Protein kinase C activation stabilizes LDL receptor mRNA via the JNK pathway in HepG2 cells[S]

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Abstract LDL is the most abundant cholesterol transport vehicle in plasma and a major prognostic indicator of atherosclerosis. Hepatic LDL receptors limit circulating LDL levels, since cholesterol internalized by the liver can be excreted. As such, mechanisms regulating LDL receptor expression in liver cells are appealing targets for cholesterol-lowering therapeutic strategies. Activation of HepG2 cells with phorbol esters enhances LDL receptor mRNA levels through transcriptional and posttranscriptional mechanisms. Here, we show that 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-induced stabilization of receptor mRNA requires the activity of protein kinase C and is accompanied by activation of the major mitogen activated protein kinase pathways. Inhibitor studies demonstrated that receptor mRNA stabilization is independent of the extracellular signal-regulated kinase or p38^{MAPK}, but requires activation of the c-Jun N-terminal kinase (JNK). An essential role for JNK in stabilizing receptor mRNA was further confirmed through small interfering RNA (siRNA) experiments and by activating JNK through two protein kinase C-independent mechanisms. Finally, prolonged JNK activation increased steady-state levels of receptor mRNA and protein, and significantly enhanced cellular LDL-binding activity. These data suggest that JNK may play an important role in posttranscriptional control of LDL receptor expression, thus constituting a novel mechanism to enhance plasma LDL clearance by liver cells.—Vargas, N. B., B. Y. Brewer, T. B. Rogers, and G. M. Wilson. **Protein kinase C activation stabilizes LDL receptor mRNA via the JNK pathway in HepG2 cells. *J. Lipid Res.* 2009. 50: 386–397.**

Supplementary key words signal transduction • mitogen-activated protein kinase • lipoprotein metabolism • mRNA turnover • gene expression

Mammalian cells require cholesterol for the synthesis of membranes, steroid hormones, and bile salts. Cholesterol is principally synthesized in the liver, but is packaged into serum lipoproteins for transport to peripheral tissues (1). LDL is the most abundant cholesterol-carrying vehicle in human plasma, and extensive epidemiological evidence maintains that elevated plasma LDL levels are a major risk factor for atherosclerosis, myocardial infarction, and related mortality (2–4). Cellular LDL uptake is mediated by the LDL receptor, an integral plasma membrane glycoprotein that is expressed in all cell types but most abundantly in the liver (5), where internalized cholesterol may be excreted either directly or after metabolic conversion into bile acids (6, 7). The importance of hepatic LDL receptors in systemic cholesterol excretion is exemplified by patients suffering from familial hypercholesterolemia, an autosomal dominant disorder whereby one or both LDL receptor alleles do not encode functional receptors (as reviewed in Refs. 1, 4, 8). Introduction of functional hepatic LDL receptors to homozygous familial hypercholesterolemia patients by liver transplantation (9) or ex vivo gene therapy (10) significantly lowers plasma cholesterol in these individuals and improves prognosis. Hepatic LDL receptor expression is also enhanced in patients taking statin drugs, which inhibit de novo cholesterol synthesis (as reviewed in Ref. 11). Since cholesterol synthesis is restricted principally to the liver, these compounds deplete intracellular cholesterol in this tissue, which in turn enhances transcription of the LDL receptor gene through activation of the sterol-regulatory element binding protein (SREBP) family of transcription factors (12). These examples demonstrate that therapeutic strategies that increase the expression of functional LDL receptors in liver cells improve LDL clearance from the circulation, which in turn

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Abbreviations: actD, actinomycin D; ARE, AU-rich element; BIM, bis-indolylmaleimide I; DiI-LDL, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-labeled human LDL; ERK, extracellular signal-regulated kinase; IL-1β, interleukin-1β; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MK2, mitogen-activated protein kinase-activated protein kinase 2; PKC, protein kinase C; qRT-PCR, quantitative RT-PCR; siRNA, small interfering RNA; SREBP, sterol-regulatory element binding protein; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TTP, tristetraprolin; UTR, untranslated region.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary material in the form of one figure and two tables.

slows atherosclerotic development and reduces the risk of coronary heart disease.

The regulation of hepatic LDL receptor expression has been extensively studied using the highly differentiated human hepatocarcinoma cell line HepG2 (13). In this cell line, LDL receptor levels are suppressed in response to cholesterol and lipoprotein loading (14). However, expression of LDL receptors is also regulated through several signal transduction pathways in HepG2 cells, including the cyclic AMP, diacylglycerol-protein kinase C (PKC), and mitogen-activated protein kinase (MAPK) pathways (15–17). Recent studies have probed the relationships between these signaling cascades in the control of receptor expression. Treating HepG2 cells with the phorbol ester 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) potently but transiently induces accumulation of LDL receptor mRNA, involving transcriptional activation of the receptor gene (18) and stabilization of its encoded mRNA (15). Induction of receptor gene transcription by TPA appears to require the α isoform of PKC and is principally mediated through the p42/44 extracellular signal-regulated kinase (ERK) signaling pathway (18). Stabilization of receptor mRNA by TPA requires sequences in the distal 3′ untranslated region (3′UTR), possibly involving elements related to the *Alu* repetitive sequence that are located in this domain of the receptor transcript (19). Recent studies also report that LDL receptor mRNA stability is enhanced in HepG2 cells following treatment with the herbal alkaloid berberine through activation of the ERK pathway, involving sequences in the proximal 3′UTR of the receptor mRNA (20).

The objective of this study was to characterize intracellular signaling pathways controlling the decay kinetics of LDL receptor mRNA. Using TPA-treated HepG2 cells as a model system, we show that stimulation of PKC stabilizes receptor mRNA concomitant with activation of the three major MAPK pathways. Unlike the inhibition of LDL receptor mRNA decay induced by berberine, stabilization of this transcript following PKC activation was not influenced by chemical inhibitors of either the ERK or $p38^{MAPK}$ pathways. Rather, experiments using selective MAPK inhibitors and small interfering RNA (siRNA) show that activation of the c-Jun N-terminal kinase (JNK) pathway is required for stabilization of receptor mRNA in response to TPA. Finally, LDL receptor mRNA was also stabilized by activation of JNK independent of PKC, leading to accumulation of receptor mRNA and protein in HepG2 cells and increased cellular LDL-binding activity. Together, these findings identify a novel mechanism to enhance the production of functional hepatic LDL receptors, but they also highlight some important distinctions between posttranscriptional mechanisms controlling the expression of LDL receptors in this cultured liver cell model.

EXPERIMENTAL PROCEDURES

Materials

Rabbit polyclonal antibodies targeting ERK, phospho-ERK, $p38\alpha^{MAPK}$, phospho-$p38\alpha^{MAPK}$, JNK, phospho-JNK, phospho-c-Jun, and phospho-mitogen-activated protein kinase-activated protein kinase 2 (MK2) were from Cell Signaling. Rabbit polyclonal antibodies against GAPDH and chicken polyclonal antibodies recognizing LDL receptor were from Abcam. Horseradish peroxidase-conjugated secondary antibodies were from SIGMA (goat anti-rabbit IgG) and Abcam (rabbit anti-chicken IgY). TPA, actinomycin D (actD), and kinase inhibitors bis-indolylmaleimide I (BIM), SP600126, SB202190, and U0126 were from Calbiochem. Interleukin-1β (IL-1β) was from R&D Systems. Tissue culture media and trypsin were from GIBCO/InVitrogen, while FBS was from Atlanta Biologicals. Lipoprotein-depleted FBS, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate-labeled human LDL (DiI-LDL), and unlabeled human LDL were from Biomedical Technologies. DNA amplification primers and probes for quantitative RT-PCR (qRT-PCR) were from Integrated DNA Technologies and siRNA duplexes from Dharmacon Research. Adenoviruses used for infection of HepG2 cells were a replication deficient human adenovirus type 5 mutant (Ad-dl312; control), and a constitutively active mutant of MKK7 (Ad-MKK7D) (21). Recombinant viruses were prepared, amplified, purified, and titered as described previously (22, 23).

Cell culture and phosphoprotein analysis

HepG2 cells were obtained from the American Type Culture Collection and maintained in MEM supplemented with 10% FBS in a humidified atmosphere containing 5% CO_2 at 37°C. To screen for phosphoproteins activated or deactivated following TPA treatment, whole-cell extracts were prepared from untreated (control) or TPA-induced (160 nM, 1 h) HepG2 cell monolayers (3×10^7 cells/sample) by washing once in 1 × phosphate buffered saline, then scraping cells directly in ice-cold lysis buffer (20 mM HEPES [pH 7.4] containing 2 mM EGTA, 5 mM EDTA, 0.5% IGEPAL-CA630, 10 μM leupeptin, 5 μM pepstatin A, 1 mM PMSF, 30 mM sodium fluoride, 20 mM disodium pyrophosphate, and 1 mM sodium orthovanadate). Soluble proteins were recovered following centrifugation at 100,000 *g* for 30 min. Relative levels of selected phosphoproteins were analyzed using the Kinetworks Phospho-Site Screen 1.3 service by Kinexus (Vancouver, Canada). There, Western blots of each extract were probed with a panel of phospho-specific antibodies. TPA-dependent changes in the level of each tested phosphoprotein were determined semiquantitatively by comparison of immunoblot band intensities.

LDL receptor mRNA decay assays

The decay kinetics of LDL receptor mRNA was measured in drug/virus-treated or control HepG2 cells using actD time-course assays. Briefly, global transcription was inhibited by addition of actD (5 μg/ml) to the culture medium. At selected time points thereafter, total RNA was purified using TRIzol reagent (InVitrogen) according to the manufacturer's instructions. Time courses were limited to 4 h to avoid complicating cellular mRNA decay pathways by actD-enhanced apoptosis (24). Levels of LDL receptor and GAPDH mRNAs were concomitantly measured in each time-course sample by multiplex, qRT-PCR using the iScript One-Step RT-PCR Kit for Probes (Bio-Rad) and primers/probes listed in supplementary Table I. Relative LDL receptor mRNA concentrations were calculated by comparison of threshold cycle numbers (C_t) to standard curves and normalized to endogenous GAPDH mRNA levels. GAPDH mRNA does not significantly decay over the 4 h actD treatment described here (15). Each data point was taken as the mean ± standard deviation from triplicate qRT-PCR reactions for each RNA sample. First-order decay constants (k) were solved by nonlinear regression (PRISM v3.03, GraphPad) of the percentage of LDL receptor mRNA remaining vs. time of actD treatment. Tabulated LDL receptor mRNA decay constants

are based on the mean ± standard deviation of n independent time-course experiments where $n \geq 3$, or the mean ± spread where $n = 2$, permitting pair-wise statistical comparisons as described below.

Western blotting

Whole-cell extracts of HepG2 cells were prepared by washing cell monolayers with phosphate buffered saline followed by scraping in 2 x SDS-PAGE buffer (250 mM Tris [pH 6.8] containing 2% SDS, 10% glycerol, and 0.05% bromophenol blue). Lysates were incubated at 100°C for 5 min, then clarified by centrifugation at 12,000 *g* for 10 min. Clarified lysates were then fractionated through 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes (Whatman) and blocked with 5% nonfat milk in Tris buffered saline containing 0.1% Tween-20 for 1 h at room temperature. Blocked membranes were incubated overnight at 4°C with primary antibodies as indicated and washed. Following 1-h incubations with peroxidase-conjugated secondary antibodies, the immunoblots were washed and developed using the Super Signal West Pico Chemiluminescent detection method (Pierce).

siRNA and adenoviral transfections

For siRNA transfections, HepG2 cells were seeded in 24-well plates at 10^5 cells/well. After 24 h, siRNA cocktails targeting JNK1 + JNK2 or an irrelevant control siRNA (50 nM total siRNA/well in 200 μl volume; siRNA sequences listed in supplementary Table I) were transfected into HepG2 cells using Dharmafect 1 reagent (Dharmacon) following the manufacturer's instructions. To maximize suppression of JNK expression, a subsequent round of siRNA transfection (JNK1/2 or control) was performed 24 h following the first. Assays to measure LDL receptor mRNA decay kinetics and cellular levels of selected MAP kinase proteins or phosphoproteins were performed 48 h after the second round of siRNA transfection.

A constitutively active mutant of the JNK-activating enzyme MKK7 (Ad-MKK7D) was expressed by replication-defective adenoviral transduction into HepG2 cells. For these experiments, HepG2 cells were seeded in 6-well plates at 5×10^5 cells/well. After 24 h, control (Ad-dl312) or MKK7D-expressing (Ad-MKK7D) adenoviruses were added to HepG2 monolayers (500 viral particles/cell) in serum-free MEM. After 1.5 h at 37°C, an equal volume of MEM containing 10% FBS was added to each culture. Cells were incubated at 37°C for 72 h posttransfection prior to analysis of LDL receptor expression/function or JNK activation.

LDL binding/uptake assay

The assay to measure specific binding and uptake of LDL by HepG2 cells was modified from previously published techniques (25, 26). Briefly, HepG2 cells were plated in 24-well plates at 10^5 cells/well in MEM containing 10% FBS. After 24 h, cells were infected with adenoviral-encoded MKK7D or control virus as described above. Seventy-two h postinfection, cells were washed with PBS before adding MEM containing 10% lipoprotein-depleted FBS and 10 μg/ml DiI-LDL lacking or containing 500 μg/ml unlabeled LDL (300 μl/well). Cells were incubated with LDL mixtures for 2 h at 37°C and then placed on ice. Nonbinding lipoproteins were removed by a rapid wash with ice-cold Tris buffered saline containing 2 mg/ml BSA, followed by two washes of 10 min each in the same solution on ice. Finally, cells were washed twice with Tris buffered saline alone before lysis in 150 μl RIPA buffer (25 mM TrisHCl [pH 7.6] containing 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS). Insoluble cellular material was removed by centrifugation at 16,000 *g* for 10 min. Retained DiI-LDL was measured by fluorescence (λ_{ex} = 540 nm; λ_{em} = 575 nm; 5 nm bandpass) using a Cary Eclipse fluorescence spectrophotometer (Varian) against a standard curve of DiI-LDL. Total protein concentrations were calculated for each sample using the BCA protein assay kit (Calbiochem) against a standard curve of BSA, permitting normalization of retained DiI-LDL to total cellular protein. Total bound DiI-LDL was measured from cells exposed to DiI-LDL alone, while cells incubated with DiI-LDL plus excess unlabeled LDL competitor revealed the extent of nonspecific DiI-LDL binding. Specific DiI-LDL binding was then resolved by subtracting nonspecific DiI-LDL binding from total measured DiI-LDL binding.

Statistics

Comparisons of LDL receptor mRNA levels, mRNA decay kinetics, or LDL-binding activities between cell populations were performed using the unpaired Student's *t*-test, with differences yielding $P < 0.05$ considered significant.

RESULTS

PKC activation stabilizes LDL receptor mRNA concomitant with activation of MAPK pathways

Previous studies indicated that the abundance and stability of LDL receptor mRNA were transiently enhanced in HepG2 cells following treatment with phorbol esters (15, 18), presumably involving activation of the PKC pathway. Using a multiplex qRT-PCR approach, we observed a similar rapid but transient induction of the LDL receptor transcript in this cell line following administration of TPA, with receptor mRNA levels increasing approximately 13-fold within 3 h, then returning to basal levels after 24 h incubation (**Fig. 1A**). LDL receptor mRNA decay kinetics was then assessed using actD time-course assays. These experiments revealed that TPA-dependent induction of LDL receptor expression also included stabilization of its mRNA, since the half-life of receptor mRNA increased from 1.1 h to 2.6 h after one h of TPA treatment (Fig. 1B and **Table 1**, $P < 0.0001$). The similarity of these findings relative to those quoted in previous reports validated the utility of the multiplex qRT-PCR approach employed in this study.

Phorbol esters are potent activators of the PKC pathway (27, 28) and can also interact with other intracellular targets (29). While activation of LDL receptor gene transcription following TPA treatment has been principally linked to the ERK pathway (18), mechanisms controlling the decay kinetics of receptor mRNA remain largely unknown. In order to identify potential signaling targets linking TPA stimulation to stabilization of receptor mRNA, lysates from control and TPA-stimulated HepG2 cells were surveyed for levels of 36 known phosphoproteins by Western blot (see supplementary Table II). Relative immunoblot band intensities suggested that phorbol ester-induced stabilization of LDL receptor mRNA was accompanied by activation of several MAPK pathways in HepG2 cells. Activation of the ERK pathway was previously noted (18) and is further supported by enhanced phosphorylation of the ERK-activating kinases, MEK1/2. Similarly, activation of the $p38^{MAPK}$ pathway was indicated by enhanced phosphorylation of $p38\alpha^{MAPK}$ and its activating kinases MEK3/6. The activity of a third

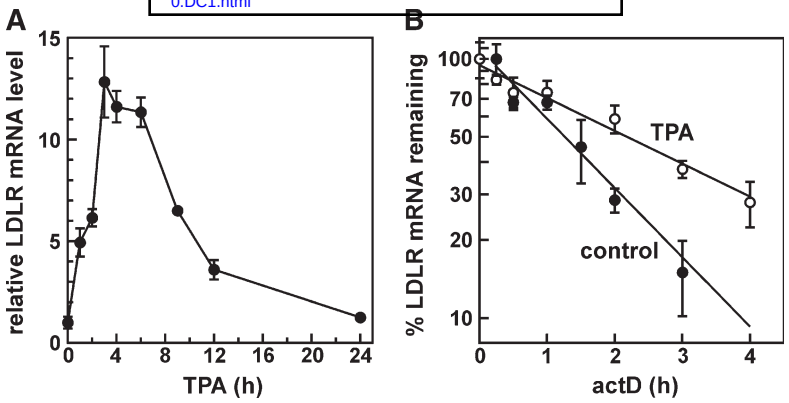

Fig. 1. Induction and stabilization of LDL receptor mRNA in HepG2 cells following treatment with 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). A: total RNA was isolated from HepG2 cells at selected time points following administration of TPA (160 nM) and analyzed for LDL receptor and GAPDH mRNA levels by multiplex quantitative RT-PCR (qRT-PCR). Points show the relative change in LDL receptor mRNA abundance normalized to GAPDH mRNA at each time point, based on the mean ± standard deviation of three independent qRT-PCR reactions. B: The decay rate of endogenous LDL receptor mRNA was measured in the absence (solid circles) or presence (open circles) of TPA (160 nM) by actinomycin D (actD) time-course assay as described under Experimental Procedures. TPA was added 1 h prior to actD where applicable. The fraction of LDL receptor mRNA remaining was plotted as a function of time following inhibition of transcription by actD. LDL receptor mRNA decay constants were resolved by nonlinear regression to a first-order decay model (lines). Average decay constants measured across replicate independent experiments are listed in Table 1.

member of the MAPK family, JNK, was also increased in HepG2 cells following TPA treatment, based on enhanced phosphorylation of both JNK and its downstream substrate, c-Jun. While the phosphorylation status of several other signaling molecules was also modulated in HepG2 cells treated with TPA, subsequent experiments focused principally on these MAPK pathways for two reasons. First, each of these signaling cascades has been linked to the regulated

TABLE 1. Regulation of LDL receptor mRNA decay kinetics in HepG2 cells

Cell Pretreatment	Kinase Target	Inducer[a]	k (h^{-1})[b]	$t_{1/2}$ (h)[c]	n
None	None	—	0.61 ± 0.01	1.1	3
None	None	TPA	0.26 ± 0.03*	2.6	3
BIM (1 μM, 3 h)	PKC inhibitor	—	0.69 ± 0.08	1.0	3
BIM (1 μM, 3 h)	PKC inhibitor	TPA	0.49 ± 0.06*	1.4	3
U0126 (5 μM, 6 h)	MEK1/2 inhibitor	—	0.82 ± 0.17	0.84	3
U0126 (5 μM, 6 h)	MEK1/2 inhibitor	TPA	0.28 ± 0.02*	2.5	3
SB202190 (5 μM, 6 h)	$p38^{MAPK}$ inhibitor	—	0.71 ± 0.10	0.97	3
SB202190 (5 μM, 6 h)	$p38^{MAPK}$ inhibitor	TPA	0.33 ± 0.05*	2.1	3
SP600125 (50 μM, 6 h)	JNK inhibitor	—	0.83 ± 0.19	0.84	3
SP600125 (50 μM, 6 h)	JNK inhibitor	TPA	0.67 ± 0.05	1.0	4
Control siRNA	None	—	0.53 ± 0.06	1.3	2
Control siRNA	None	TPA	0.23 ± 0.01*	3.0	2
JNK1/2 siRNA	JNK depletion	—	0.50 ± 0.09	1.4	3
JNK1/2 siRNA	JNK depletion	TPA	0.55 ± 0.07	1.3	2
Control virus (72 h)	none	—	0.95 ± 0.19	0.73	3
MKK7D virus (72 h)	JNK activator	—	0.37 ± 0.03[d]	1.9	3
None[e]	None	—	0.73 ± 0.08	0.95	3
None	None	IL-1β	0.38 ± 0.07*	1.8	3
SP600125 (50 μM, 6 h)	JNK inhibitor	IL-1β	0.63 ± 0.05	1.1	3

MAPK, mitogen-activated protein kinase; PKC, protein kinase C; siRNA, small interfering RNA; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate.

[a] Where indicated, TPA (160 nM, 1 h) or IL-1β (5 ng/ml, 15 min) were added to HepG2 cells prior to inhibition of transcription with actD.

[b] First-order mRNA decay constants were resolved for each cell population by actinomycin time-course assay as described under Experimental Procedures. Listed decay constants represent the mean ± SD for $n \geq 3$ independent time-course experiments, or mean ± spread where $n = 2$. Cell pretreatments where LDL receptor mRNA was significantly stabilized following addition of TPA or IL-1β ($P < 0.05$) are indicated by asterisks.

[c] mRNA half-lives were calculated from mean k values as $t_{1/2} = \ln 2/k$.

[d] LDL receptor mRNA significantly stabilized in cells expressing MKK7D vs. control virus ($P < 0.01$).

[e] Total FCS concentrations were reduced to 0.5% when HepG2 cells were stimulated with IL-1β. For comparative purposes, cells lacking IL-1β were incubated in 0.5% FCS for 15 min prior to administration of actD in these experiments.

decay of specific mRNAs. For example, combined action of the ERK and p38^{MAPK} pathways controls tumor necrosis factor α mRNA degradation in macrophages through the AU-rich element (ARE)-binding protein tristetraprolin (TTP) (30), which may involve regulating the stability and/or subcellular distribution of this protein (31, 32) or the stability of TTP mRNA (33). ERK is also required for stabilization of p21 mRNA in nonsmall cell lung carcinoma cells following treatment with prostaglandin A_2 (34). The JNK pathway influences the stability of several transcripts in cardiomyocytes, including mRNAs encoding the protein phosphatase 2A regulatory subunit, B56α (35), and microsomal prostaglandin E_2 synthase-1 (36). Second, the ERK and p38^{MAPK} pathways have each been implicated in control of LDL receptor gene transcription (18, 37), raising the possibility that these signaling pathways may coordinately direct receptor production at multiple gene regulatory steps.

To determine whether TPA-dependent activation of these MAPK pathways and concomitant stabilization of LDL receptor mRNA required the activity of PKC, HepG2 cells were treated with the PKC inhibitor BIM for 3 h prior to TPA stimulation. BIM is a structural homolog of staurosporine that potently inhibits PKC isozymes α, β_1, δ, and ε (38). MAPK activation was monitored by Western blot using phospho-specific antibodies. In cells lacking BIM, TPA treatment did not significantly alter the total cellular abundance of ERK, p38^{MAPK}, or JNK, but dramatically enhanced levels of their phosphorylated forms (**Fig. 2A**), consistent with the results of the phosphoprotein survey (see supplementary Table II). Pretreatment with BIM significantly inhibited but did not completely abrogate stimulation of the ERK and JNK pathways, indicating a significant PKC-dependent component in their activation following TPA treatment. However, phosphorylation of p38^{MAPK} was not detectably impaired by BIM pretreatment. Several possibilities can be envisioned that may account for this observation. For example, induction of the p38^{MAPK} pathway by TPA may be independent of PKC in HepG2 cells, or employ one or more PKC isoforms that are not inhibited by BIM. Alternatively, BIM might not completely inhibit PKC under the conditions tested, which would preclude rigorous suppression of some downstream pathways.

ActD time-course assays revealed that the constitutive stability of LDL receptor mRNA was not affected by the presence of BIM alone (Fig. 2B and Table 1, $t_{1/2}$ = 1.0 h). However, pretreatment with BIM dramatically attenuated stabilization of the receptor transcript in response to TPA. Receptor mRNA decay remained significantly retarded in BIM+TPA-treated cells relative to cells treated with BIM alone ($P = 0.027$), but the magnitude of the TPA-stabilizing effect was diminished to 40% in the presence of BIM vs. 140% in the absence of the PKC inhibitor (Table 1). These data confirm that PKC activity significantly contributes to stabilization of LDL receptor mRNA in HepG2 cells following TPA treatment. However, the ability of BIM to substantially diminish TPA-dependent stabilization of receptor mRNA without detectably inhibiting p38^{MAPK} phosphorylation provides the first evidence that stabilization of LDL receptor mRNA through PKC does not involve the p38^{MAPK} pathway.

PKC-dependent stabilization of LDL receptor mRNA does not require the ERK or p38^{MAPK} pathways

In the next series of experiments, kinase-specific inhibitors were used to identify specific MAPK pathways regulating TPA-dependent stabilization of LDL receptor mRNA. Similar to the BIM-based experiments, HepG2 cells were preincubated with specific MAPK inhibitors followed by treatment with or without TPA. Preliminary time-course experiments indicated that 6-h inhibitor pretreatments maximally suppressed TPA-dependent activation of each targeted pathway without inducing significant cell death (data not shown). U0126 is an inhibitor of the MEK1/2 kinases, which phosphorylate and activate ERK (39, 40). Western blot analyses showed that TPA-induced phosphorylation of ERK was almost completely blocked in the presence of this inhibitor (**Fig. 3A**). However, inhibition of the ERK pathway had no significant effect on the constitutive decay rate of LDL receptor mRNA (Table 1) or its stabilization following TPA treatment (Fig. 3B, P = 0.006 vs. U0126 alone). A role for the p38^{MAPK} pathway was tested

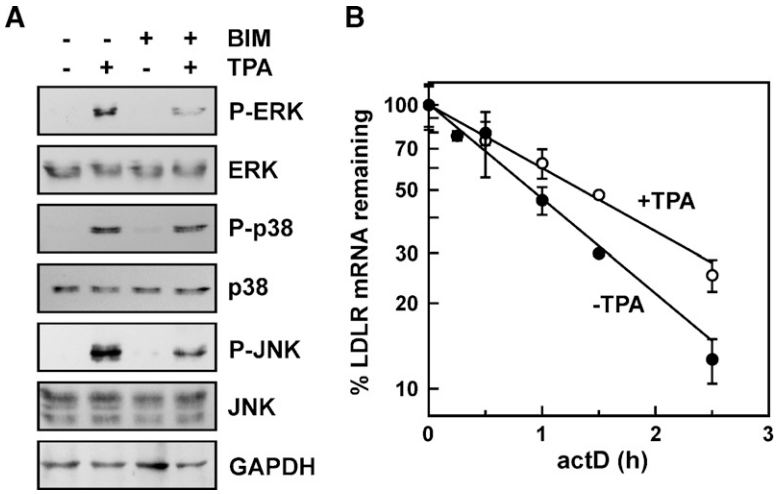

Fig. 2. Role of protein kinase C (PKC) in activation of the major mitogen-activated protein kinase (MAPK) pathways and stabilization of LDL receptor mRNA. A: HepG2 cells were treated with or without TPA (160 nM) for 1 h either in the absence or presence of the PKC inhibitor bis-indolylmaleimide I (BIM) (1 μM) added 3 h prior to TPA as indicated. Specific MAPK proteins and phosphoproteins were detected in whole-cell lysates by Western blot. A Western blot for GAPDH was used to normalize protein loading between samples (bottom). B: Representative actD time-course assays measuring LDL receptor mRNA turnover kinetics in HepG2 cells treated with BIM either with (open circles) or without (solid circles) subsequent TPA stimulation as described above. LDL receptor mRNA levels were measured at selected time points following actD administration permitting resolution of receptor mRNA decay kinetics as described in Fig. 1.

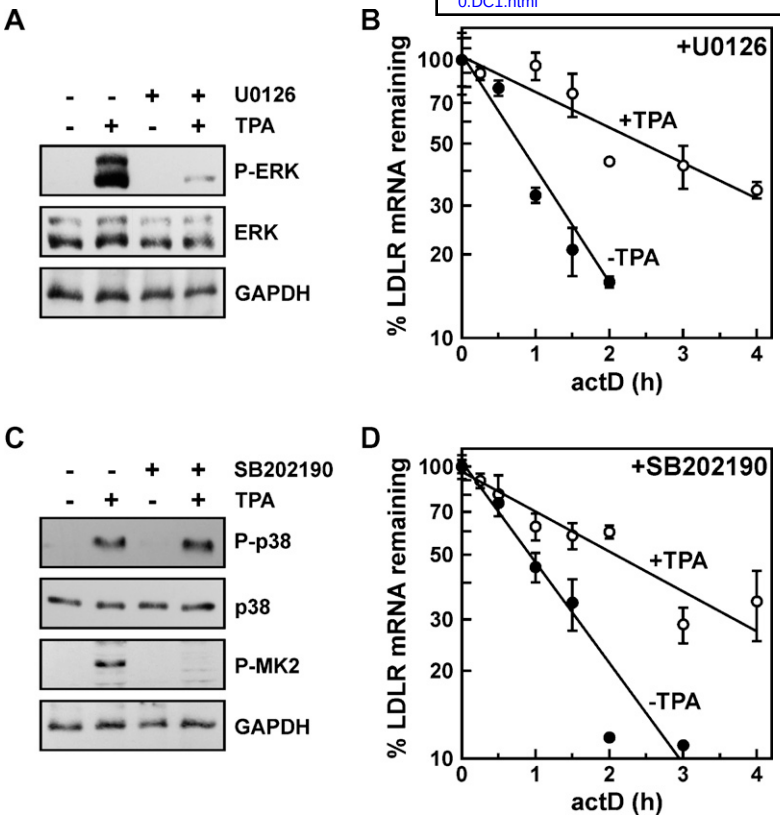

Fig. 3. Roles of the extracellular signal-regulated kinase (ERK) and p38^{MAPK} pathways in the regulation of LDL receptor mRNA decay kinetics. Whole-cell lysates were collected from HepG2 cells treated with or without TPA (160 nM, 1 h) either in the absence or presence of the MEK1/2 inhibitor U0126 (5 μM) (A), or the p38^{MAPK} inhibitor SB202190 (5 μM) (C) added 6 h prior to TPA where indicated. The activation status of the ERK and p38^{MAPK} pathways was monitored by Western blot using antibodies recognizing the proteins or phosphoproteins listed (right). Protein loading was normalized by Western blot for GAPDH (bottom). LDL receptor mRNA decay kinetics were measured in HepG2 cells treated with U0126 (5 μM, 6 h) (B) or SB202190 (5 μM, 6 h) (D) prior to addition of TPA (160 nM, 1 h; open circles). Parallel cultures did not receive TPA following inhibitor treatments (solid circles). Receptor mRNA decay constants were resolved as described in Fig. 1.

by pretreating HepG2 cells with SB202190, a direct inhibitor of p38^{MAPK} (39, 40). Neither the abundance nor the phosphorylation status of p38^{MAPK} was affected by this inhibitor in the presence or absence of TPA (Fig. 3C). However, pretreatment with SB202190 completely blocked the ability of activated p38^{MAPK} to phosphorylate its substrate MK2 indicating that it robustly inhibits downstream effects of p38^{MAPK} stimulation (Fig. 3C). Similar to inhibition of the MEK/ERK pathway, pretreating HepG2 cells with the p38^{MAPK} inhibitor did not alter basal LDL receptor mRNA turnover kinetics, not did it impair TPA-dependent stabilization of the receptor transcript (Fig. 3D and Table 1, $P = 0.0041$ vs. SB202190 alone). Together, these data indicate that neither the ERK nor p38^{MAPK} pathways are required for stabilization of LDL receptor mRNA in TPA-stimulated HepG2 cells.

PKC-dependent stabilization of LDL receptor mRNA requires the JNK pathway

Involvement of the JNK pathway in control of LDL receptor mRNA decay kinetics was assessed using SP600125, a direct inhibitor of the ubiquitously expressed JNK isoforms, JNK1 and JNK2 (41). Pretreatment of HepG2 cells with this compound markedly inhibited JNK phosphorylation following TPA treatment, and efficiently blocked phosphorylation of the JNK substrate c-Jun (**Fig. 4A**). ActD time-course assays revealed that constitutive turnover of receptor mRNA was not influenced by inhibition of the JNK pathway (Table 1). By contrast, TPA-dependent stabilization of this transcript was completely abrogated in the presence of the JNK inhibitor (Fig. 4B and Table 1), indicating that JNK activity participates in posttranscriptional events controlling LDL receptor expression downstream of PKC. We next tested whether JNK-dependent mRNA stabilization contributes to elevation of receptor mRNA levels in TPA-stimulated HepG2 cells. TPA treatment increased LDL receptor mRNA levels by approximately 13-fold after 3 h (Fig. 4C). However, pretreatment with the JNK inhibitor decreased TPA-induced accumulation of the receptor transcript by almost 50% relative to treatment with TPA alone ($P = 0.015$). Inhibition of the ERK pathway, which blocks TPA-dependent activation of receptor transcription (18), suppressed induction of receptor mRNA more dramatically ($P = 0.005$ for U0126 + TPA vs. TPA alone), but not completely. Even in the absence of ERK activity, stimulation of HepG2 cells with TPA induces a 2- to 2.5-fold increase in receptor mRNA levels relative to untreated cells ($P = 0.006$). Together, these data indicate that both transcriptional (ERK-directed) and posttranscriptional (JNK-directed) mechanisms contribute to accumulation of LDL receptor mRNA in HepG2 cells following treatment with TPA.

To validate a role for JNK in the regulation of LDL receptor mRNA turnover, this pathway was activated in HepG2 cells independently of PKC by treatment with the cytokine IL-1β. Elevated levels of IL-1β and other cytokines occur in inflammatory syndromes including sepsis and severe trauma, and are associated with a hypocholesterolemic state (42–44) that may involve enhanced production of hepatic LDL receptors (45, 46). In HepG2 cells, acute IL-1β exposure induces expression of LDL receptor mRNA concomitant with activation of selected MAPK pathways, including JNK (47). To determine whether IL-1β-directed

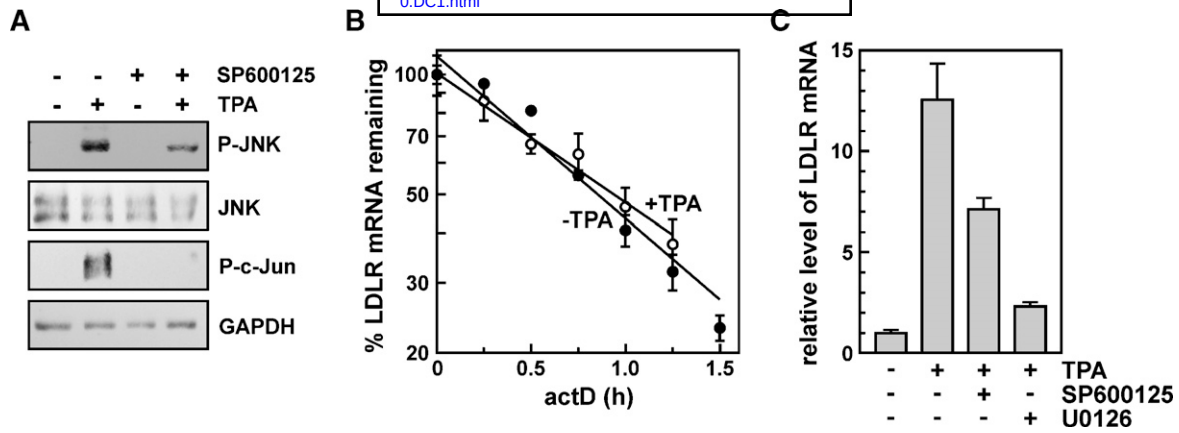

Fig. 4. Control of LDL receptor mRNA decay by inhibition of JNK. A: Activation of the JNK pathway in HepG2 cells was monitored by Western blot using whole-cell lysates from cells treated with or without the JNK inhibitor SP600125 (50 μM) added 6 h prior to TPA (160 nM, 1 h) as indicated. A Western blot of GAPDH was used to normalize protein loading (bottom). B: Representative plots of LDL receptor mRNA decay in HepG2 cells treated with (open circles) or without (solid circles) TPA (160 nM, 1 h) following preincubation with SP600125 (50 μM, 6 h). Decay constants were resolved as described in Fig. 1. C: Total RNA was purified from HepG2 cells treated with or without TPA (160 nM) for 1 h either in the absence or presence of SP600125 (50 μM) or U0126 (5 μM) added 6 h prior to TPA as indicated. Relative LDL receptor mRNA levels were measured by qRT-PCR as described in Fig. 1. Bars indicate the mean ± SD measurements from three independent RNA samples.

enhancement of LDL receptor mRNA levels included JNK-dependent stabilization of receptor mRNA, parallel HepG2 cultures were preincubated with or without the JNK inhibitor SP600125 prior to stimulation with IL-1β. Western blots indicated that IL-1β treatment activated JNK to a degree similar to that observed following stimulation by TPA, based on phosphorylation of both JNK and its substrate c-Jun (**Fig. 5A**). ActD time-course assays showed a nearly 2-fold stabilization of LDL receptor mRNA in IL-1β-treated cells (Fig. 5B and Table 1; $P = 0.005$ vs. low serum). However, pretreating HepG2 cells with SP600125 effectively blocked activation of JNK and stabilization of receptor mRNA following IL-1β treatment, indicating that posttranscriptional control of receptor expression following stimulation with IL-1β also requires the JNK pathway.

In a second series of experiments, to validate the role of JNK in stabilization of LDL receptor mRNA, endogenous JNK1 and JNK2 were depleted from HepG2 cells using siRNA cocktails. JNK1 and JNK2 were coordinately targeted because of the high degree of conservation (83% amino acid sequence identity) and functional redundancy between these enzymes (48, 49). Western blot analyses indicated that endogenous JNK proteins were suppressed to undetectable levels following transfection with JNK1/2 siRNAs (**Fig. 6A**). Consistent with this observation, stimulation with TPA revealed no evidence of JNK activation in

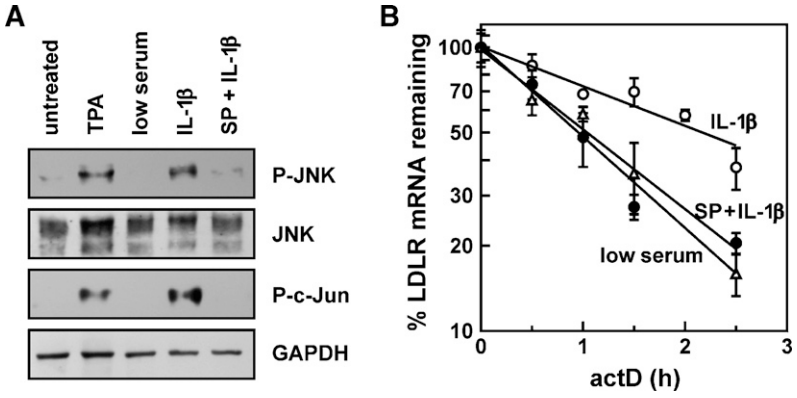

Fig. 5. JNK-dependent stabilization of LDL receptor mRNA following stimulation with IL-1β. A: Western blots to monitor activation of the JNK pathway in HepG2 cells using whole-cell lysates from untreated cells or following addition of TPA (160 nM, 1 h). Additional cultures were treated with low serum (0.5% FBS, 15 min) or IL-1β (5 ng/ml in 0.5% FBS, 15 min). In the right lane, HepG2 cells were treated with the JNK inhibitor SP600125 (50 μM) for 6 h prior to addition of IL-1β (5 ng/ml in 0.5% FBS, 15 min). B: Representative plots of LDL receptor mRNA decay in cells treated with low serum (0.5% FBS, 15 min; solid circles), IL-1β (5 ng/ml in 0.5% FBS, 15 min; open circles), or pretreated with SP600125 (50 μM, 6 h) prior to addition of IL-1β (5 ng/ml in 0.5% FBS, 15 min; triangles). ActD time-course data sets were resolved as described in Fig. 1.

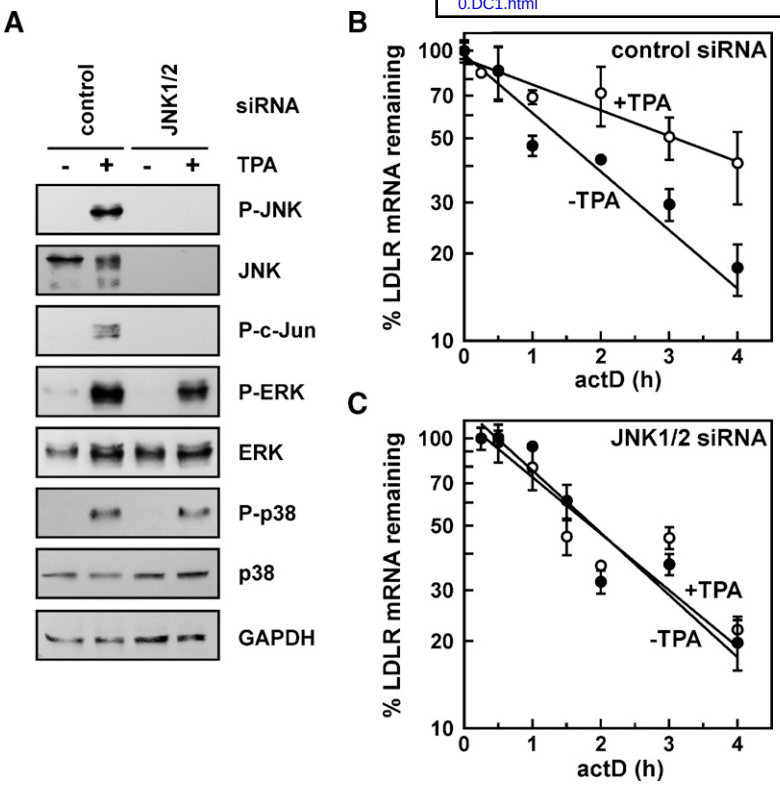

Fig. 6. Decay kinetics of LDL receptor mRNA following small interfering RNA (siRNA)-directed repression of JNK. A: HepG2 cells were transfected using a two-hit strategy with an siRNA cocktail targeting JNK1/2 or nontargeting siRNAs (control) as described under Experimental Procedures. Forty-eight h posttransfection, cells were treated with or without TPA (160 nM, 1 h) as indicated. The efficiency of JNK knockdown was assessed by Western blot analyses of whole-cell lysates using anti-JNK antibodies (JNK panel) and abrogation of TPA-dependent activation of the JNK pathway by probing for phospho-JNK (P-JNK panel) and phosphorylation of its substrate, c-Jun (P-c-Jun panel). The activation status of the ERK and $p38^{MAPK}$ pathways was also monitored in these cells by Western blot using antibodies recognizing each protein and its activated, phosphorylated form as indicated (right). A GAPDH control was included to assess protein loading (bottom). ActD time-course assays were used to measure LDL receptor mRNA decay kinetics in HepG2 cells treated with (open circles) or without (solid circles) TPA (160 nM, 1 h), 48 h following two-hit transfections with control (B) or JNK1/2-specific (C) siRNAs. Receptor mRNA decay kinetics were resolved as described in Fig. 1.

JNK1/2 siRNA-treated cells, because phosphorylated forms of neither JNK nor its substrate c-Jun were detected. By contrast, JNK was well expressed in cells treated with control siRNA, and was readily activated by TPA treatment in these cells. To further evaluate the specificity of JNK repression in cells transfected with the JNK1/2 siRNAs, expression and activation of the other major MAPK enzymes were also assessed. Western blots showed that JNK1/2 siRNAs minimally impacted levels of both ERK and $p38^{MAPK}$ (Fig. 6A). Also, both factors were readily phosphorylated in these cells following TPA treatment, indicating that the ERK and $p38^{MAPK}$ pathways were not significantly perturbed by siRNA-directed depletion of JNK1/2.

In cells transfected with control siRNA, LDL receptor mRNA decayed with a half-life of 1.3 h, slightly but not significantly longer than the 1.1 h half-life observed in untreated cells (Fig. 6B and Table 1). The addition of TPA stabilized receptor mRNA by a factor of 2.3 ($P = 0.022$), virtually identical to the degree of stabilization observed in untransfected cells. However, in JNK1/2-depleted cells, this transcript decayed with a half-life of 1.3–1.4 h regardless of TPA treatment (Fig. 6C), indicating that the JNK pathway does not contribute to basal turnover of LDL receptor mRNA, but is required for its stabilization following activation of PKC.

Sustained JNK activation posttranscriptionally induces expression of functional LDL receptors

If activation of the JNK pathway is requisite for stabilization of LDL receptor mRNA in response to treatment with TPA or IL-1β, we reasoned that direct activation of JNK independent of these stimuli would also prolong the cellular lifetime of this transcript. A constitutively active mutant of the JNK-activating kinase, MKK7 (termed MKK7D), was introduced into HepG2 cells by adenoviral transduction. Parallel cultures were infected with a control virus containing an empty expression cassette. Cell lysates were prepared 72 h postinfection and assayed for JNK activity by Western blot. Introduction of the MKK7D mutant dramatically stimulated phosphorylation of JNK with relatively minor effects on overall JNK levels. Activity of the phosphorylated JNK was verified by enhanced phosphorylation of its substrate, c-Jun (**Fig. 7A**). Activation of JNK by MKK7D stabilized LDL receptor mRNA 2.6-fold relative to cells infected with the control virus (Fig. 7B and Table 1, $P = 0.007$). By contrast, the mRNA encoding vascular endothelial growth factor decayed with comparable kinetics in cells infected with either control ($k = 0.50 \pm 0.04\ h^{-1}$, $n = 3$; $t_{1/2} = 1.4$ h) or MKK7D-expressing viruses ($k = 0.46 \pm 0.05\ h^{-1}$, $n = 3$; $t_{1/2} = 1.5$ h; see supplementary Fig. I), indicating that JNK activation does not induce general mRNA stabilization in HepG2 cells.

Unlike the transient stabilization of receptor mRNA observed following TPA treatment (*15*), JNK activation could be sustained for several days following introduction of MKK7D, permitting the influence of this pathway on the accumulation of LDL receptor mRNA and protein to be assessed. MKK7D-dependent stabilization of receptor mRNA resulted in a 2-fold increase in LDL receptor mRNA levels after 72 h (Fig. 7C, $P = 0.02$), which in turn substantially enhanced production of the receptor protein, observed for both the preprocessed (≈ 120 kDa) and fully processed (≈ 160 kDa) forms (Fig. 7D). To determine whether the increased levels of LDL receptors observed

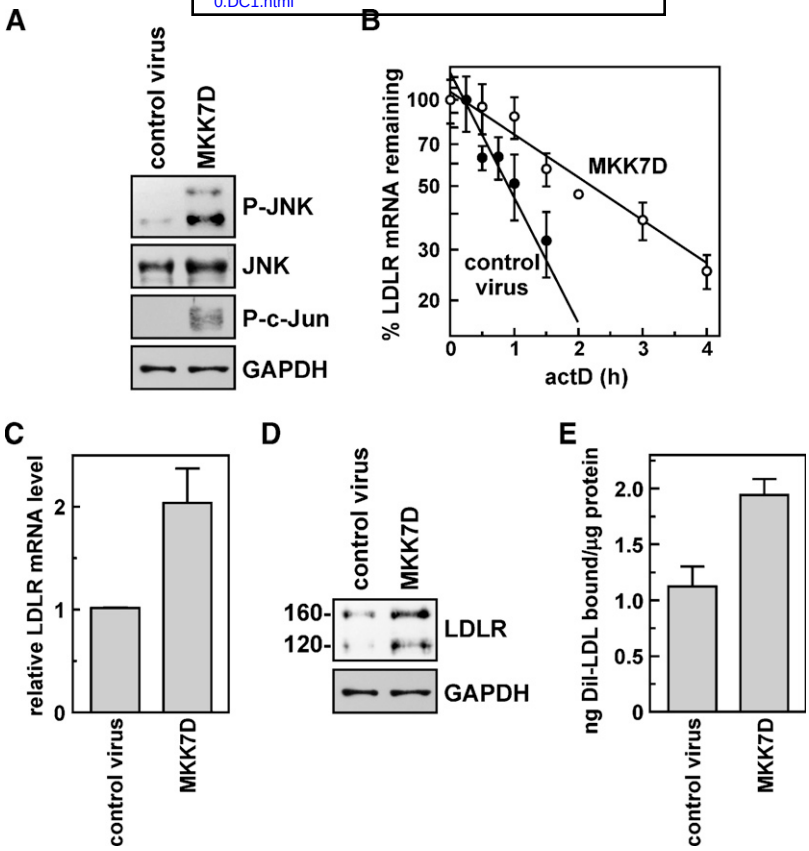

Fig. 7. Regulation of LDL receptor mRNA expression by direct activation of JNK. A: HepG2 cells were infected with an adenovirus encoding a constitutively active mutant of the JNK-activating enzyme MKK7 (*MKK7D*) or an empty expression cassette (control virus) as described under Experimental Procedures. Seventy-two h postinfection, the abundance and activation status of JNK was assessed by Western blot as described in Fig. 6, using GAPDH as a loading control. B: Representative actD time-course assays showing the decay kinetics of LDL receptor mRNA in cells infected with control (solid circles) or MKK7D-encoding (open circles) adenoviruses. Constants describing receptor mRNA turnover rates were resolved as described in Fig. 1. Total LDL receptor mRNA (C), and protein (D) levels were measured in HepG2 cells infected with control or MKK7D-expressing adenoviruses. Receptor mRNA levels were resolved by multiplex qRT-PCR normalized to GAPDH. Bars represent the mean ± standard deviation of three independent samples. Receptor protein levels were assessed by Western blot. An independent replicate experiment yielded similar results. E: The specific LDL-binding activity of HepG2 cells was measured 72 h after infection with control or MKK7D-expressing adenoviruses using the fluorescent lipoprotein 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate-labeled human LDL (DiI-LDL) as described under Experimental Procedures. Bars indicate the mean ± standard deviation of the specific LDL-binding activity for each cell population ($n = 5$).

in JNK-stimulated HepG2 cells were functional, the LDL-binding activity of these cells was measured using the fluorescently-labeled lipoprotein, DiI-LDL. HepG2 cells expressing MKK7D for 72 h displayed a 75% increase in specific DiI-LDL binding relative to cells infected with control virus (Fig. 7E, $P = 0.007$), verifying that enhancement of LDL receptor mRNA stability by sustained JNK activation significantly increases the production of functional LDL receptors in HepG2 cells.

DISCUSSION

Activation of HepG2 cells with the phorbol ester TPA rapidly induces expression of LDL receptors by enhancing transcription from the receptor gene and the stability of its encoded mRNA (Refs. 15, 18 and this work). Each of these mechanisms contributing to accumulation of LDL receptor mRNA requires PKC, because PKC inhibitors block both the transcriptional induction (50) and stabilization of receptor mRNA (Fig. 2) observed in response to this stimulus. Concomitant with enhancement of LDL receptor mRNA synthesis and stability, TPA stimulation activates several MAPK signaling systems in HepG2 cells, including the ERK, $p38^{MAPK}$, and JNK pathways (**Fig. 8**). Increased transcriptional activity following TPA treatment is mediated by the ERK pathway and requires Sp1 and sterol regulatory element sites within the proximal promoter of the LDL receptor gene (50). Activation of $p38^{MAPK}$ may exert a negative influence on ERK-dependent induction of receptor gene transcription, because the $p38^{MAPK}$ inhibitor SB202190 augments LDL receptor mRNA levels in HepG2 cells, accompanied by enhancement of ERK phosphorylation (37). However, two experiments described in this study

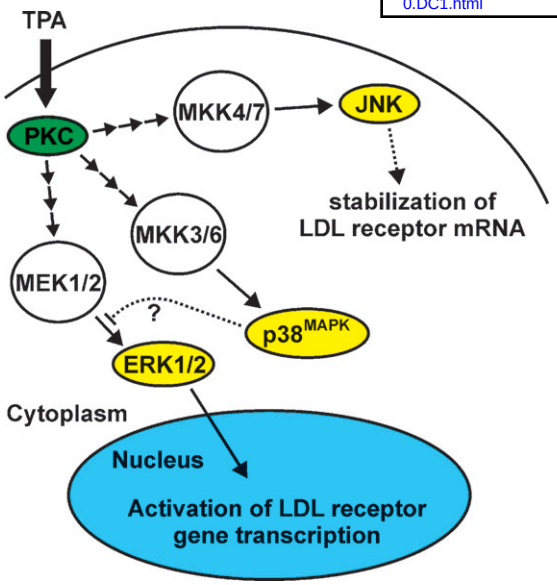

Fig. 8. Links between MAP kinase signaling cascades and control of LDL receptor expression in HepG2 cells. Stimulation of HepG2 cells with TPA rapidly activates the three major MAPK pathways. While TPA is a potent activator of several PKC isoforms, it remains possible that some effects of TPA on the MAPK cascades are independent of PKC. This may be particularly true for the $p38^{MAPK}$ pathway, because pretreatment with the PKC inhibitor BIM did not detectably alter activation of $p38^{MAPK}$ following TPA treatment (Fig. 2). Details describing different steps in the induction of LDL receptor expression following activation of the MAPK pathways are related in the text.

demonstrated that TPA-induced stabilization of receptor mRNA was independent of either the ERK or $p38^{MAPK}$ pathways in HepG2 cells. First, chemical inhibitors of $p38^{MAPK}$ or the ERK-activating kinases MEK1/2 did not prevent LDL receptor mRNA stabilization following TPA treatment (Fig. 3B, D) despite essentially quantitative suppression of these signaling pathways (Fig. 3A, C). Second, TPA did not inhibit decay of receptor mRNA in cells transfected with JNK1/2 siRNAs, even though the ERK and $p38^{MAPK}$ pathways remained fully active in these cells (Fig. 6). By contrast, several observations indicated that JNK plays a major role in cellular control of LDL receptor mRNA decay. First, pretreating HepG2 cells with a JNK inhibitor blocked stabilization of LDL receptor mRNA in response to treatment with TPA (Fig. 4) or IL-1β (Fig. 5). Similarly, TPA-dependent control of receptor mRNA decay kinetics was abrogated in cells following depletion of endogenous JNK using siRNA (Fig. 6). Finally, direct activation of JNK using a constitutively active mutant of MKK7 stabilized LDL receptor mRNA in HepG2 cells independent of TPA or IL-1β stimulation (Fig. 7). These findings indicate a significant divergence in the signaling pathways inducing LDL receptor expression at the transcriptional vs. posttranscriptional levels, and define mRNA stabilization via the JNK pathway as a novel mechanism to enhance production of functional LDL receptors (Fig. 8).

Recent findings indicate that treating HepG2 cells with the herbal alkaloid berberine can also stimulate expression of LDL receptors by inhibiting receptor mRNA decay and that administration of berberine to hypercholesterolemic patients leads to reductions in total serum cholesterol, triglycerides, and LDL-cholesterol (20). These data highlight the utility of enhancing hepatic LDL receptor mRNA stability as a hypolipidemic strategy, but also present several features indicating that the mRNA stabilizing mechanism induced by berberine is distinct from that employed by the PKC/JNK pathway described in this study. First, enhancement of LDL receptor mRNA levels by berberine might function independent of PKC because this induction was not affected by treatment with the PKC inhibitor calphostin C (20). Second, berberine-induced receptor mRNA stabilization required activation of the ERK pathway (20, 51). By contrast, inhibition of receptor mRNA decay following TPA treatment was independent of ERK activity (Figs. 3, 6). This apparent discrepancy could be related to the differential time frames of receptor mRNA induction following treatment with these stimuli. Stabilization of LDL receptor mRNA occurs within minutes of TPA administration and is independent of new gene expression (15). Conversely, induction of receptor mRNA levels following treatment with berberine was only apparent after 4 or more h (20). These temporal differences suggest that inhibition of receptor mRNA decay by berberine may be a secondary effect of ERK-dependent changes in the expression or activity of selected signaling and/or RNA-binding factors that are not apparent in the first few hours of ERK activation following TPA treatment. Finally, stabilization of receptor mRNA by berberine requires sequences in the proximal 3′UTR of the transcript, including three sites with similarity to AREs (20). By contrast, TPA-induced stabilization of receptor mRNA requires elements in the *Alu*-rich region of the distal 3′UTR, which does not include ARE-like sequences (19). Together, these data suggest that berberine and the PKC/JNK pathway stabilize LDL receptor mRNA through different mechanisms, but also raise the possibility that stimulating both of these pathways may yield additive effects on LDL receptor expression in cells.

MAPK pathways are emerging as critical interfaces between extracellular stimuli and intracellular mRNA decay mechanisms. Several groups have linked the ERK and $p38^{MAPK}$ signaling systems to the regulation of mRNA turnover through AREs, particularly involving the ARE-binding protein TTP; this topic is discussed at length elsewhere (30–32, 52). However, mechanisms controlling mRNA decay through JNK are less well resolved. For example, stabilization of IL-2 mRNA during T-cell activation is JNK-dependent (53), requiring sequences in the extreme 5′ UTR that associate with the proteins nucleolin and YB-1 (54). However, association of these proteins with 5′UTR sequences inhibits translation of some mRNAs, possibly by competing with eIF4E for the 5′-cap structure (55, 56). As such, it is unclear whether JNK regulates IL-2 mRNA decay directly through the nucleolin/YB-1 binding events, or as a secondary consequence of modulating translational efficiency, because processes controlling the degradation of some mRNAs are sensitive to their translational status (as reviewed in Refs. 57, 58). Stabilization of some other ARE-containing mRNAs is also blocked or attenuated by

inhibition of the JNK pathway (59–61), possibly by modulating ARE-targeted mRNA decay mechanisms. For example, JNK-dependent stabilization of IL-3 mRNA in mast cells requires the ARE sequence (59). Also, a recent study suggests that stabilization of inducible nitric oxide synthase mRNA by JNK in lung epithelial cells may be accompanied by induction of TTP expression (61). While activation of JNK is also responsible for stabilizing LDL receptor mRNA in HepG2 cells (this study), these effects are likely independent of ARE sequences (as previously discussed). Finally, JNK activity is not uniformly associated with mRNA stabilization, because activation of JNK in rat cardiomyocytes accelerates decay of the mRNA encoding the B56α subunit of protein phosphatase 2A (35). These emerging data indicate that JNK activation can differentially regulate the stability of specific target mRNAs, likely employing a diverse array of mechanisms. Conceivably, these mechanisms may include modulation of the mRNA-binding and/or -stabilizing/destabilizing activities of distinct *trans*-acting factors or populations of factors. By this model, downstream effects on the decay kinetics of individual mRNA targets may vary depending on the placement or identity of specific *cis*-acting elements within each transcript and/or their cellular context.

In conclusion, we have identified the JNK pathway as an important regulator of LDL receptor mRNA stability in HepG2 cells, and have shown that sustained activation of this signaling system enhances the production of functional LDL receptors in these cells. Due to the fact that JNK is activated in response to pro-inflammatory cytokines like IL-1β (Fig. 5) (47), JNK-dependent stabilization of hepatic LDL receptor mRNA may thus constitute one mechanism contributing to the plasma hypocholesterolemia associated with septic shock or trauma (42–44). However, given the critical role of hepatic LDL receptors in shuttling plasma cholesterol to the bile acid pathway for excretion, it is hoped that downstream components of this JNK-activated cascade will ultimately provide targets for novel cholesterol-lowering therapeutic strategies. Furthermore, the role of distal 3′UTR sequences in the control of LDL receptor mRNA turnover by the PKC/JNK pathway and its functional independence from ERK signaling distinguish this regulatory mechanism from those responsible for enhancing receptor expression in response to either berberine or statin treatment. We anticipate that increasing hepatic levels of functional LDL receptors through multiple complementary pathways will be clinically useful in lowering plasma LDL concentrations and associated atherosclerotic risk.

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