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

Noelle B. Vargas,* Brandy Y. Brewer,* Terry B. Rogers,* and Gerald M. Wilson1,*†

Department of Biochemistry and Molecular Biology,* and Marlene and Stewart Greenebaum Cancer Center,†

University of Maryland School of Medicine, Baltimore, MD 21201

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 p38MAPK, 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 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

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.

†To whom correspondence should be addressed.

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

This work was supported by National Institutes of Health grants R01 CA102428 (to G.M.W.) and P01 HL070709 (to T.B.R.) and a Scientist Development Grant from the American Heart Association (to G.M.W.). N.B.V received support from National Institutes of Health grant F31 HL087731, an NIGMS Initiative for Minority Student Development Grant (R25-GM55036), and Procter and Gamble.

Manuscript received 17 June 2008 and in revised form 30 September 2008 and in re-revised form 20 October 2008.

Published, JLR Papers in Press, October 20, 2008.
DOI 10.1194/jlr.M800316-JLR200

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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 MAPK, phospho-p38 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 (a-d), 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 (DiL-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). Re-combinant 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% CO₂ 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 (3×10⁶ 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% IGE- PAL-Ca630, 10 μM leupeptin, 5 μM pepstatin A, 1 mM PMSE, 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 (Ct) 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 ≥ 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 nitrocellu-
lose 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 ade-
oviral transduction into HepG2 cells. For these experiments,
HepG2 cells were seeded in 6-well plates at 5 x 10^5 cells/well.
After 24 h, control (Ad-dl512) or M KK7D-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 procedures
(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 M KK7D or control virus as de-
scribed 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 fluo-
rescence (λex = 540 nm; λem = 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 nor-
malization 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 bind-
ing. Specific DiI-LDL binding was then resolved by subtracting non-
specific 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 sta-
bility of LDL receptor mRNA were transiently enhanced in HepG2 cells following treatment with phorbol esters
(15, 18), presumably involving activation of the PKC path-
way. 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 10-
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 in-
duction of LDL receptor expression also included stabilization
of its mRNA, since the half-life of receptor mRNA in-
creased 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 vali-
dated the utility of the multiplex qRT-PCR approach em-
ployed in this study.

Phorbol esters are potent activators of the PKC pathway
(27, 28) and can also interact with other intracellular tar-
gets (29). While activation of LDL receptor gene transcrip-
tion following TPA treatment has been principally linked
to the ERK pathway (18), mechanisms controlling the de-
cay 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 sev-
eral MAPK pathways in HepG2 cells. Activation of the ERK
pathway was previously noted (18) and is further sup-
ported by enhanced phosphorylation of the ERK-activating
kinases, MEK1/2. Similarly, activation of the p38MAPK
pathway was indicated by enhanced phosphorylation of p38α
and its activating kinases MEK3/6. The activity of a third

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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

![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.

![Supplemental Material can be found at:](http://www.jlr.org/content/suppl/2008/10/22/M800316-JLR2008-D1/DC1.html)

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

<table>
<thead>
<tr>
<th>Cell Pretreatment</th>
<th>Kinase Target</th>
<th>Inducer</th>
<th>k (h⁻¹)</th>
<th>t₁/₂ (h)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>—</td>
<td>0.61 ± 0.01</td>
<td>1.1</td>
<td>3</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>TPA</td>
<td>0.26 ± 0.05*</td>
<td>2.6</td>
<td>3</td>
</tr>
<tr>
<td>BIM (1 μM, 3 h)</td>
<td>PKC inhibitor</td>
<td>—</td>
<td>0.69 ± 0.08</td>
<td>1.0</td>
<td>3</td>
</tr>
<tr>
<td>BIM (1 μM, 3 h)</td>
<td>PKC inhibitor</td>
<td>TPA</td>
<td>0.49 ± 0.06*</td>
<td>1.4</td>
<td>3</td>
</tr>
<tr>
<td>U0126 (5 μM, 6 h)</td>
<td>MEK1/2 inhibitor</td>
<td>—</td>
<td>0.82 ± 0.17</td>
<td>0.84</td>
<td>3</td>
</tr>
<tr>
<td>U0126 (5 μM, 6 h)</td>
<td>MEK1/2 inhibitor</td>
<td>TPA</td>
<td>0.28 ± 0.02*</td>
<td>2.5</td>
<td>3</td>
</tr>
<tr>
<td>SB202190 (5 μM, 6 h)</td>
<td>p38 MAPK inhibitor</td>
<td>—</td>
<td>0.71 ± 0.10</td>
<td>0.97</td>
<td>3</td>
</tr>
<tr>
<td>SB202190 (5 μM, 6 h)</td>
<td>p38 MAPK inhibitor</td>
<td>TPA</td>
<td>0.33 ± 0.05*</td>
<td>2.1</td>
<td>3</td>
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<tr>
<td>SP600125 (50 μM, 6 h)</td>
<td>JNK inhibitor</td>
<td>—</td>
<td>0.83 ± 0.19</td>
<td>0.84</td>
<td>3</td>
</tr>
<tr>
<td>SP600125 (50 μM, 6 h)</td>
<td>JNK inhibitor</td>
<td>TPA</td>
<td>0.67 ± 0.05</td>
<td>1.0</td>
<td>4</td>
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<tr>
<td>Control siRNA</td>
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<td>—</td>
<td>0.53 ± 0.06</td>
<td>1.3</td>
<td>2</td>
</tr>
<tr>
<td>Control siRNA</td>
<td>TPA</td>
<td>—</td>
<td>0.29 ± 0.01*</td>
<td>3.0</td>
<td>2</td>
</tr>
<tr>
<td>JNK1/2 siRNA</td>
<td>JNK depletion</td>
<td>—</td>
<td>0.50 ± 0.09</td>
<td>1.4</td>
<td>3</td>
</tr>
<tr>
<td>JNK1/2 siRNA</td>
<td>JNK depletion</td>
<td>TPA</td>
<td>0.55 ± 0.07</td>
<td>1.3</td>
<td>2</td>
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<tr>
<td>Control virus (72 h)</td>
<td>None</td>
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<td>0.95 ± 0.19</td>
<td>0.75</td>
<td>3</td>
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<tr>
<td>MKK7D virus (72 h)</td>
<td>JNK activator</td>
<td>—</td>
<td>0.37 ± 0.03*</td>
<td>1.9</td>
<td>3</td>
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<tr>
<td>None</td>
<td>None</td>
<td>—</td>
<td>0.73 ± 0.08</td>
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<td>SP600125 (50 μM, 6 h)</td>
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<td>IL-1β</td>
<td>0.38 ± 0.07*</td>
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<td>SP600125 (50 μM, 6 h)</td>
<td>JNK inhibitor</td>
<td>IL-1β</td>
<td>0.63 ± 0.05</td>
<td>1.1</td>
<td>3</td>
</tr>
</tbody>
</table>

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

*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.

*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 ≥ 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.

*LDL receptor mRNA significantly stabilized in cells expressing MKK7D vs. control virus (P < 0.01).

*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.
Somatic prostaglandin E2 synthase-1 (36). Second, the ERK pathway activation following TPA treatment was monitored by Western blot using phospho-specific antibodies. In cells lacking BIM, TPA stimulation produced ERK phosphorylation, which was not detectable in BIM-treated cells (Fig. 2A, solid circles). In contrast, the phosphorylation of p38MAPK was not significantly inhibited by BIM pretreatment. Several possibilities were considered that may account for this observation. For example, inactivation of the p38MAPK pathway by BIM 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, t1/2 = 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 p38MAPK phosphorylation provides the first evidence that stabilization of LDL receptor mRNA through PKC does not involve the p38MAPK pathway.

PKC-dependent stabilization of LDL receptor mRNA does not require the ERK or p38MAPK 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 U0126, which is consistent with results from the BIM-based experiments. However, phosphorylation of p38MAPK was not detectably impaired by U0126 pretreatment. Several possibilities were considered that may account for this observation. For example, induction of the p38MAPK 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.

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by pretreating HepG2 cells with SB202190, a direct inhibitor of p38MAPK (39, 40). Neither the abundance nor the phosphorylation status of p38MAPK was affected by this inhibitor in the presence or absence of TPA (Fig. 3C). However, pretreatment with SB202190 completely blocked the ability of activated p38MAPK to phosphorylate its substrate MK2 indicating that it robustly inhibits downstream effects of p38MAPK stimulation (Fig. 3C). Similar to inhibition of the MEK/ERK pathway, pretreating HepG2 cells with the p38MAPK inhibitor did not alter basal LDL receptor mRNA turnover kinetics, nor 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 p38MAPK 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
enhancement of LDL receptor mRNA levels included JNK-dependent stabilization of LDL 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
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 p38MAPK (Fig. 6A). Also, both factors were readily phosphorylated in these cells following TPA treatment, indicating that the ERK and p38MAPK 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 1.5 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 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.

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 p38MAPK 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.
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, p38MAPK, 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 p38MAPK may exert a negative influence on ERK-dependent induction of receptor gene transcription, because the p38MAPK inhibitor SB202190 augments LDL receptor mRNA levels in HepG2 cells, accompanied by enhancement of ERK phosphorylation (37). However, two experiments described in this study
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 hours (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 A-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 p38MAPK 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 hypercholesterolemia 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.

We thank Dr. Yevgeniya Lukyanenko and Ms. Shirley Gaa for assistance with the adenoviral transduction experiments, and Drs. Paul Shapiro and Alexey Belkin for helpful discussions.

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