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Insulin Silences Apolipoprotein B mRNA Translation by Inducing Intracellular Traffic into Cytoplasmic RNA Granules

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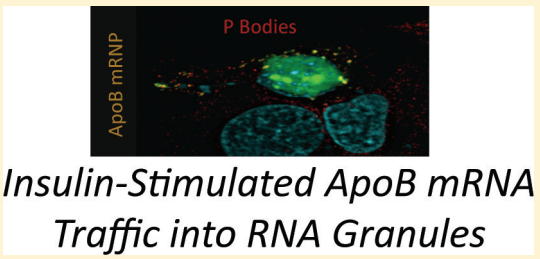
ABSTRACT: Insulin is a potent inducer of global mRNA translation and protein synthesis, yet it negatively regulates apolipoprotein B (apoB) mRNA translation, via an unknown mechanism. ApoB mRNA has a long half-life of 16 h, suggesting intracellular storage as mRNPs likely in the form of RNA granules. The availability of apoB mRNA for translation may be regulated by the rate of release from translationally silenced mRNPs within cytoplasmic foci called processing bodies (P bodies). In this report, we directly imaged intracellular apoB mRNA traffic and determined whether insulin silences apoB mRNA translation by entering cytoplasmic P bodies. We assessed the colocalization of apoB mRNA and β-globin mRNA (as a control) with P body (PB) markers using a strong interaction between the bacteriophage capsid protein MS2 and a sequence specific RNA stem−loop structure. We observed statistically significant increases in the localization of apoB mRNA into P bodies 4−16 h after insulin treatment (by 72−89%). The movement of apoB mRNA into cytoplasmic P bodies correlated with reduced translational efficiency as assessed by polysomal profiling and measurement of apoB mRNA abundance. PB localization of β-globin mRNA was insensitive to insulin treatment, suggesting selective regulation of apoB mRNA by insulin. Overall, our data suggest that insulin may specifically silence apoB mRNA translation by reprogramming its mRNA into P bodies and reducing the size of translationally competent mRNA pools. Translational control via traffic into cytoplasmic RNA granules may be an important mechanism for controlling the rate of apoB synthesis and hepatic lipoprotein production.



Apolipoprotein B 100 (apoB) is the main structural protein component of very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL). High plasma levels of LDL and VLDL have been shown to be associated with an increased risk of atherosclerosis, which is the major cause of cardiovascular disease worldwide.[1] ApoB synthesis is known to be metabolically regulated at multiple levels through post-transcriptional, cotranslational, and post-translational mechanisms.[2] Kinetic analysis of the decay of [^{3}H]uridine-labeled apoB mRNA demonstrated that the half-life of apoB mRNA was 16 h.[3] ApoB mRNA levels remain stable over a wide variety of conditions that considerably alter the apoB protein level, suggesting control at the level of translation and post-translational mechanisms.[4] Early studies demonstrated that insulin-mediated inhibition of apoB synthesis is at least partially mediated by translational control.[5,6] A number of other studies have since confirmed translational regulation of apoB synthesis by insulin and other metabolic regulators.[7,8]

The focus of this study was to further investigate the mechanisms involved in the translational control of apoB and the potential role of insulin in this regard. Insulin promotes global translation, yet it has an inhibitory effect on the translation of apoB mRNA. This unusual response of apoB mRNA to insulin has recently been linked to its 5′UTR sequence.[9,10] Computational analysis predicted the formation of highly structured secondary hairpin structures at the 5′UTR of apoB mRNA that are essential for the optimal translation of this molecule.[9] ApoB mRNA has a 5′UTR of 128 nucleotides and a 3′UTR of 304 nucleotides. Several structural features of apoB mRNA UTR sequences suggest the presence of potential *cis* elements that may interact with putative *trans*-acting protein factors. Analysis of the apoB UTR sequences using Mfold, to predict RNA secondary structure, revealed elements within the 5′- and 3′UTRs of apoB mRNA with potential to form secondary structure.[9] Deletion constructs of the UTR regions of apoB revealed that the 5′UTR was necessary and sufficient for insulin to inhibit apoB synthesis.[9] An insulin sensitive 110 kDa protein factor that interacts with the 5′UTR of apoB mRNA was reported by our laboratory to mediate translational control.[10] Insulin inhibited the interaction of p110 with the 5′UTR region, a process involving signaling through the phosphatidylinositol 3-kinase (PI3-K)−mTOR pathway.[11,12] Interestingly, translational control of apoB mRNA via the 5′UTR and the binding of the 110 kDa protein factor was also found to be regulated by the protein kinase C (PKC) signaling cascade.[12] Using dual (bicistronic) luciferase constructs, we also examined the role of internal ribosomal entry sites (IRES) with respect to the 5′UTR of the apoB mRNA and found that the apoB 5′UTR possesses IRES activity and the basal translational activity of the apoB mRNA may be partly cap-independent.[11]

It has recently been proposed that the main regulators of gene expression at the translational level are cytoplasmic RNA

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granules[13] that include germinal granules, stress granules (SGs), processing bodies (P bodies), and neural granules. The former is found in germ cells, and the latter three exist in somatic cells. RNA granules, composed of various RNAs and a subset of different proteins, are responsible for the localization, stability, and translation of mRNAs.[14] P bodies, which were the focus of this study, are dynamic RNA−protein structures found in the cytoplasm of eukaryotes from yeast to mammals. PB assembly correlates positively with the pool of translationally silent mRNA in a cell.[15−18] P bodies hold the components of the mRNA decay machinery pathway and are composed of Dcp1/Dcp2 (decapping enzymes), Dhh1/RCK/p54, Pat1, Scd6/RAP55, Edc3, the Lsm1−7 complex (activators of decapping), Xrn1 (5′−3′ exonuclease),[14,19] nonsense-mediated decay machinery, the miRNA repression system,[20,21] staufen, and FMRP (RNA binding proteins mediating mRNA transport).[18]

The major aims of this study were to visualize apoB mRNA traffic in cultured hepatocytes, to reveal possible localization in cytoplasmic P bodies, and to elucidate the potential role of these RNA granules in translational control of the apoB message by insulin. We further examined the effect of insulin on the distribution of apoB mRNA between translationally competent polysomal and nonpolysomal pools and correlated this shift with the appearance of the message in RNA granules.

EXPERIMENTAL PROCEDURES

Cell Culture. The HepG2 human hepatoma cell line was obtained from American Type Culture Collection (ATCC) and maintained in complete alpha modification of eagles medium (AMEM) (Wisent, Inc., Montreal, QC) supplemented with 10% fetal bovine serum (FBS) (Wisent, Inc.). Cells were plated into T-75 flasks and kept in a Nuaire incubator at 37 °C under a 95% air/5% CO_2 mixture. The medium was renewed twice a week, and cells were subcultured on a weekly basis usually after reaching 90% confluency.

Plasmid Preparation. A modified version of pGL3-Control Vector (Promega Corp., Madison, WI) containing the 5′UTR of the apoB gene followed by the sequence encoding 15% of full-length apoB from the N-terminus and 3′UTR of apoB was constructed as previously described.[9] The pSL-MS2bs-24X vector holds 24 tandem repeats of MS2 binding sites upon transcription and was a generous gift from R. H. Singer's laboratory (Albert Einstein College of Medicine, Yeshiva University, Bronx, NY). The pMS2-GFP-NLS plasmid encodes MS2 protein fused to GFP containing a nuclear localization signal (NLS) at its C-terminus[22] and was also kindly provided by R. H. Singer's laboratory. The mammalian expression vector, pCMV-myc-cyto (Invitrogen, Carlsbad, CA), was used as a backbone to construct the pCMV-5′UTR-apoB15%-3′UTR-MS2-24X-cyto plasmid. EF1a-β-globin mRNA-MS2-bs was used as a positive control[23] and purchased from N. Kedersha's laboratory (Harvard Medical School, Brigham and Women's Hospital, Boston, MA).

Transformation and Plasmid DNA Amplification. Subcloning efficiency DH5α competent cells (Invitrogen) were used to amplify all plasmids using the heat shock method according to the manufacturer's procedures. Midiprep plasmid isolation was conducted using the Endotoxin Free Plasmid Isolation Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purity of the plasmid DNA was assessed by calculating the ratio of optical density units at A_{260} to A_{280}, which was between 1.8 and 2.

Construction of the pCMV-MS2bs-24X-cyto Plasmid. The gene transcribing 24 tandem repeats of MS2bs from the pSL-MS2bs-24X vector was inserted into the pCMV-myc-cyto plasmid using NcoI and NotI restriction enzymes and T4 DNA ligase (New England Biolabs).

Construction of the pCMV-5′UTR-apoB15%-3′UTR-MS2-24X-cyto Chimeric Plasmid. To construct this plasmid, the 5′UTR-apoB15%-3′UTR sequence was obtained from the modified pGL3 vector. To create proper restriction sites, we designed 18mer polymerase chain reaction (PCR) primers (Table 1) to create an ApaI restriction site before the 5′UTR end and a BamHI site after the 3′UTR end. A clamp of three random bases was added at both extreme ends of the oligonucleotide primers to provide a sufficient grip for the restriction enzyme. PfuUltra II fusion HS DNA polymerase enzyme (Stratagene, La Jolla, CA) was used to amplify the sequence. This fragment was then inserted in place on the pCMV-MS2bs-24X-cyto plasmid. All constructs were confirmed by direct DNA sequencing.

Table 1. Names and Sequences of Primers Used for the Construction of the pCMV-5′UTR-apoB15%-3′UTR-MS2-24X-cyto Plasmid

primer name	primer sequence
5′ApaI-Forw	AGAGGGCCCTTATTCCCACCGGGACCT
BamH13′-Rev	AGAGGATCCCCGCCCCGACTCTAGATA

Transient Transfection. HepG2 cells were transiently cotransfected with 5.5 μg of pMS2-GFP-SV40 NLS plasmid and 14.5 μg of either pCMV-5′UTR-apoB15%-3′UTR-MS2-24X-cyto (reporter plasmid) or EF1a-β-globin mRNA-MS2bs plasmid (control) using Lipofectamine 2000 reagent (Invitrogen) and the reverse transfection method according to the manufacturer's recommendations. Transfected HepG2 cells were seeded on collagen-coated coverslips into six-well plates. Cells were kept at 37 °C for ∼16 h prior to insulin treatment.

Insulin Treatment. Sixteen hours post-transfection, the GFP signal was detected under an epifluorescence microscope (Zeiss). At this time, cells were serum starved and treated with insulin (100 nM) (Eli Lilly, Canada Inc., Toronto, ON) for 1, 4, 8, and 16 h.

Immunostaining Experiments. Cells were rinsed twice with PBS and fixed immediately using 4% paraformaldehyde in a 1× PBS solution for 15 min. Paraformaldehyde was then aspirated, and −20 °C methanol was added for 10 min. Cells were washed with 1× PBS and blocked in 1× PBS containing 5% fetal bovine serum (FBS) and 0.02% sodium azide (Sigma Aldrich, St. Louis, MO) for 1 h. Mouse anti-human GE-1/hedls antibody (P70 S60 kinase α) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was diluted 1:1000 in the blocking solution and added to the fixed cells for 1 h. After two washes with 1× PBS (10 min each), donkey anti-mouse rhodamine red secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was diluted 1000-fold in the blocking solution and added for 1 h. Three washes with 1× PBS were performed (10 min each), and cells were incubated with DAPI (4′,6-diamidino-2-phenylindole·2HCl) (1 mg/mL stock, 1:1000 dilution) (Santa Cruz Biotechnology, Inc.) for 15 min to stain the nucleus. After three washes with 1× PBS (10 min each),

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cells were mounted in Dako flurescent mounting medium (Dako North America, Inc., Carpinteria, CA), and slides were protected from light and kept at 4 °C.[24] All staining steps were performed on a gentle shaker at room temperature.

Confocal Microscopy Imaging. Images were acquired from fixed cells using spinning disk confocal microscopy (Zeiss) and velocity software. DAPI confocal, GFP confocal, and Cy3 confocal laser channels were used to detect the nucleus, GFP signal, and P bodies, respectively, with sensitivities of ~200 and exposures not longer than 1 s. Images were then deconvolved to remove the out of focus light (90% similarity between the deconvolved image and the original; 20 rounds of deconvolution). Sensitivity is a feature of the camera referring to the electronic gain on the electron multiplier CCD. To obtain fluorescent images of cells, we used a special high-sensitivity camera (Hamamatsu model 9100) to acquire images.

Statistical Analysis of Imaging Data. Velocity software version 5 was used to quantify the colocalization of P bodies and apoB mRNA at different time points, and the significance of the findings was validated by the Pearson's correlation coefficient.

Polysome Profiling. To determine the effect of insulin on the global translation of HepG2 cells, we obtained polysomal profiles of HepG2 cells at different time points of insulin treatment. Cells were serum starved briefly and treated with 100 nM insulin for 15 min, 1 h, 4 h, or 16 h. To stabilize the polysomes, cycloheximide (CHX) (Sigma Aldrich) was added to the medium at a final concentration of 100 μg/mL half an hour prior to harvest. After three washes with ice-cold 1× PBS and 100 μg/mL CHX, cells were lysed in a freshly made lysis buffer [0.015 M Tris-HCl (pH 8.0), 0.3 M NaCl, 0.005 M $MgCl_2$, 1% Triton X-100, 0.0005 M dithiothreitol (DTT), 1 mg/mL heparin sodium salt, and 0.1 mg/mL CHX] for 10 min on ice. Cell lysates were centrifuged at 12000 rpm and 4 °C for 10 min. Two milliliters of the supernatant containing the cytoplasmic extract was layered over 12 to 55% linear sucrose (Sigma Aldrich) gradients. Sucrose gradients were freshly made using a sucrose gradient maker (BioComp Gradient Mate model 117, BioComp Instruments, Inc.) in polysome buffer containing 0.02 M Tris-HCl (pH 8.0), 0.14 M KCl, 0.005 M $MgCl_2$, 0.0005 M DTT, 0.5 mg/mL heparin sodium salt, and 0.1 mg/mL CHX. Gradients were subjected to ultracentrifugation at 40000 rpm and 4 °C for 2.5 h in a Beckman SW41 Ti rotor. Gradients were then fractionated using an ISCO gradient fractionation system equipped with a UA-6 detector following an upward displacement method. Four 3 mL fractions were obtained from each gradient and stored at −80 °C. While the fraction on top contained monosomes and ribosomal subunits, heavy polysomes were found in the bottom layers. The fourth fraction contained the heaviest polysomes. Total RNA was then extracted from each fraction and subjected to real-time PCR (RT-PCR) analysis.

RNA Isolation and RT-PCR. To assess the amount of apoB mRNA associated with polysomes, which is an indication of translational activity, total RNA was isolated from each fraction using Trizol (Invitrogen) and RNeasy minikit (Qiagen, Mississauga, ON) reagents following the manufacturer's suggested procedure. Two micrograms of total RNA from each fraction was reverse transcribed using a high-capacity cDNA reverse transcription kit with RNase inhibitor (Applied Biosystems, Foster City, CA) and random hexamer primers according to the manufacturer's protocol. RT-PCR analysis was performed on an ABI Prism 7700 instrument (Applied Biosystems) using Power SYBR Green reagent (Applied Biosystems), apoB, internal, and positive control primers. Threshold cycle (Ct) values were obtained in duplicate for each sample. The mean of four experiments in duplicate was calculated. Melting curves were analyzed to ensure that fluorescence signals reflected only specific amplicons.

Statistical Analysis of the RNA Distribution. For every primer set, the Ct value of each fraction was subtracted from the maximum Ct value of the four fractions. This was called ΔCt. Because after each cycle the amount of DNA is doubled theoretically, to calculate the relative changes, $2^{\Delta Ct}$ was calculated. The distribution of mRNA in each fraction across the entire fractions was then graphed as the percentage of the mRNA in each fraction divided by the total mRNA (the sum of all four fractions).[25]

RESULTS

Detection of P Bodies in HepG2 Cells. We made use of a strong interaction between the bacteriophage MS2 capsid protein and MS2 binding site to visualize apoB mRNA and to examine the influence of insulin on the colocalization of apoB mRNA with P bodies. HepG2 cells were transiently cotransfected with two plasmids (Figure 1). The first was a chimeric DNA construct that was transcribed to a reporter mRNA containing 15% of the full-length apoB linked to its UTRs (5′UTR-apoB15%-3′UTR) that was fused to the MS2 binding site. The MS2 binding site included 24 tandem repeats of 19-nucleotide RNA stem−loop structures. This RNA can be recognized by phage capsid MS2 protein that was encoded by the second plasmid. The second plasmid made encoded a MS2 protein fused to GFP with a nuclear localization signal at the C-terminus. Upon expression, the GFP−MS2 coat protein binds the stem−loop structure as a dimer.[26] This allows the detection of a specific RNA that is bound to the stem−loop structure. The presence of NLS helped in the elimination of false positive signals in the cytoplasm by sequestering any unbound MS2−GFP protein in the nucleus. Using the spinning disk confocal microscopy technique allowed us to study the colocalization of apoB mRNA with P bodies at different time points of insulin treatment.

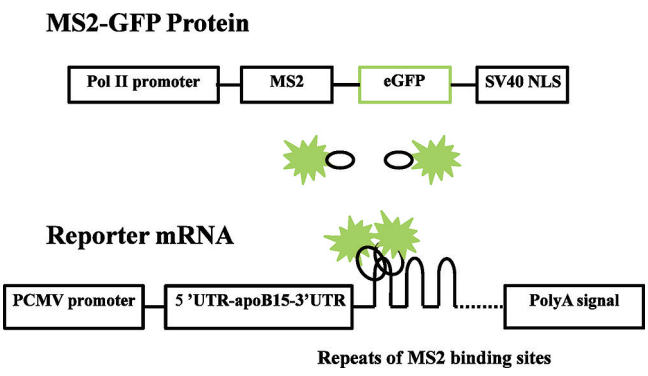

Figure 1. Construction map of MS2−GFP and apoB mRNA reporter plasmids. A system composed of two plasmids allowed us to detect apoB mRNA in HepG2 cells. In this system, one plasmid encoded an MS2 binding protein fused to GFP (top) and the other was transcribed to a reporter mRNA containing part of the apoB mRNA sequence followed by MS2 binding sites (bottom).

To detect P bodies in HepG2 cells, we applied mouse anti-human GE-1/hedls antibody to fixed cells. This antibody identifies a major protein component of P bodies, human enhancer of decapping larger subunit. Donkey anti-mouse rhodamine red-conjugated antibody was used to visualize the signal (Figure 2A). To confirm this, we also utilized an antibody against a second P body (PB) protein, hDcp1a (human decapping protein 1), and very similar results were obtained (Figure 2B). To further validate that these are in fact P bodies, cells were subjected to 1 mM puromycin for 30 min (Figure 2C). Puromycin is known to promote the formation of P bodies by disrupting the translational machinery. Results from eight different slides indicated that puromycin significantly increased P body count and size by 99 and 25%, respectively ($P < 0.005$).

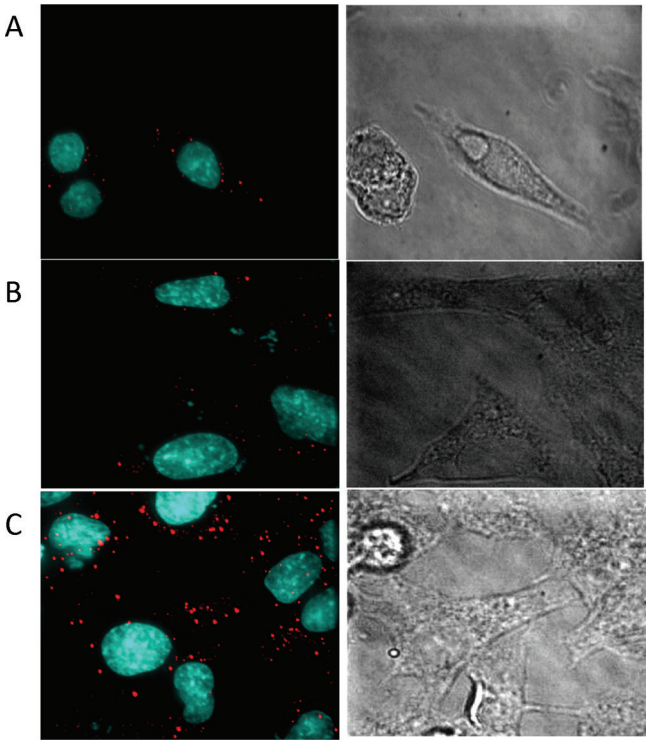

Figure 2. Detection of P bodies in the cytoplasm of HepG2 cells. HepG2 cells were fixed and immunostained for two essential protein components of P bodies, hedls (A) and hDcp1a (B) (red). DAPI was used to stain the nucleus (blue). Puromycin was used to further verify the detection of P bodies, and HepG2 cells were treated with 1 mM puromycin for 0.5 h, fixed, and immunostained for hDCP1 (C). DIC (differential interference contrast) images of the same fields are shown at the right.

Insulin Induces Colocalization of ApoB mRNA with P Bodies. We transiently cotransfected HepG2 cells with pCMV-5′UTR-ApoB15%-3′UTR-MS2bs-24X-cyto and MS2-GFP-NLS constructs as described above (Figure 1). Transfected cells were then treated with insulin at different time points, fixed, and immunostained for P body marker, GE-1/hedls. Upon expression, MS2 binding protein fuses to GFP by recognizing its binding site on the reporter mRNA as illustrated in Figure 1. Because of the presence of a nuclear localization signal downstream of the GFP, any unbound MS2−GFP protein was sequestered in the nucleus. We used the spinning disk confocal microscopy technique to image the colocalization of apoB mRNA with P bodies. Long-term insulin treatment (4, 8, and 16 h) (Figure 3C−E) significantly increased the level of colocalization of apoB mRNA with P bodies. On the other hand, acute insulin treatment (1 h) did not have any significant effect on the localization of apoB mRNA with P bodies (Figure 3B).

To compare the colocalization of apoB mRNA with P bodies at different time points of insulin treatment, Velocity software version 5 was used. Pearson's correlation coefficients were assessed on the basis of the intensity of green and red pixels that overlapped as an indication of the colocalization. A number between 0 and 1 was assigned to each cell, with 0 indicating no colocalization and 1 representing complete colocalization. Numbers obtained from eight different slides were averaged and graphed as represented in Figure 3F. Our data suggested that insulin strongly promotes apoB mRNA localization with P bodies after treatment for 4, 8, and 16 h.

Insulin Does Not Affect the Colocalization of β-Globin mRNA with P Bodies. β-Globin mRNA has already been shown to localize in P bodies.[23] A plasmid containing β-globin mRNA fused to the MS2 binding site and the MS2−GFP−NLS construct were co-expressed in HepG2 cells. As shown in Figure 4, although there was marked colocalization of β-globin mRNA with the P body marker, no significant change was observed following insulin treatment. Thus, in contrast to apoB mRNA, β-globin mRNA showed a high degree of colocalization with P bodies independent of insulin (Figure 4F).

The Time Course of Insulin-Induced ApoB mRNA Colocalization with P Bodies Coincides with a Reduced Level of Association of ApoB mRNA with Polysomes. We also determined the effect of insulin on the association of apoB mRNA with polysomes (a direct indicator of translational activity) under the same time course conditions used to assess localization in RNA granules. Polysomal profiles were obtained by fractionating monosomes, light polysomes, and heavy polysomes using standard sucrose gradient fractionation methods. ApoB mRNA levels in each fraction were subsequently measured using RT-PCR. The relative percentage of apoB mRNA in each fraction was calculated and graphed. Data from four independent experiments suggested that very short-term insulin treatments (15 min and 1 h) increased the level of association of apoB mRNA with polysomes (Figure 5), while long-term insulin exposure (4 and 16 h) showed an inhibitory effect on the translation of apoB and shifted the mRNA toward the lighter polysomes and monosome-rich fractions (Figure 5). The time course of these changes in polysomal association of apoB mRNA closely coincided with the changes observed above (Figure 3) in PB colocalization, suggesting that insulin may suppress apoB mRNA translation by reducing the level of polysomal association and increasing the level of mRNA storage in the form of RNA granules.

Under Conditions That Inhibit ApoB mRNA Polysomal Association, Insulin Acutely Stimulates Global Translation. As a control, we also examined the effect of insulin on global translation in HepG2 cells by performing detailed polysomal profiling. As shown in Figure 6, short-term insulin treatment (15 min) promoted global translation, as depicted by the rightward shift of the polysomal profile (indicating the formation of heavy polysomes), similar to its effect on apoB mRNA. However, after insulin treatment for 4 h, although the association of apoB mRNA with polysomes was inhibited, the level of global translation was noticeably increased as indicated

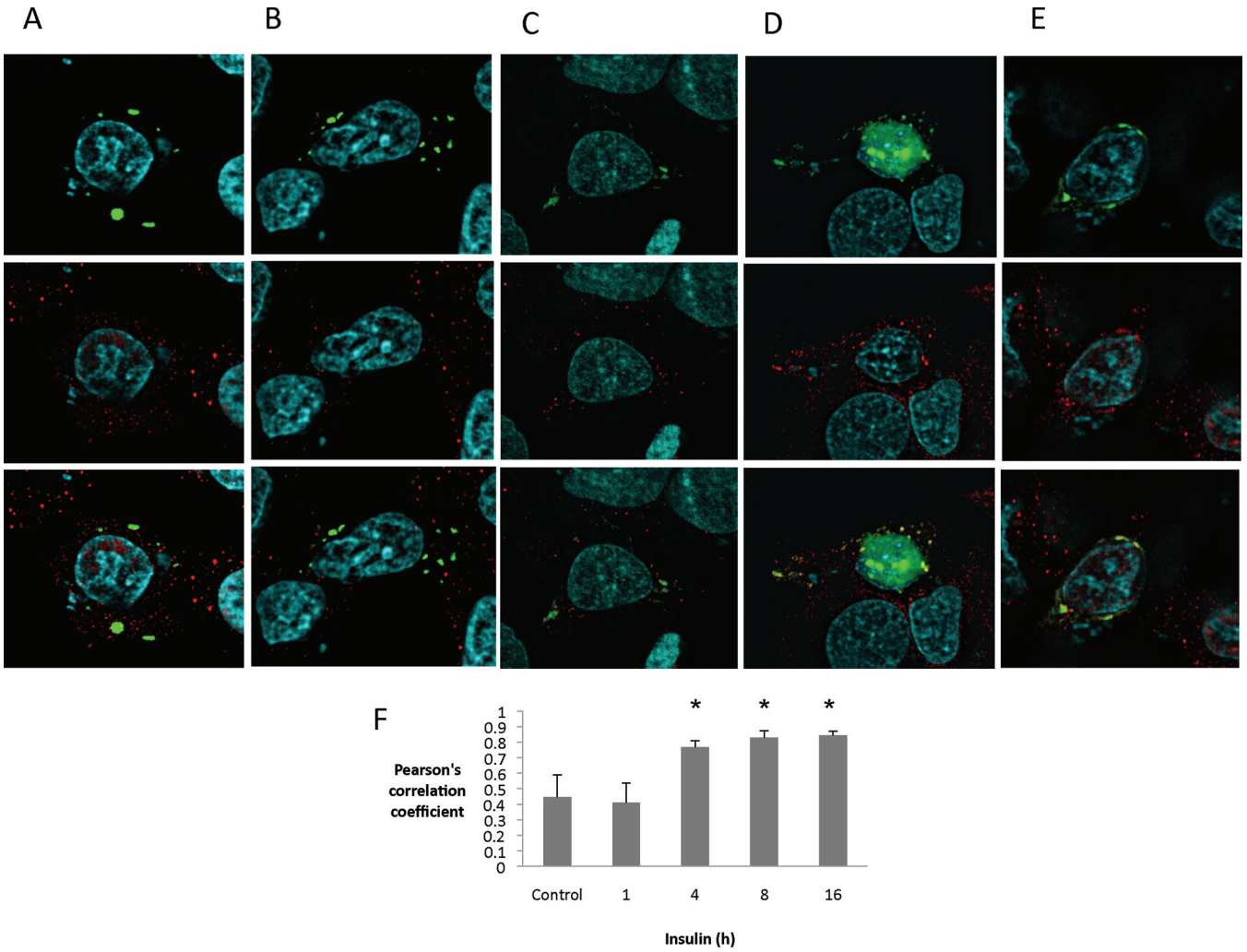

Figure 3. Visualizing apoB mRNA traffic in HepG2 cells. Insulin-induced colocalization with P bodies. HepG2 cells were transiently cotransfected with pCMV-5′UTR-ApoB15%-3′UTR-MS2bs-24X-cyto and MS2-GFP-NLS plasmids. Sixteen hours post-transfection, cells were serum sratved briefly (A) and treated with insulin for 1 (B), 4 (C), 8 (D), and 16 h (E). Cells were then fixed and immunostained for hedls, one of the main protein components of P bodies. Green represents apoB mRNA; P bodies are colored red, and DAPI was used to stain the nucleus (blue). Panel F shows the quantification of apoB mRNA colocalization with P bodies. Velocity software version 5 was used to measure Pearson's correlation coefficients. A number between 0 and 1 was allotted to each cell, with 0 indicating no colocalization and 1 specifying perfect colocalization. Asterisk indicates statistically significant increase compared to control.

by the presence of heavy polysomes. After 16 h, the effect of insulin on global translation was lost, while its inhibition of apoB mRNA translation persisted (Figure 6).

■ DISCUSSION

Although insulin normally activates global translation of cellular protein synthesis, it has a specific inhibitory effect on apoB mRNA translation. This suggests that insulin induces a unique signaling cascade that leads to specific inhibition of apoB mRNA translation despite global translational stimulation. We have previously demonstrated that insulin modulates apoB mRNA translation via changes in the binding of a *trans*-acting 110 kDa protein factor to the 5′UTR.[10] This putative RNA-binding protein (termed p110) was found to specifically bind the 5′UTR of apoB mRNA, with its level of binding reduced in the presence of insulin.[10] This observation highlights the importance of RNA−protein interactions that regulate the fate and activity of apoB mRNA intracellularly. There is now a growing body of evidence that eukaryotic mRNAs (particularly those with longer half-lives) exist in association with protein complexes in the form of RNA granules that can govern both mRNA decay and translational activity. The focus of this study was to investigate the potential role of cytoplasmic RNA granules (P bodies) in insulin-mediated translational regulation of apoB. P bodies control the translation of many mRNAs in eukaryotic cells, and we postulated that apoB mRNA is subcellularly compartmentalized in the form of ribonucleoprotein complexes in RNA granules, which act as a reservoir for translatable mRNA, a process that can potentially be inhibited by insulin.

Confocal studies revealed that long-term insulin exposure promotes the colocalization of apoB mRNA with P bodies with increases of 72% (after 4 h), 85% (after 8 h), and 89% (after 16 h) in the level of PB colocalization compared to that of non-insulin-treated controls. However, a shorter (1 h) insulin treatment did not appear to induce observable changes in PB colocalization. The mechanisms behind the delay in the insulin response is unclear but may suggest the need for fresh synthesis of one or more factors involved in the traffic of apoB mRNA

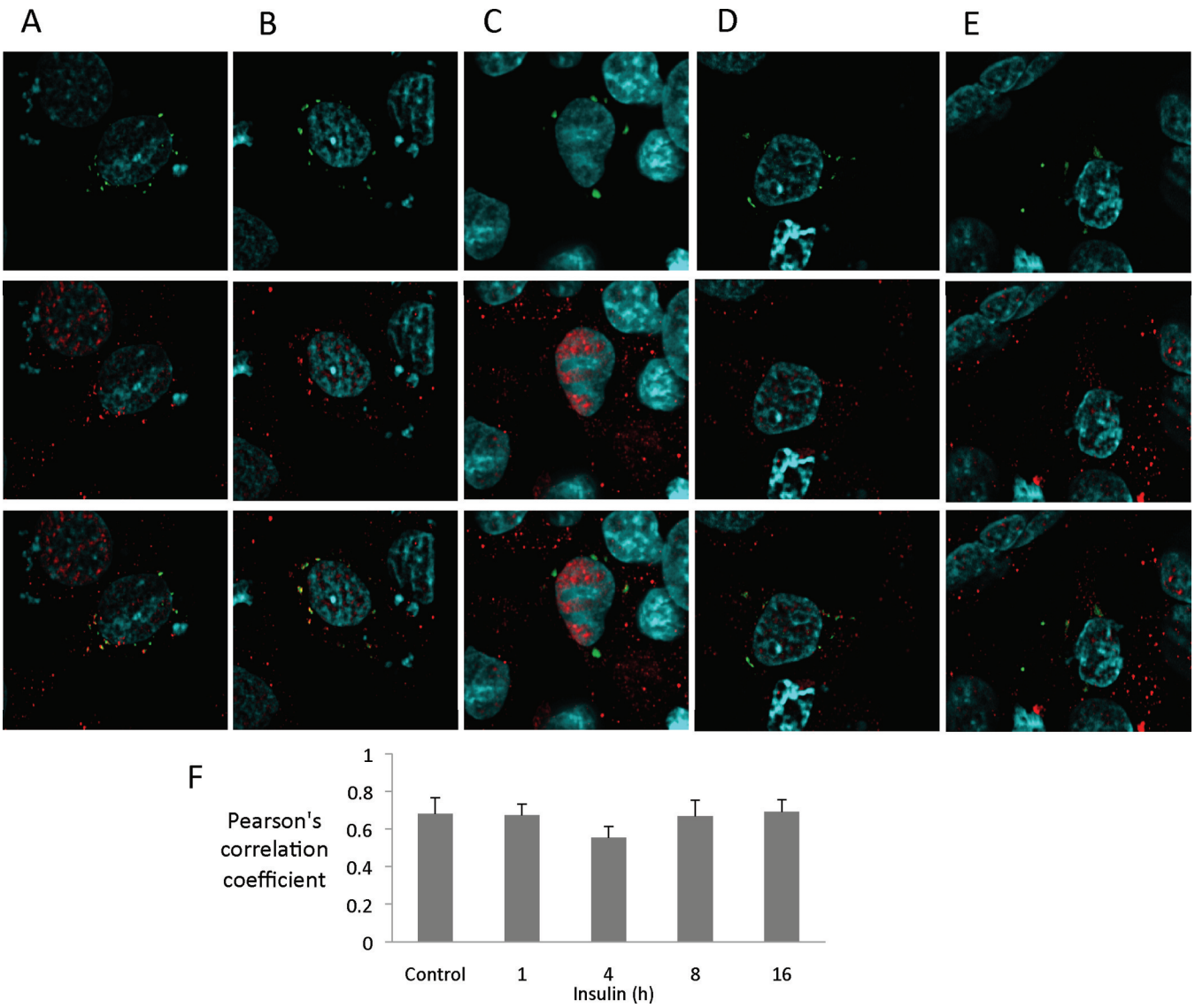

Figure 4. Effect of insulin on the colocalization of β-globin mRNA with P bodies. HepG2 cells were cotransfected with EF1a-β-globin mRNA-MS2-bs and MS2-GFP-NLS plasmids; cells were then serum starved briefly (A) and treated with insulin for 1 (B), 4 (C), 8 (D), and 16 h (E). Cells were then fixed and immunostained for hedls, one of the protein components of P bodies. Green represents β-globin mRNA; P bodies are colored red, and DAPI was used to stain the nucleus (blue). Panel F shows quantification of β-globin mRNA colocalization with P bodies. Velocity software version 5 was used to calculate Pearson's correlation coefficients. A number between 0 and 1 was allotted to each cell, with 0 indicating no colocalization and 1 specifying perfect colocalization.

into RNA granules. As a control, when HepG2 cells were transfected with the pMS2-GFP-NLS plasmid alone, the entire GFP signal was confined to the nucleus because of the presence of a nuclear localization signal at the C-terminus of the protein. In these experiments, we employed a recently established method that has been successfully used to visualize exogenously expressed mRNAs in living cells. In this technique, a fluorescent protein, such as GFP, is fused to RNA phage MS2 coat protein with a nuclear localization signal at the C-terminus.[27] The RNA of interest is constructed to contain tandem repeats of the specific phage RNA sequence that binds MS2 coat protein. This method was first utilized by Bertrand and colleagues to monitor the asymmetrical movement of ASH1 mRNA in dividing *Saccharomyces cerevisiae* cells[27] but has since been applied to several mammalian cell lines. Fusco et al. and Rook et al. employed this approach to monitor the movement of the cytoplasmic RNA particles in COS and nervous cells.[22,28] Forrest and Gavis examined the dynamic colocalization of endogenous nanos RNA in *Drosophila* oocytes,[29] and Kedersha et al. monitored the presence of specific mRNA transcripts in SGs and P bodies.[23] In addition, this method has been applied to study retroviral RNA trafficking.[30,31]

It is important to note that although Singer's method is a very useful tool in imaging an exogenous mRNA in living cells it has some limitations. For example, to obtain a strong GFP signal the presence of 24 repeats of MS2 binding sites is mandatory,[22] and it is not possible to obtain a good signal using fewer repeats of MS2 binding sites. Because two molecules of GFP interact with each of the stem–loop structures,[22] a large amount of GFP accumulates in a small area. This in fact increases the intensity of the signal at the expense of lowering the sensitivity. As observed in confocal images, apoB mRNA and PB marker did not colocalize in all P bodies. This is expected because each eukaryotic cell holds many P bodies

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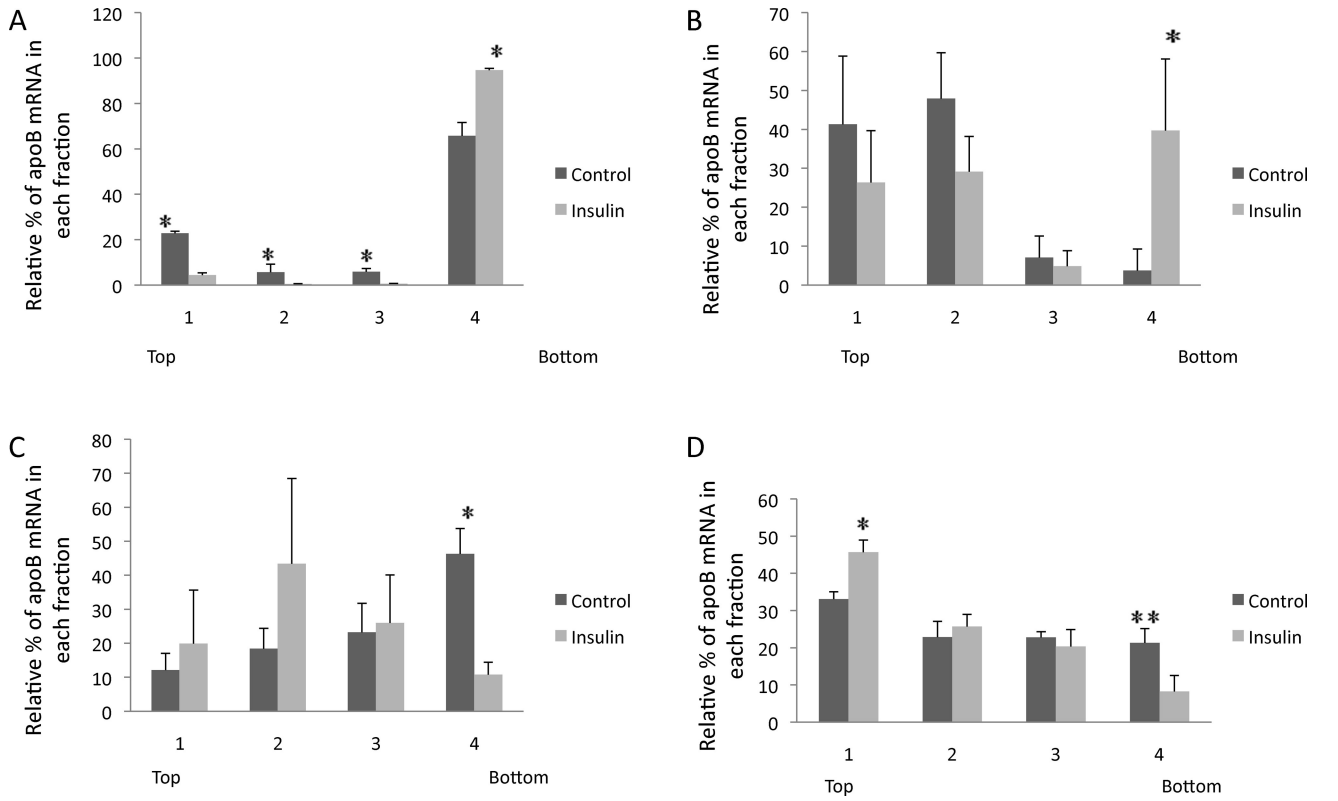

Figure 5. Effect of short-term and long-term insulin treatment on association of apoB mRNA with polysomes. HepG2 cells were serum starved (control) and treated with insulin for 15 min (A) and 1 (B), 4 (C), and 16 h (D). Cytoplasmic extracts of HepG2 cells were density fractionated at 40000 rpm for 2.5 h. The relative percentage of apoB mRNA in each fraction was then measured by RT-PCR. (Fraction 1 was the lightest top fraction, and fraction 4 contained the heaviest polysomes at the bottom of the gradient.) Asterisks denote *P* values of <0.05.

containing a variety of different kinds of mRNAs. A single PB does not contain all mRNAs, and also a single species of mRNA does not exist in all P bodies. Also, in some of the images we obtained, the GFP signal was observed in both the nucleus and the cytoplasm. This was due to the high level of MS2−GFP protein expression. Because of the presence of the NLS, the surplus GFP that was not bound to the mRNA was sequestered in the nucleus. This in fact was predictable from the onset and did not interfere with the interpretation of the imaging data.

The monoclonal antibody used to recognize cytoplasmic P bodies exhibited some nuclear staining in addition to the cytoplasm. This is due to the reactivity of this antibody with p70 S6 kinase protein. The double specificity of this antibody was confirmed using recombinant hedls, and this antibody was used to visualize P bodies in p70 S6 kinase knockout cells.[24] The anti-hedls antibody readily detects cytoplasmic P bodies, and most of the non-hedls signal is nuclear and did not impede the recognition of cytoplasmic P bodies.[24]

To determine whether the time course of insulin-mediated colocalization of apoB mRNA with P bodies coincides with alterations in the translational activity of the message, we also conducted sucrose gradient fractionation of polysomes and measured apoB mRNA levels associated with translationally active polysomes using RT-PCR. Our results suggested that insulin initially increases the rate of apoB mRNA translation at 15 min and 1 h, very similar to its effect observed on global translation. Interestingly, however, following a longer insulin exposure (4 h), apoB mRNA shifted toward lighter polysomes and monosome fractions, suggesting inhibition of translational activity. At the same time point, global mRNA translation was still stimulated by insulin treatment. With an even longer insulin treatment (16 h), both apoB mRNA association with heavy polysomes and total global polysomal activity were reduced. These data suggest that apoB mRNA translation is uniquely inhibited by insulin under conditions that stimulate global mRNA translation. The time course of translational inhibition correlates with movement of apoB mRNA into cytoplasmic P bodies.

The mechanism controlling the movement of apoB mRNA into cytoplasmic RNA granules is currently unknown but is likely governed by specific binding to *trans*-acting RNA binding proteins. Mature mRNAs in the cytoplasm of eukaryotic cells are associated with a complex network of ribonucleoprotein particles (mRNPs).[32,33] Early studies of mRNPs showed the presence of two major proteins,[34] a 70 kDa poly(A) binding protein (PABP)[35] and a 50 kDa protein (p50) responsible for the repressed, nonactive state of mRNAs, such as globin mRNA within free mRNP particles.[36] More recent studies have identified a large number of other protein components of mRNPs, including RNA binding proteins, RNA helicases, and translational factors.[37]

All RNA granules contain translationally silenced mRNAs. New evidence indicates a dynamic interaction between these RNA granules and translationally active polysomal mRNAs,[38] suggesting that the availability of some mRNAs could be regulated by the rate of release from translationally silenced mRNAs within RNA granules. It is currently unknown whether apoB mRNA translation can be controlled by the release of translatable apoB mRNA transcripts from cytoplasmic stores of mRNPs that are translationally repressed. However, the long

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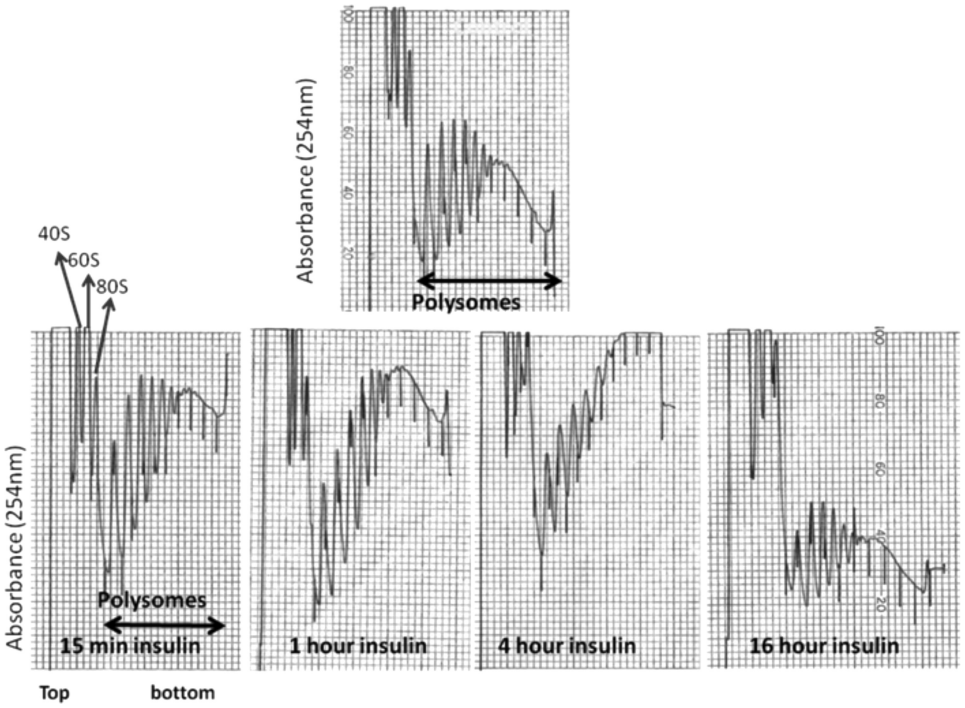

Figure 6. Insulin acutely stimulates global mRNA translation in HepG2 cells. To assess the general effect of insulin on the cellular protein translation, HepG2 cells were serum starved briefly and treated with insulin for 15 min and 1, 4, and 16 h. Cytoplasmic extracts were then subjected to density fractionation at 40000 rpm for 2.5 h, and polysome profiles were obtained. Each panel shows the abundance of ribosomal subunits and monosomes (left; top of the gradient) and polysomes of various sizes (the heaviest polysomes containing multiple ribosomes sink to the bottom of the gradient). Different peaks represent different sized polysomes (mRNAs complexed to one, two, or more polysomes). The presence of heavy polysomes is a direct indicator of higher translational activity.

half-life of apoB mRNA (16 h) clearly suggests that a significant proportion of the message may be stored in the cell prior to translation. Interestingly, apoB mRNA polysome complexes have been reported to exhibit unusual physical properties and exhibit unique sedimentation behaviors more characteristic of nonpolysomal mRNPs, [39] further suggesting the existence of apoB mRNA in RNA granules. We thus hypothesize that certain stimuli such as insulin may inhibit apoB mRNA translation by reducing the rate of release of translatable mRNA transcripts from stored mRNPs.

We and others have previously shown regulation of apoB secretion by multiple factors and mechanisms, including TNF-α, LXR-α, NF-κB, MEK-ERK, and several other mediators. It is currently unknown whether the underlying mechanisms in some or all of these cases involve modulation of localization of apoB mRNA to P bodies. We postulate that those factors that modulate insulin signal transduction may act predominantly at the translational level, while others may function post-translationally by altering apoB stability and degradation. Further studies are needed to delineate the role of apoB mRNA traffic in the control of apoB secretion under various metabolic conditions.

In conclusion, our data suggest that long-term insulin treatment may decrease the rate of apoB mRNA translation by promoting the localization of the mRNA into P bodies. The cellular response to hormonal stimuli is normally rapid, and like other hormonal responses, hepatocytes respond to insulin promptly by decreasing the rate of secretion of apoB protein via changes in protein stability and/or degradation. With longer insulin exposures, hepatocytes reduce the rate of apoB synthesis by reprogramming apoB mRNA and forcing the translationally competent apoB mRNA toward P bodies for storage.

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ABBREVIATIONS

apoB, apolipoprotein B; GFP, green fluorescent protein; IRES, internal ribosome entry site; mRNP, messenger ribonucleoprotein; PB, processing bodies; SG, stress granule; UTR, untranslated region; VLDL, very low-density lipoprotein.

REFERENCES

(1) Hansson, G. K., and Libby, P. (2006) The immune response in atherosclerosis: A double-edged sword. *Nat. Rev. Immunol. 6*, 508–519.

(2) Fisher, E. A., and Ginsberg, H. N. (2002) Complexity in the secretory pathway: The assembly and secretion of apolipoprotein B-containing lipoproteins. *J. Biol. Chem. 277*, 17377–17380.

(3) Pullinger, C. R., North, J. D., Teng, B. B., Rifici, V. A., Ronhildde Brito, A. E., and Scott, J. (1989) The apolipoprotein B gene is

constitutively expressed in HepG2 cells: Regulation of secretion by oleic acid, albumin, and insulin, and measurement of the mRNA half-life. *J. Lipid Res. 30*, 1065−1077.

(4) Dashti, N., Williams, D. L., and Alaupovic, P. (1989) Effects of oleate and insulin on the production rates and cellular mRNA concentrations of apolipoproteins in HepG2 cells. *J. Lipid Res. 30*, 1365−1373.

(5) Sparks, J. D., and Sparks, C. E. (1990) Insulin modulation of hepatic synthesis and secretion of apolipoprotein B by rat hepatocytes. *J. Biol. Chem. 265*, 8854−8862.

(6) Adeli, K., and Theriault, A. (1992) Insulin modulation of human apolipoprotein B mRNA translation: Studies in an in vitro cell-free system from HepG2 cells. *Biochem. Cell Biol. 70*, 1301−1312.

(7) Levy, E., Sinnett, D., Thibault, L., Nguyen, T. D., Delvin, E., and Menard, D. (1996) Insulin modulation of newly synthesized apolipoproteins B-100 and B-48 in human fetal intestine: Gene expression and mRNA editing are not involved. *FEBS Lett. 393*, 253−258.

(8) Pan, M., Liang, J., Fisher, E. A., and Ginsberg, H. N. (2000) Inhibition of translocation of nascent apolipoprotein B across the endoplasmic reticulum membrane is associated with selective inhibition of the synthesis of apolipoprotein B. *J. Biol. Chem. 275*, 27399−27405.

(9) Pontrelli, L., Sidiropoulos, K. G., and Adeli, K. (2004) Translational control of apolipoprotein B mRNA: Regulation via cis elements in the 5′ and 3′ untranslated regions. *Biochemistry 43*, 6734−6744.

(10) Sidiropoulos, K. G., Pontrelli, L., and Adeli, K. (2005) Insulin-mediated suppression of apolipoprotein B mRNA translation requires the 5′ UTR and is characterized by decreased binding of an insulin-sensitive 110-kDa 5′ UTR RNA-binding protein. *Biochemistry 44*, 12572−12581.

(11) Sidiropoulos, K. G., Meshkani, R., Avramoglu-Kohen, R., and Adeli, K. (2007) Insulin inhibition of apolipoprotein B mRNA translation is mediated via the PI-3 kinase/mTOR signaling cascade but does not involve internal ribosomal entry site (IRES) initiation. *Arch. Biochem. Biophys. 465*, 380−388.

(12) Sidiropoulos, K. G., Zastepa, A., and Adeli, K. (2007) Translational control of apolipoprotein B mRNA via insulin and the protein kinase C signaling cascades: Evidence for modulation of RNA-protein interactions at the 5′UTR. *Arch. Biochem. Biophys. 459*, 10−19.

(13) Anderson, P., and Kedersha, N. (2009) RNA granules: Post-transcriptional and epigenetic modulators of gene expression. *Nat. Rev. Mol. Cell Biol. 10*, 430−436.

(14) Anderson, P., and Kedersha, N. (2006) RNA granules. *J. Cell Biol. 172*, 803−808.

(15) Teixeira, D., Sheth, U., Valencia-Sanchez, M. A., Brengues, M., and Parker, R. (2005) Processing bodies require RNA for assembly and contain nontranslating mRNAs. *RNA 11*, 371−382.

(16) Liu, J., Valencia-Sanchez, M. A., Hannon, G. J., and Parker, R. (2005) MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nat. Cell Biol. 7*, 719−723.

(17) Pillai, R. S., Bhattacharyya, S. N., Artus, C. G., Zoller, T., Cougot, N., Basyuk, E., Bertrand, E., and Filipowicz, W. (2005) Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science 309*, 1573−1576.

(18) Martin, K. C., and Ephrussi, A. (2009) mRNA localization: Gene expression in the spatial dimension. *Cell 136*, 719−730.

(19) Eulalio, A., Behm-Ansmant, I., and Izaurralde, E. (2007) P bodies: At the crossroads of post-transcriptional pathways. *Nat. Rev. Mol. Cell Biol. 8*, 9−22.

(20) Franks, T. M., and Lykke-Andersen, J. (2008) The control of mRNA decapping and P-body formation. *Mol. Cell 32*, 605−615.

(21) Shyu, A. B., Wilkinson, M. F., and van Hoof, A. (2008) Messenger RNA regulation: To translate or to degrade. *EMBO J. 27*, 471−481.

(22) Fusco, D., Accornero, N., Lavoie, B., Shenoy, S. M., Blanchard, J. M., Singer, R. H., and Bertrand, E. (2003) Single mRNA molecules demonstrate probabilistic movement in living mammalian cells. *Curr. Biol. 13*, 161−167.

(23) Kedersha, N., Stoecklin, G., Ayodele, M., Yacono, P., Lykke-Andersen, J., Fritzler, M. J., Scheuner, D., Kaufman, R. J., Golan, D. E., and Anderson, P. (2005) Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. *J. Cell Biol. 169*, 871−884.

(24) Kedersha, N., and Anderson, P. (2007) Mammalian stress granules and processing bodies. *Methods Enzymol. 431*, 61−81.

(25) Bachand, F., Lackner, D. H., Bahler, J., and Silver, P. A. (2006) Autoregulation of ribosome biosynthesis by a translational response in fission yeast. *Mol. Cell. Biol. 26*, 1731−1742.

(26) Beckett, D., and Uhlenbeck, O. C. (1988) Ribonucleoprotein complexes of R17 coat protein and a translational operator analog. *J. Mol. Biol. 204*, 927−938.

(27) Bertrand, E., Chartrand, P., Schaefer, M., Shenoy, S. M., Singer, R. H., and Long, R. M. (1998) Localization of ASH1 mRNA particles in living yeast. *Mol. Cell 2*, 437−445.

(28) Rook, M. S., Lu, M., and Kosik, K. S. (2000) CaMKIIα 3′ untranslated region-directed mRNA translocation in living neurons: Visualization by GFP linkage. *J. Neurosci. 20*, 6385−6393.

(29) Forrest, K. M., and Gavis, E. R. (2003) Live imaging of endogenous RNA reveals a diffusion and entrapment mechanism for nanos mRNA localization in *Drosophila*. *Curr. Biol. 13*, 1159−1168.

(30) Basyuk, E., Boulon, S., Skou, P. F., Bertrand, E., and Vestergaard, R. S. (2005) The packaging signal of MLV is an integrated module that mediates intracellular transport of genomic RNAs. *J. Mol. Biol. 354*, 330−339.

(31) Moore, M. D., Nikolaitchik, O. A., Chen, J., Hammarskjold, M. L., Rekosh, D., and Hu, W. S. (2009) Probing the HIV-1 genomic RNA trafficking pathway and dimerization by genetic recombination and single virion analyses. *PLoS Pathog. 5*, e1000627.

(32) Keene, J. D. (2001) Ribonucleoprotein infrastructure regulating the flow of genetic information between the genome and the proteome. *Proc. Natl. Acad. Sci. U.S.A. 98*, 7018−7024.

(33) Spirin, A.S. (1966) "Masked" forms of mRNA. *Curr. Top. Dev. Biol. 1*, 1−38.

(34) Jain, S. K., Pluskal, M. G., and Sarkar, S. (1979) Thermal chromatography of eukaryotic messenger ribonucleoprotein particles on oligo (dT)-cellulose. Evidence for common mRNA-associated proteins in various cell types. *FEBS Lett. 97*, 84−90.

(35) Blobel, G. (1973) A protein of molecular weight 78,000 bound to the polyadenylate region of eukaryotic messenger RNAs. *Proc. Natl. Acad. Sci. U.S.A. 70*, 924−928.

(36) Minich, W. B., Maidebura, I. P., and Ovchinnikov, L. P. (1993) Purification and characterization of the major 50-kDa repressor protein from cytoplasmic mRNP of rabbit reticulocytes. *Eur. J. Biochem. 212*, 633−638.

(37) Angenstein, F., Evans, A. M., Ling, S. C., Settlage, R. E., Ficarro, S., Carrero-Martinez, F. A., Shabanowitz, J., Hunt, D. F., and Greenough, W. T. (2005) Proteomic characterization of messenger ribonucleoprotein complexes bound to nontranslated or translated poly(A) mRNAs in the rat cerebral cortex. *J. Biol. Chem. 280*, 6496−6503.

(38) Balagopal, V., and Parker, R. (2009) Polysomes, P bodies and stress granules: States and fates of eukaryotic mRNAs. *Curr. Opin. Cell Biol. 21*, 403−408.

(39) Chen, X., Sparks, J. D., Yao, Z., and Fisher, E. A. (1993) Hepatic polysomes that contain apoprotein B mRNA have unusual physical properties. *J. Biol. Chem. 268*, 21007−21013.