membrane fractions and nuclear extracts from sterol-fed HepG2 cells that were treated with MK-2206 with anti-SREBP-2 antibodies. Western blot analysis of the membrane fractions with an antibody directed against the C-terminal region of SREBP-2 and the nuclear extracts with an anti-N-terminal SREBP-2 antibody revealed that MK-2206 increases the amounts of both CTF-SREBP-2 and NTF-SREBP-2 with a concomitant reduction in full-length (FL)- SREBP-2 levels (Fig. 4D). This result showed that MK-2206 stimulates the proteolytic cleavage of FL-SREBP-2. Once in the nucleus, NTF-SREBPs are rapidly degraded by the ubiquitin-proteasome system [32]. Therefore, MK-2206 could be assumed to induce the amount of NTF-SREBP-2 in the nucleus by not only enhancing FL-SREBP-2 processing but also through increasing its stability. To address this possibility, HepG2 cells transiently transfected with a FLAG-tagged NTF-SREBP-2 (2xFLAG-SREBP-2) were treated with MK-2206 and then examined by immunoblotting with anti- FLAG antibodies. As shown in Fig. 4E, MK-2206 did not affect the amount of exogenously expressed NTF-SREBP-2, suggesting that MK-2206 does not alter the stability of NTF-SREBP-2. Taken together, these results show that MK-2206 capacitates the cells for increased expression of LDLR by enhancing the processing of nascent SREBP-2 and thereby increasing the amount of NTF-SREBP-2 in the nucleus.

Apart from its activation in a sterol-dependent manner, SREBP-2 has been shown to be proteolytically activated under ER stress or apoptotic conditions independently of intracellular cholesterol content [33–36]. Therefore, we felt it important to examine whether MK-2206-induced processing of SREBP-2 and the subsequent upregulation of LDLR occurs as a result of induction of ER stress or apoptosis. To this end, CHO and HepG2 cells were treated with various concentrations of MK-2206 and then analyzed for the splicing of *XBP1* mRNA and PARP cleavage as indicators of ER stress and apoptosis, respectively [37–39]. As shown in Supplementary Fig. 7 and 8, MK-2206 did not induce ER stress or apoptosis. Thus, neither ER stress nor apoptosis accounts for the MK-2206-mediated activation of SREBP-2 and induction of LDLR. Furthermore, the data in Supplementary Fig. 8 and 9 show that MK-2205 does not appreciably affect the viability of HepG2 cells under the experimental condition used.

3.4. MK-2206 increases LDLR expression in primary hepatocytes

To examine whether MK-2206 also induced the expression of LDLR in primary cells, we treated primary adult human hepatocytes with various concentrations of MK-2206 or 10 μmol/L mevastatin and then examined them for the expression of LDLR protein by Western blotting. As expected, mevastatin induced the expression of LDLR (Fig. 5). Importantly, similar to its effect on the cell lines used in this study, MK-2206 exerted a stimulating effect on the expression of LDLR by primary human hepatocytes.

4. Discussion

This study was initiated to investigate the potential role of AKT in regulation of LDLR expression. Here, we show that MK-2206, an allosteric inhibitor of AKT, enhances the cellular uptake of LDL-C through induction of *LDLR* mRNA and LDLR protein. Furthermore, our results show that although MK-2206-mediated induction of LDLR requires the activity of SREBP-2, it occurs independently of intracellular cholesterol status, a property that distinguishes MK-2206 from statins. Based on the rapid kinetics of MK-2206-induced induction of *LDLR* and the observation that it occurs in a protein synthesis-independent manner, we propose that MK-2206 circumvents the dependency on low intracellular cholesterol levels to induce the expression of *LDLR* by triggering a signaling cascade that promotes the activation of SREBP-2. Thus, this property of MK-2206 enables it to upregulate LDLR even when the expression of LDLR is subject to sterol negative feedback regulation.

The observation that MK-2206 activates SREBP-2 and induces the expression of LDLR was an unexpected finding that is in disagreement with the notion that AKT activity is required for activation of SREBP-2 and induction of LDLR levels [12,13,40]. By contrast, our finding is in line with the result of Portsmann et al. showing a lack of relationship between activation of AKT and SREBP-2 processing [14]. This, together with the high selectivity of MK-2206 for AKT [17] and the abrogation of the LDLR-inducing effect of MK-2206 by AKT1-DD expression, prompts us to speculate that allosteric inhibition rather than stimulation of AKT promotes the activation of SREBP-2.

Our results demonstrate that MK-2206 increases the amount of nuclear SREBP-2 by enhancing the proteolytic processing of nascent SREBP-2, in a manner that is independent of HMGCR inhibition, ER stress or apoptosis. A plausible mechanism for this effect of MK-2206 would be that MK-2206 facilitates incorporation of SREBP-2-SCAP into COPII-coated vesicles, thus enhancing the export of nascent SREBP-2 from ER to the Golgi for proteolytic cleavage. MK-2206-induced degradation of INSIG proteins could account for increase in loading of SREBP-2-SCAP into COPII-coated vesicles. However, because induction of *LDLR* mRNA within 2 h after MK-2206 treatment indicates a rapid processing and activation of nascent SREBP-2, we favor the possibility that MK-2206 might influence the conformation of SCAP or INSIGs through a post-translational modification mechanism causing SREBP-2-SCAP to dissociate from INSIGs. We would like to emphasize that elucidation of MK- 2206-triggered molecular events that culminate in activation of SREBP-2 is of paramount importance as a detailed understanding of this signaling cascade may allow its modulation for the purpose of induction of LDLR.

AKT activity is crucial for normal functioning of several cellular processes, including, but not limited to, insulin signaling [15]. Therefore, it can be assumed that prolonged exposure to MK-2206 might produce serious adverse effects, such as hyperglycemia. This assumption is justified by the results of clinical trials with MK-2206 in cancer patients, showing incidence of hyperglycemia with a rate of approximately 9–30% [21,22,41,42]. Interestingly, this MK-2206-

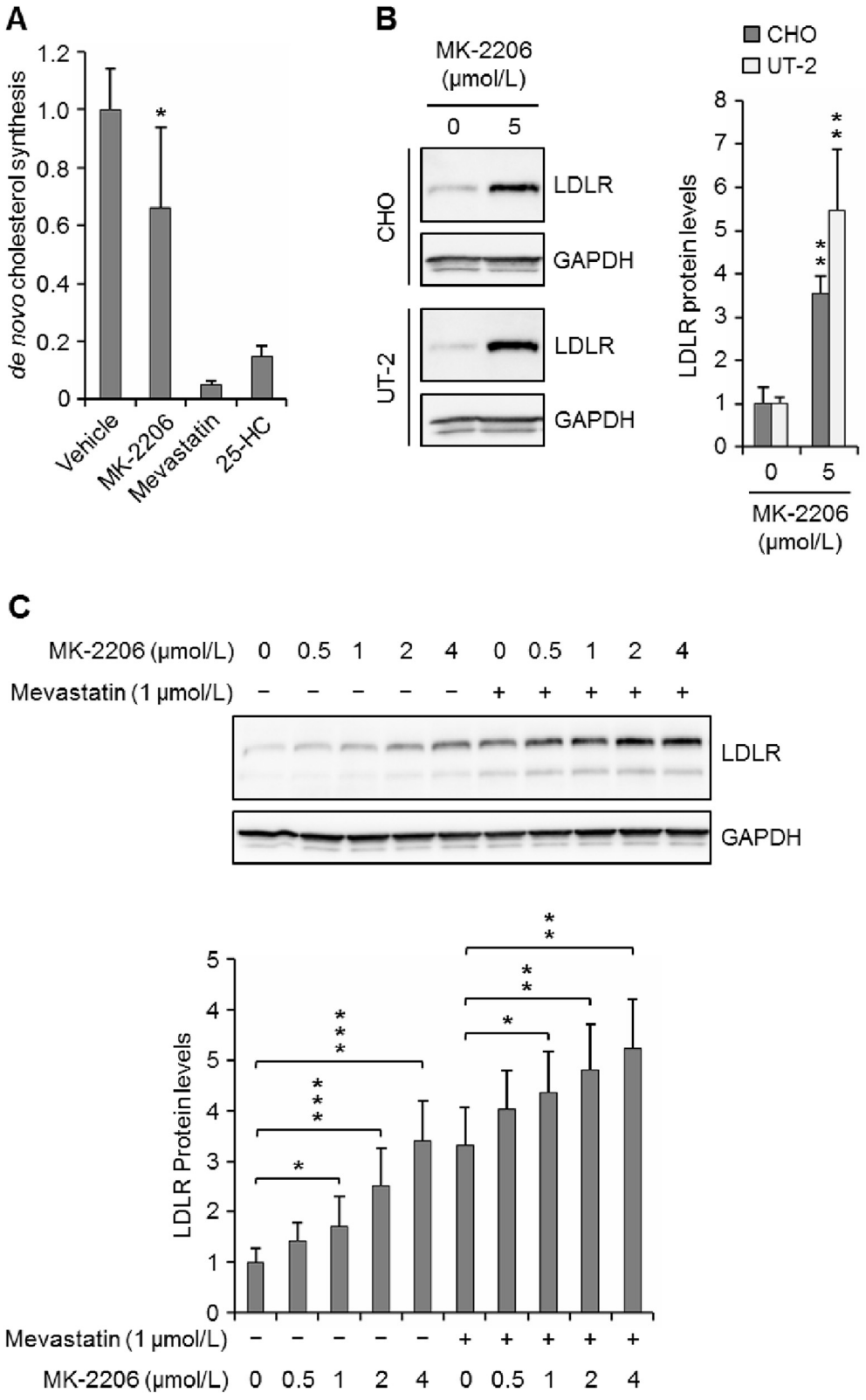

Fig. 3. MK-2206 increases the expression of LDLR independent of the cholesterol biosynthesis pathway and improves the LDLR-inducing effect of mevastatin.
(A) Sterol-starved HepG2 cells were treated with vehicle, 5 μmol/L MK-2206, 10 μmol/L mevastatin or 10 μmol/L 25- hydroxycholesterol (25-HC) for 14 h before processing for determination of cholesterol biosynthesis as described in materials and methods. The obtained values were then plotted relative to the value for vehicle-treated cells (n = 4). (B) CHO and UT-2 were treated with MK-2206 for 14 h before harvesting and analysis by immunoblotting. Left panel shows one representative blot (n = 3). Right panel: the immunoblots data were plotted relative to matched vehicle-treated controls. (C) Sterol-starved HepG2 cells were treated with vehicle or mevastatin for 24 h before exposure to the indicated concentrations of MK-2206 for a further 14 h. Cells were then harvested and subjected to Western blot analysis. Upper panel shows one representative blot from six experiments. Lower panel: the immunoblots data were plotted relative to vehicle-treated cells. Error bars represent SD. One, two and three asterisks indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, compared with matched vehicle-treated cells (A and B).

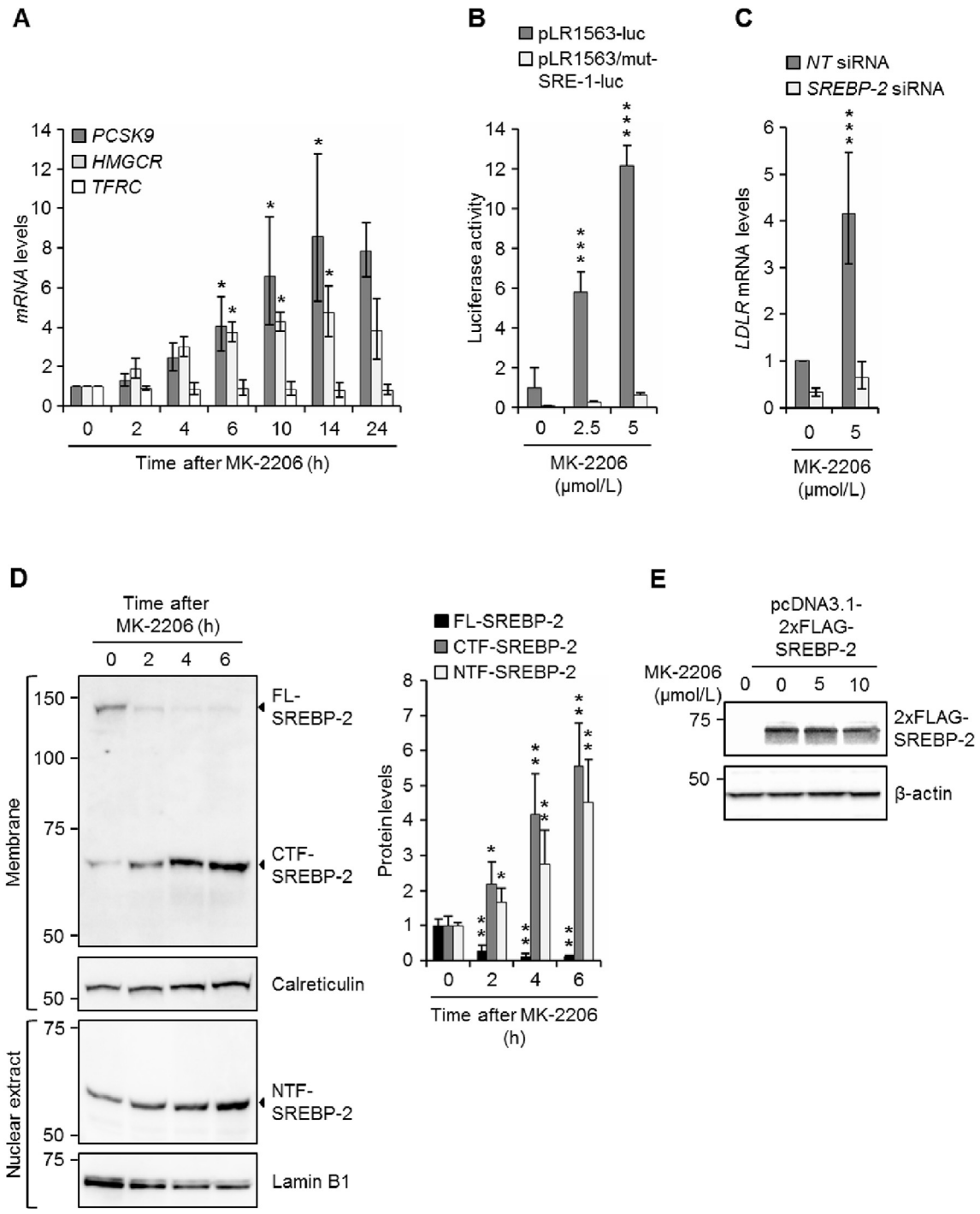

Fig. 4. MK-2206-mediated induction of LDLR requires SREBP-2.
(A) Sterol-fed HepG2 cells were treated with 5 μmol/L MK-2206 before harvesting at the indicated times for determination of *PCSK9, HMGCR* and *TFRC* mRNA levels by qPCR. The obtained values were then plotted relative to the values of respective controls harvested at time 0 (n = 3). (B) Sterol-fed HepG2 cells were transfected with either wt (pLR1563-luc) or mutant (pLR1563/mutSRE-1-luc) *LDLR* promoter and Renilla luciferase reporter. At 24 h after transfection, cells were treated with the indicated concentrations of MK-2206 for 14 h and then harvested for analysis of *LDLR* promoter activity. The plot shows the average of data pooled from four independent experiments relative to the value obtained from vehicle-treated cells that were transfected with pLR1563-luc. (C) Sterol-fed HepG2 cells were transfected with a non-targeting siRNA (*NT* siRNA) or an SREBP-2-specific siRNA. Cells were treated with MK-2206 at 24 h post-transfection and then harvested after 14 h for determination of *LDLR* mRNA levels by qPCR. *LDLR* mRNA levels were plotted relative to those obtained from vehicle-treated, *NT* siRNA-transfected cells (n = 4). (D) Left panel: sterol-fed HepG2 cells were treated with 5 μmol/L MK-2206 for the indicated time points, harvested and subjected to subcellular fractionation before being analyzed by Western blotting. Membrane fractions were blotted with an antibody raised against the C-terminus of SREBP-2 and anti-calreticulin. Nuclear extracts were blotted with an antibody directed against the N-terminus of SREBP-2 and anti-lamin B1. FL-SREBP-2, full-length SREBP-2; CTF-SREBP-2, C-terminal fragment of SREBP-2; NTF-SREBP-2, N-terminal fragment of SREBP-2. One representative blot of three is shown. Right panel: the western blots were analyzed by densitometry to obtain FL-SREBP-2/calreticulin, CTF-SREBP-2/calreticulin and NTF/lamin B1 ratios. The obtained values were then plotted relative to the values of respective controls harvested at time 0. mRNA data are displayed with error bars representing the 95% confidence interval. Otherwise, error bars represent SD. One, two and three asterisks indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, compared with matched vehicle-treated cells (B and C) or matched cells harvested at 0 h (A and D). (E) Sterol-fed HepG2 cells were transfected with vector encoding the 2xFLAG-tagged N-terminal domain of SREBP-2 (pcDNA3.1-2xFLAG-SREBP-2). At 24 h post-transfection, cells were treated with MK-2206 for 14 h and then harvested for analysis by Western blotting with anti-FLAG and anti-β-actin antibodies. One representative blot is shown (n = 3).

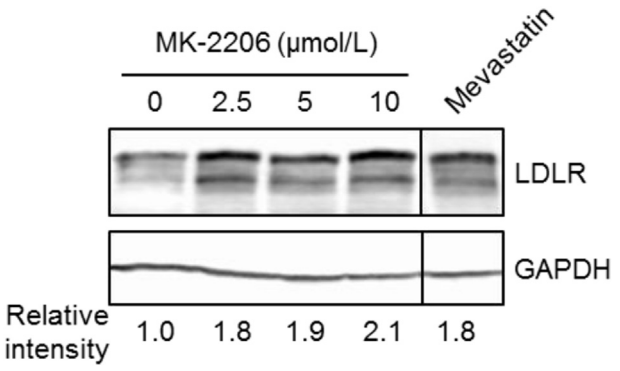

Fig. 5. MK-2206 induces the expression of LDLR in primary human hepatocytes. Primary human hepatocytes were treated with vehicle, MK-2206 or 10 μmol/L mevastatin for 14 h before analysis by Western blotting. After quantification by densitometry, the LDLR/GAPDH ratio was calculated to obtain the LDLR band relative intensity. One representative experiment of two is shown.

related hyperglycemia was mainly mild and transient. Nonetheless, while appreciating the concern over the suitability of MK- 2206 as a potential therapeutic option for hypercholesterolemia, we believe that the results from clinical studies supporting the favorable safety profile of MK-2206 [21,23,43], together with the results presented in this study, merit *in vivo* studies to examine whether concentrations of MK-2206 that exert a cholesterol-lowering effect, both singularly and in combination with statins, elicit any side effects, and if so, whether they are of such severity that overwhelm the therapeutic effect of MK-2206.

Conflicts of interest

SN is listed as inventor on a patent application relating to MK-2206. The other authors do not have anything to disclose.

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Author contributions

KB designed the research, performed experiments, analyzed data, and contributed to the writing of the manuscript. HS performed experiments. TPL helped with data review and writing of the manuscript. SN provided the concept, designed the research, performed experiments, analyzed data, and wrote the first draft and the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.atherosclerosis.2018.07.009.

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