Distinct Roles of Heterogeneous Nuclear Ribonucleoprotein K and microRNA-16 in Cyclooxygenase-2 RNA Stability Induced by S100b, a Ligand of the Receptor for Advanced Glycation End Products

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Advanced glycation end products play major roles in diabetic complications. They act via their receptor RAGE to induce inflammatory genes such as cyclooxygenase-2 (COX-2). We examined the molecular mechanisms by which the RAGE ligand, S100b, induces COX-2 in monocytes. S100b significantly increased COX-2 mRNA accumulation in THP-1 monocytes at 2 h via mRNA stability. This was further confirmed by showing that S100b increased stability of luciferase-COX-2 3’-UTR mRNA. Chromatin immunoprecipitation and RNA immunoprecipitation revealed that S100b decreased occupancy of the DNA/RNA-binding protein, heterogeneous nuclear ribonucleoprotein K (hnRNPK), at the COX-2 promoter but simultaneously increased its binding to the COX-2 3’-UTR. S100b treatment promoted the translocation of nuclear hnRNPK to cytoplasm, whereas a cytoplasmic translocation-deficient hnRNPK mutant inhibited S100b-induced COX-2 mRNA stability. Small interfering RNA-mediated specific knockdown of hnRNPK blocked S100b-induced COX-2 mRNA stability, whereas on the other hand, overexpression of hnRNPK increased S100b-induced COX-2 mRNA stability. S100b promoted the release of entrapped COX-2 mRNA from cytoplasmic processing bodies, sites of mRNA degradation. Furthermore, S100b significantly down-regulated the expression of a key microRNA, miR-16, which can destabilize COX-2 mRNA by binding to its 3’-UTR. MiR-16 inhibitor oligonucleotides increased, whereas, miR-16 mimic oligonucleotides decreased COX-2 mRNA stability in monocytes, further supporting the inhibitory effects of miR-16. Interestingly, hnRNPK knockdown increased miR-16 binding to COX-2 3’-UTR, indicating a cross-talk between them. These new results demonstrate that diabetic stimuli can efficiently stabilize inflammatory genes via opposing actions of key RNA-binding proteins and miRs.

The cyclooxygenase-2 (COX-2) enzyme catalyzes the conversion of arachidonic acid to prostaglandins and related eicosanoids. COX-2 is significantly induced by cytokines and growth factors and can lead to the formation of potent inflammatory prostaglandins such as prostaglandin E2 as well as the vasodilatory and protective prostacyclin (1, 2). COX-2 and its proinflammatory products have been implicated in the pathogenesis of atherosclerosis and islet dysfunction related to diabetes (3–7). Several studies have demonstrated that diabetic stimuli such as high glucose and ligands of the receptor for advanced glycation end products (RAGE), including AGEs and S100b, can increase COX-2 and inflammatory genes in vascular cells and monocytes (8–13). This is associated with increased monocyte activation, migration, and adhesion to the endothelium, events related to inflammation and the pathogenesis of atherosclerosis. RAGE is a well studied cell surface receptor that interacts with multiple ligands including AGEs and proinflammatory peptides such as S100b belonging to the S100/calgranulin family (14, 15). Animal studies have demonstrated key roles for RAGE and its ligands including S100b and AGEs in inflammation and diabetic atherosclerosis (15–17).

Previously, we reported that RAGE ligation by exogenous AGEs or by S100b can increase COX-2 expression and activity in THP-1 monocytic cells as well as in primary human blood monocytes (PBMC). COX-2 induction by S100b after 4 h of treatment was transcriptionally regulated and involved key NF-κB elements in the distal COX-2 promoter (18). However, in those studies, we noted that S100b could also increase COX-2 mRNA expression at an early time point of 2 h via unknown post-transcriptional mechanisms and were therefore investigated in the current study.

Transcription of the COX-2 gene is regulated via cis-acting elements that bind to transcription factors such as NF-κB (19, 20).
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20). However, COX-2 can also be regulated by post-transcriptional mechanisms such as mRNA stabilization (21, 22). In mammalian cells, conserved adenosine- and uridine-rich (AU-rich) elements (AREs) found in the 3′-untranslated regions (UTRs) of many inflammatory genes, including COX-2, can promote rapid mRNA destabilization and degradation (23). AREs present within the COX-2 3′-untranslated region (COX-2 3′-UTR) regulate COX-2 mRNA decay through association with specific RNA-binding proteins such as RNA stability factor HuR and the translational repressor protein TIA-1 that target specific AREs. These RNA-protein (RNP) complexes regulate COX-2 mRNA degradation, stabilization, and translation (24–27).

Increasing evidence suggests that microRNAs (miRs) also play key roles in ARE-mediated destabilization of inflammatory gene mRNAs (28). Binding of miR-16 to AREs of mRNA transcripts like tumor necrosis factor-α, interleukin-6, and COX-2 could promote their degradation (29). Although several studies have examined transcriptional mechanisms regulating pathological genes under diabetic conditions, much less is known about the mechanisms involved in mRNA stability or the role of miRs in regulating ARE-containing mRNAs.

In the present study, we show for the first time that miR-16 and a key DNA/RNA-binding protein, namely heterogeneous nuclear ribonucleoprotein K (hnRNPK), play opposing roles in S100b-induced COX-2 mRNA stabilization. Our results suggest that, at early time points after treatment of monocytes with S100b, increased stabilization of COX-2 mRNA occurs via movement of hnRNPK from the COX-2 promoter to the 3′-UTR, whereas miR-16 binding to the 3′-UTR is simultaneously reduced. These data illustrate an efficient mechanism by which diabetic conditions can rapidly augment the stability of inflammatory genes without the need for increased transcription.

EXPERIMENTAL PROCEDURES

Materials—The following antibodies were used for the co-immunoprecipitation and Western blotting analyses: anti-GFP, anti-hnRNPK (Cell Signaling, Danvers, MA), anti-luciferase, and anti-α-actin (Sigma). miR-16 siRNAs, inhibitor and mimics were from Dharmacon (Lafayette, CO). Control and hnRNPK siRNAs were from Ambion (Austin, TX). Bovine brain S100b peptide was obtained from Calbiochem. RAGE antibody was from Dr. A. M. Schmidt, Columbia University, New York, NY.

Cell Culture and Treatments—Human THP-1 monocytic cells were obtained from the American Type Culture Collection or from the National Cell Culture Center (Minneapolis, MN) and cultured as described (18) in RPMI 1640 medium supplemented with heat-inactivated 10% fetal calf serum and 5.5 mM d-glucose (normal glucose, NG). Where indicated, THP-1 cells were treated with S100b (40 μg/ml) protein.

Isolation of PBMC—Human PBMC were isolated as described earlier using an Institutional Review Board-approved protocol (18). PBMC were plated in 6-well dishes (1 × 10^6 cells/well) and treated with S100b as indicated, and total RNA were isolated as described below.

RNA Preparation and Relative RT-PCR—Total RNA was isolated and reverse-transcribed, and relative RT-PCR was performed using gene-specific primers paired with QuantumRNA 18 S internal standards as described earlier (18, 30). The 5′ and 3′ primers for firefly luciferase (Luc) mRNA were 5′-ACGGATTACAGGGATTTAGTC-3′ and 5′-AGGCTCTCAGAAAACAGGCTCTTC-3′, respectively. Gene expression was expressed as -fold stimulation over NG after normalizing with paired 18 S RNA levels.

Analysis of microRNA (miR) Expression—The microRNAs were isolated using the mirVana quantitative RT-PCR miRNA detection kit as per the manufacturer’s protocol (Ambion, Austin, TX). MiR-16 levels were quantified with Has-miR-16 quantitative RT-PCR primer sets (Ambion). For Northern blot detection of miR-16, total RNA (40 μg) was fractionated on 12.5% 7M urea gel at 125 volts for 100 min. RNAs were transferred overnight using capillary transfer on to a Zeta probe membrane (Bio-Rad) in 2× SSC solution. The membranes were covalently cross-linked by UV irradiation for 10 min with UV Stratallinker 1800 (Stratagene, San Diego, CA). An antisense miR-16 oligonucleotide probe was labeled using T4-Poly nucleotide kinase and [γ-32P]ATP to be used as probe. Membranes were hybridized overnight with the probe in 7% SDS, 0.2 mM Na2PO4, pH 7.0, at 35 °C, washed three times with 2× SSC containing 0.1% SDS at 37 °C, once with 1× SSC containing 0.1% SDS, and once with 0.5× SSC containing 0.1% SDS at 37 °C. Washed membranes were exposed to x-ray film at −70 °C for overnight.

Measurement of mRNA Stability—For Luc mRNA stability assays, Luc reporter constructs containing the indicated COX-2 3′-UTR regions (21) were transiently transfected into THP-1 cells using Nucleofection equipment (Amaza). Cells were allowed to recover for 1 day and treated without or with S100b (40 μg/ml) for 2 h, and then transcription was stopped by adding actinomycin D (Act D, 10 μg/ml). Samples (5 × 10^6 cells/ml) were collected at the indicated time intervals in RNA-STAT60 reagent. Total RNA was isolated, and Luc mRNA levels were quantified by real-time PCR.

Real-time Quantitative PCR—Real-time quantitative PCRs (QPCRs) were performed using SYBR green reagent kits with gene-specific primers on the Applied Biosystems 7300 real-time PCR system (Foster City, CA). The 5′ and 3′ primers for Luc mRNA amplification were 5′-TCTCTGGCATGCCAGA-ACATCT-3′ and 5′-ACGGATTACAGGGATTTAGTC-3′, respectively. Primers for control GAPDH were forward, 5′-ATCT-3′ and reverse, 5′-CACTATTCTCCTCTGGAGATGGTG-3′. Each sample was run in triplicate. The relative RNA amount was calculated after normalization with the internal control glyceraldehyde-3-phosphate dehydrogenase according to the method described earlier (29).

Preparation of Cytoplasmic and Nuclear Extracts—Cytoplasmic and nuclear extracts from control (NG) and S100b (40 μg/ml)-treated THP-1 cells were prepared as described before (30).

Plasmid DNA/siRNA Transfections and Luc Assays—THP-1 cells (1.2 × 10^6/transfection) were transfected with indicated siRNA or miR oligonucleotides (1 nM each) and plasmids (1 μg each) using an Amaza Nucleofector (Amaza Biosystem) according to the manufacturer’s protocols and plated in six-well plates. Following an overnight recovery period, the
transfected cells were cultured in either medium alone (NG) or medium containing S100b (40 μg/ml) as indicated. Luc activity in cell lysates (20 ul) was determined using a Luc assay kit (Promega, Madison, WI) according to the manufacturer’s instructions.

RNA-Electrophoretic Mobility Shift Assay (RNA-EMSA) and UV Cross-linking—RNA-EMSA and UV-cross-linking were performed as described earlier (31). In vitro transcribed 32P-labeled COX-2 3’-UTR RNA and its deletion fragments (115, 420, and Δ1000 bp) were used as probes. After the UV cross-linking, the RNA body was digested with RNases (RNase A and RNase U2), and the proteins were fractionated on 12% SDS-PAGE. 32P-labeled RNP complexes visualized by autoradiography.

Chromatin Immunoprecipitation (ChIP) Assays—ChIP assays were performed as described earlier (32). ChIP-enriched DNA was analyzed by PCR and QPCR using COX-2 promoter-specific primers. Distal promoter region primers were: forward primer, 5’-CTTATGAGATGTCGACTA-3’, and reverse primer, 5’-CGAGAGCCCTGCAAGCTG-3’. Proximal promoter region primers were: forward primer, 5’-TGTCGGA-CCTGAGCGTGGAGA-3’, and reverse primer, 5’-GCCTGCTACCCAGAAGAACCT-3’. Data were normalized using input samples.

RNA-Immunoprecipitation (RNA-IP)—THP-1 cells were lysed using Buffer A (10 mM Tris-Cl, pH 7.9, 60 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol) containing 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and Complete protease inhibitors (Roche Applied Science). Lysates were clarified by centrifugation for 10 min at 12,000 rpm, and RNAs (Promega, 40 units/ml) was added. Cell lysates were immunoprecipitated using GFP or hnRNPK antibodies (2 μg each) and 30 μl of a 50% suspension of protein A-agarose (Upstate Biotechnology) for 2 h at RT. Then protein A agarose beads were collected by centrifugation at 1000 × g and washed four times with phosphate-buffered saline containing 150 mM NaCl. RNA was extracted from the beads by phenol-chloroform extraction and reverse-transcribed using a reverse transcriptase kit (Applied Biosystems, Foster City, CA). The resulting cDNA was amplified using the following primers for human COX-2: forward, 5’-ATCTACCCTCTCAAGTCC-3’, and reverse, 5’-TACCAAGGCGGAGGATTACCAG-3’.

Immuno-fluorescent Staining—THP-1 cells were treated with or without S100b as indicated, and at the end of the incubation period, cells were washed and immobilized on a polylysine-coated glass slide for 30 min. Cells were fixed in 4% paraformaldehyde for 30 min and permeabilized with phosphate-buffered saline containing 0.05% Triton X-100. The slides were then blocked (10 min) in phosphate-buffered saline containing 4% human albumin and immunostained with hnRNPK antibody (1:250 dilution) followed by appropriate Texas Red-conjugated secondary antibody (1:200 dilution). Slides were viewed using a fluorescent microscope (Olympus IX50 with DP70 camera).

In Situ Hybridization—THP-1 cells were transfected with GFP-Dcp1a expression plasmid and immobilized on a polylysine glass slide for 30 min. After fixing with paraformaldehyde (4%), cells were treated with 70% ethanol, rehydrated in 2× SSC, and used for hybridization. Cells were hybridized with 15 ng of the fluorescent oligonucleotides complementary to the COX-2 coding region in 50 μl of hybridization solution containing 2× SSC, 30% formamide,10% dextran sulfate, 0.2 mg/ml bovine serum albumin, and 250 μg/ml yeast tRNA. The sequence of the probe used was 5’-gTacctaggtttgtgaaag-T*agtctggcatcaatT*c-3’. Residues marked as T* are the amino-modified thymidines that were conjugated to Alexa Fluor 555. Slides were washed three times with 2× SSC and 30% formamide for 30 min at 37 °C. Slides were counterstained for DNA with DAPI (Sigma), mounted using antifade Vectashield (Vector Laboratories, Burlingame, California), and viewed under a fluorescence microscope.

In Vitro Pull-down Assays to Evaluate miR-16 Binding—Biotin UTP-labeled RNA of COX-2–3’-UTRs were prepared by in vitro transcription using T7 RNA polymerase for 2 h at 37 °C with 1:10 ratio of biotin-labeled UTP:UTP (Applied Biosystems, Foster City, CA). The RNA samples were then run on a 5% 7 M urea PAGE. RNA was extracted from the excised gel samples followed by phenol:chloroform extraction and precipitated with ethanol. The biotin-labeled RNA was further purified by another round of phenol:chloroform extraction and ethanol precipitation. MiR-16 and antisense miR-16 were 32P-labeled at the 5’ end with T4 polynucleotide kinase. These labeled miRs (5 × 10^6 cpm) were each added to a 30-μl reaction mixture containing 15 μl of THP-1 cell cytoplasmic extracts (CE), 1 mM ATP, 0.2 mM GTP, 40 units/ml RNasin, 5 mM EGTA, 30 μg/ml creatine kinase, 25 mM creatine phosphate, and 200 pmol of biotin-labeled RNA. The binding reaction was carried out for 10 min at 30 °C. The biotin-labeled RNA was pulled down with streptavidin-coated Dynabeads that had been preblocked with CEs. The amount of miR bound to the RNA was determined using a scintillation counter.

Data Analyses—Data are expressed as mean ± S.E. of multiple experiments. Paired Student’s t tests were used to compare two groups and analysis of variance with Dunnnett’s post tests for multiple comparisons.

RESULTS

COX-2–3’-UTR Plays an Important Role in S100b-mediated Stabilization of COX-2 mRNA—We previously reported that S100b could induce COX-2 expression at 4 h in human monocytes through NF-κB-mediated transcriptional activation of the COX-2 promoter (18) and at 2 h due to mRNA stability regulated via unknown mechanisms. We therefore evaluated the mechanisms involved in S100b-induced COX-2 mRNA stability. Human THP-1 monocytic cells were first transiently transfected with plasmids containing Luc reporter under the control of human COX-2 promoter regions (−7140/+127, −1430/+127, and −860/+127), stimulation with S100b for 2 h, and Luc activities were determined. S100b did not induce COX-2 promoter transcriptional activation at 2 h with any of these constructs (Fig. 1A), unlike at 4 h (18).

Evidence shows that COX-2 mRNA stability can be regulated by the presence of a 3’-UTR with multiple copies of AREs targeted by RNA-interacting factors (21, 24–27). We therefore hypothesized that the S100b-induced COX-2 stabilization may involve similar mechanisms. Because COX-2 mRNA can be
FIGURE 1. S100b stimulates COX-2 mRNA stability at 2 h in THP-1 monocytes. A, S100b does not induce transcriptional activation of the COX-2 promoter at 2 h. THP-1 cells were transfected with reporter plasmids containing Luc gene under the control of the indicated COX-2 promoter fragments or a control plasmid pGL3-Luc. On the next day, luciferase activity in cell lysates was determined without (NG) or with S100b (40 μg/ml) treatment for 2 h (mean ± S.E., n = 3). B, Luc reporter plasmids with COX-2–3′-UTR. Luc gene expressing from cytomegalovirus promoter was fused with full-length (1477 bp) or various deletions of COX-2 3′-UTR. Filled circles represent AU-rich (AUUUA) sequences. Curly brackets represent miR-16 binding sites. C, increased activity of Luc gene containing COX-2–3′-UTR by S100b. THP-1 cells were transfected with indicated Luc-COX-2–3′-UTR reporter plasmids (shown in B). On the next day, Luc activity in cell lysates was determined without (NG) or with S100b treatment for 2 h (mean ± S.E., *, p < 0.014, **, p < 0.001, versus NG, n = 3). D, S100b increases Luc protein levels. Cell lysates from control (−) or S100b (+)-treated THP-1 cells transfected with indicated plasmids were immunoblotted with Luc or β-actin antibodies. E, S100b increases Luc mRNA levels in THP-1 cells transfected with pZeo/luc-COX-2–1477 plasmid relative to control (NG). Luc mRNA levels were determined by RT-QPCR, and results were expressed as fold over control (mean ± S.E., *, p < 0.0063 versus NG, n = 3). F–G, the role of COX-2 3′-UTR in S100b-induced mRNA stability. THP-1 cells were transfected with the indicated Luc-COX-2–3′-UTR plasmids, and on the next day, they were treated with or without S100b for 2 h. At the end of the S100b treatment (0 min), Act D (10 μg/ml) was added to each sample, and aliquots of THP-1 cells were collected at 30-, 60-, and 120-min time intervals. Luc mRNA levels were quantified by RT-QPCR using glyceraldehyde-3-phosphate dehydrogenase as internal control. The percentages of mRNA (relative to zero time point) remaining after Act D treatment were plotted against time. The values shown are mean ± S.E. (n = 3). Filled squares represent S100b-treated, and open squares represent untreated (NG) cells. H, S100b induces formation of RNP complexes with the COX-2 3′-UTR. An autoradiogram showing RNP complexes formed with COX-2 3′-UTR regions using CE from control (−) and S100b (+)-treated THP-1 cells is shown. RNA-EMSA was performed with CE from THP-1 cells using the indicated 32P-labeled COX-2 3′-UTR probes (numbers above each lane). RNP complexes were UV-cross-linked, excess RNA was digested with RNases, and samples were fractionated on SDS-PAGE (10%) gels. Probe indicates free probe without any extract. The arrow indicates S100b-specific RNP complexes.
increased by both stability (at 2 h) as well as promoter activation at > 4 h (18), measuring COX-2 mRNA will not reflect the true RNA stability component. Therefore, we used reporter plasmids containing the COX-2 3′-UTR at the 3′ end of the Luc gene expressed from a cytomegalovirus promoter (21) and used Luc as a surrogate for the COX-2. Under these conditions, measuring Luc mRNA half-life (steady-state Luc mRNA levels) after treatment with Act D to stop transcription would reflect COX-2 mRNA stabilization by S100b due to the COX-2 3′-UTR. THP-1 cells were transfected with indicated Luc-COX-2 3′-UTR plasmids (Fig. 1B), treated with or without S100b, and Luc activities in lysates were measured. Results showed that basal Luc activity was dramatically reduced in cells expressing Luc containing full-length COX-2 3′-UTR (pZeo/Luc-COX-2–1477) and 420 bp (pZeo/Luc-COX-2–420) when compared with those transfected without COX-2 3′-UTR (pZeo/Luc) (Fig. 1C), demonstrating the destabilizing effect of the COX-2 3′-UTR on Luc mRNA (or COX-2 mRNA). S100b treatment reversed this effect and significantly increased Luc activity of both pZeo/Luc-COX-2–1477 and pZeo/Luc-COX-2–420 by 3-fold with no effect on pZeo/Luc transfected cells (Fig. 1C). In contrast, Luc with COX-2 3′-UTR region in the antisense orientation (pZeo/Luc-COX-2–420AS) or with 3′-UTR harboring a deletion at the 5′end (pZeo/Luc-COX-2Δ1000) displayed higher basal Luc activity when compared with the 1477- and 420-bp constructs. S100b treatment also failed to increase their Luc activities (Fig. 1C). These results demonstrate that the cis-elements located within 5′ end 420 bp of the 3′-UTR are involved in S100b-induced COX-2 mRNA stability in monocytes. Furthermore, S100b-induced Luc activity at 2 h in THP-1 cells transfected with pZeo/Luc-COX-2–420 was significantly blocked by pretreatment with a RAGE antibody relative to IgG alone (data not shown), demonstrating the role of RAGE-mediated signaling in COX-2 stability.

Immunoblotting of transfected cell lysates with Luc antibody showed increased Luc protein levels in S100b-treated THP-1 cells transfected with pZeo/Luc-COX-2–1477 and pZeo/Luc-COX-2–420 when compared with the respective untreated cells (Fig. 1D) but not in cells transfected with pZeo/Luc or pZeo/Luc-COX-2–420AS (Fig. 1D). S100b also significantly increased levels of Luc-COX-2–1477 mRNA in THP-1 cells (Fig. 1E). These results confirm that the observed changes in Luc activity reflect Luc protein and mRNA levels in transfected cells.

Next, we further confirmed the involvement of the COX-2 3′-UTR in Luc mRNA (or COX-2 mRNA) stability in the THP-1 cells treated with S100b. THP-1 cells transfected with pZeo/Luc-COX-2–1477 were treated with S100b for 2 h, and transcription was inhibited by treatment with Act D. Luc mRNA levels were then determined by RT-QPCR at various time intervals up to 2 h after Act D treatment. As shown in Fig. 1F, stability of Luc mRNA with 1477-bp 3′-UTR was greatly enhanced by S100h (filled squares) when compared with untreated control (open squares). S100b also increased stability of Luc mRNA in cells transfected with pZeo/Luc-COX-2–420 but not pZeo/Luc-COX-2–420AS (Fig. 1G). These results further established that S100b increases mRNA stability via regulatory elements located within the 420 bp region of the COX-2 3′-UTR.

Next, we evaluated the role of specific transacting protein factor(s) that regulate mRNA stability by binding to cis-elements in the COX-2 3′-UTR. CE from control and S100b-stimulated THP-1 cells were used to perform RNA-EMSA and UV cross-linking using in vitro transcribed and 32P-labeled RNAs containing various regions of the COX-2 3′-UTR as probes as described under “Experimental Procedures.” As shown in Fig. 1H, stable RNP complexes were formed only with CE from S100b-treated cells using 1400- (lane 6), 420–, (lane 3) and 115-nt (lane 2)-3′-UTR probes when compared with untreated cells (lane 5) but not with the 1000-nt probe containing 420-nt deletion (lane 4). Lane 1 shows free probe without CE. These results demonstrate that S100b can promote the formation of RNP complexes at the COX-2 3′-UTR in monocytes and that the 115-nt region at the 5′ end was sufficient.

S100b Can Inhibit the Occupancy of hnRNPK at the COX-2 Promoter Region—We next evaluated whether binding of transacting factors to the COX-2 promoter was repressing transcription at 2 h after S100b treatment. We had used DNA affinity chromatography linked to liquid chromatography/tandem mass spectrometry as described (33) to examine the factors involved in pretranscriptional complex at the human COX-2 promoter at the 2-h time point. Biotinylated 250-bp distal (−580/−330 bp) and 280-bp proximal (−280/+1) COX-2 promoter fragments containing consensus binding sites for transcription factors such as NF-κB were used as probes (Fig. 2A). These studies showed that hnRNPK, an RNA/DNA-binding protein, was bound to both the proximal and the distal regions of the COX-2 promoter in nuclear extracts from control THP-1 cells. In contrast, the binding of hnRNPK to the distal region was absent in nuclear extracts from S100b-treated THP-1 cells.4 These results prompted us to evaluate hnRNPK further in the current study and test whether hnRNPK could act as a repressor of transcription and/or a key factor in S100b-mediated COX-2 mRNA stabilization at the early 2 h time point.

An additional rationale for evaluating hnRNPK was that it is a DNA/RNA-binding protein that is involved in the regulation of transcription, translation, mRNA stability, chromatin remodeling, and signal transduction (34). These diverse roles of hnRNPK suggest that it can regulate transcription upon binding to promoters and can act as a post-transcriptional regulator when it binds to the 3′-UTR of mRNAs. However, its role in gene expression and mRNA stability in diabetic cells is not known. Therefore, we performed further experiments to determine the specific role of hnRNPK protein in COX-2 gene regulation under diabetic conditions.

To first verify hnRNPK binding to the COX-2 promoter, ChIP assays were performed using hnRNPK antibody. ChIP-enriched DNA was amplified using primers spanning the COX-2 proximal (−280/+1) and distal (−580/−330) promoter regions. Results showed that in untreated THP-1 cells, hnRNPK occupancy was evident at both distal and proximal

4 N. Shanmugam, T. Bane, T. Lee, and R. Natarajan, unpublished results.
COX-2 promoter regions (Fig. 2B). However, S100b treatment clearly reduced this hnRNPK occupancy at the distal promoter site (Fig. 2B, upper panel) by 4-fold (Fig. 2C), without affecting its binding at the proximal site on the COX-2 promoter (Fig. 2B, lower panel, and C). Input DNA (Fig. 2B, lanes 3 and 4) and ChIP with IgG (Fig. 2B, right panel) were used as controls. These ChIP assay results demonstrate that S100b could induce loss of hnRNPK from the COX-2 distal promoter region at the 2-h time point, and this might promote subsequent transcriptional activation.
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hnRNPK was predominantly present only in the nucleus in untreated cells (upper panel, lanes 1–4), whereas S100b-treated cells showed hnRNPK (GFP) bands in both nuclear and cytoplasmic extracts (upper panel, lanes 5–8), confirming nuclear to cytoplasmic translocation. Actin (middle panel) was used as a general loading control, and histone-H3 (bottom panel) was used as a specific loading control for nuclear fractions. These results support the notion that signaling mechanisms activated by diabetic stimuli such as S100b can displace nuclear hnRNPK from the COX-2 promoter and promote its export to the cytoplasm to facilitate its binding to the 3′-UTR of COX-2-mRNA for subsequent stabilization.

Role of hnRNPK in S100b-induced COX-2 mRNA Stability in Monocytes—We next evaluated whether hnRNPK specifically regulates S100b-induced COX-2 mRNA stability using loss- and gain-of-function approaches with hnRNPK siRNAs and overexpression, respectively. Immunoblotting revealed that siRNAs targeting hnRNPK (Fig. 5A, si-hnRNPK) could efficiently knock down hnRNPK protein (>80%) in THP-1 cells relative to control siRNA (Fig. 5A, si-Ctrl). Next, we examined Luc mRNA stability in THP-1 cells co-transfected with pZeo/Luc-COX-2–1477 plasmid along with si-hnRNPK or si-Ctrl. As shown in Fig. 5B, S100b increased Luc mRNA half-life relative to control (S100b versus NG) as expected, but this was clearly attenuated in hnRNPK knockdown cells (S100b-k/d versus NG-k/d). Furthermore, as shown in Fig. 5C, the S100b-induced Luc activity was also completely abolished in hnRNPK knockdown cells (si-hnRNPK).

We next examined the effects of hnRNPK overexpression. THP-1 cells were co-transfected with GFP or GFP-hnRNPK expression vectors along with pZeo/Luc-COX-2–1477 UTR vector, and 48 h later, S100b-induced Luc mRNA stability was determined in the presence of Act D. GFP-hnRNPK overexpression was confirmed by immunoblotting with GFP antibody (Fig. 5E). Overexpression of hnRNPK increased basal Luc mRNA stability relative to GFP (Fig. 5F, NG-hnRNPK versus NG), and this was further enhanced by S100b (Fig. 5F, S100b-hnRNPK versus S100b). Thus, under basal conditions, hnRNPK can act as a S100b-specific transacting factor to increase COX-2 mRNA stability.

To further support our ChIP assay data in Fig. 2B and that hnRNPK might also act as transcriptional repressor, THP-1 cells were co-transfected with si-hnRNPK or si-Ctrl oligonucleotides along with pZeo/Luc-COX-2–860 promoter plasmid in which Luc reporter gene was under the control of the COX-2 promoter, and Luc activity was determined 72 h later. Results showed that a 2-h treatment with S100b did not transactivate COX-2 promoter in si-Ctrl cells but significantly increased it in THP-1 cells transfected with si-hnRNPK (Fig. 5D), supporting our hypothesis that hnRNPK acts as a transcriptional repressor at the COX-2 promoter. Thus, hnRNPK exhibits dual functions on COX-2 expression in monocytes as a transcriptional repressor under basal conditions and a 3′-UTR-binding protein to increase COX-2 mRNA stability in S100b-treated cells.

Evidence shows that hnRNPK shuttles between the nucleus and cytoplasm, and this is regulated by MEK/ERK-mediated phosphorylation of amino acids residues serines 284 and 353 in
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the nuclear-shuttling domain (38, 39). We previously reported that S100b activates the ERK signaling pathway in THP-1 cells (18). Therefore, we next tested the role of MEK/ERK pathway in hnRNPK translocation by co-transfecting THP-1 cells with control GFP- or vector-expressing mutant hnRNPK in which Ser-284/353 were mutated to Ala (39) along with pZeo/Luc-COX-2–1477 and analyzed Luc mRNA stability in the presence of Act D. This mutant hnRNPK (Ala-284/353) greatly reduced S100b-induced Luc mRNA stability (Fig. 5G) without affecting stability under basal conditions. Thus, in S100b-treated cells, activated ERK might regulate hnRNPK translocation from the nucleus to cytoplasm to then increase COX-2 mRNA stability.

Increased COX-2 mRNA Stability by S100b via Novel Mechanisms Involving the Inhibition of miR-16

Recent studies showed that miR-16 could promote degradation of COX-2 mRNA in HeLa cells by binding to AREs in the 3′-UTRs of COX-2 and other genes (29). Therefore, we hypothesized that diabetic stimuli such as S100b can increase COX-2 mRNA sta-

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hnRNPK and microRNA-16 in COX-2 mRNA Stability

FIGURE 7. Negative regulatory role of miR-16 in S100b-induced COX-2 mRNA stability. A, nucleotide sequence of pre-miR-16-1 and pre-miR-16-2 with mature miR-16 sequences shown in bold letters. B, nucleotide sequences of specific siRNAs targeting miR-16-1 and miR-16-2. C, Northern blot confirming efficacy of siRNA-mediated knock down of miR-16 in THP-1 cells. D, Luc activity in THP-1 cells co-transfected with pZeo/luc-COX-2–1477 along with si-miR-16-1 or si-miR-16-2, without (NG) or with S100b treatment (Ctrl (NG) versus si-miR-16-1 (NG), p < 0.01; Ctrl (NG) versus si-miR-16-2 (NG), p < 0.05). E and F, Luc mRNA half-life was determined (as described in the legend for Fig. 1) in THP-1 cells co-transfected with pZeo/Luc-COX-2–1477 along with control or miR-16 inhibitor oligonucleotides (miR-16 K/d) (E) and control or miR-16 mimic oligonucleotides (miR-16e) (F) (mean ± S.E., n = 3).

bility not only via their effects on hnRNPK but also via concomitant decreases in the expression of miR-16 or in the interaction of miR-16 with the COX-2 3′-UTR. RT-QPCR analysis showed a significant 2-fold reduction in miR-16 levels in S100b-treated THP-1 cells when compared with untreated control (Fig. 6A). This down-regulation of miR-16 by S100b was further confirmed by Northern blot analysis using a 32P-labeled miR-16 probe (Fig. 6B, lanes 2 and 3 versus lanes 1 and 4). S100b also reduced miR-16 levels in isolated PBMC from normal human volunteers (PBMCs 1 and 2, Fig. 6C). These results show for the first time that diabetic stimuli such as S100b can down-regulate the expression of miR-16 in monocytes.

To demonstrate the functional involvement of miR-16, we next examined whether knockdown of miR-16 with specific siRNAs can enhance COX-2 mRNA stability. Bioinformatics analysis showed that two copies of miR-16 are expressed from chromosome 13 (miR-16-1) and chromosome 3 (miR-16-2) (Fig. 7A). Thus, THP-1 cells were transiently co-transfected with double-stranded siRNAs targeting pre-miR-16-1 (si-miR-16-1) or pre-miR-16-2 (si-miR-16-2) (Fig. 7B), along with pZeo/Luc-COX-2–1477 UTR and Luc activities were determined. Northern Blots confirmed that both siRNAs efficiently knocked down miR-16 (Fig. 7C). Further, both siRNAs led to a 2.5-fold increase in basal Luc activity when compared with control (Ctrl), which was not further enhanced by S100b (Fig. 7D).

We also evaluated the effects of miR-16 inhibitor and miR-16 mimic oligonucleotides on COX-2 mRNA stability. Luc mRNA showed increased stability when pZeo/Luc-COX-2–1477 was co-transfected with the miR-16 inhibitor (miR-16 K/d) in both basal and S100b-treated THP-1 cells (Fig. 7E) when compared with control. In contrast, S100b failed to increase Luc mRNA stability when pZeo/Luc-COX-2–1477 was co-transfected with the miR-16 mimic (Fig. 7F, NG-miR-16 e versus S100b-miR-
Role of Cytoplasmic Processing Bodies (P-bodies) in COX-2 mRNA Stability—Our data show that ARE-containing COX-2 mRNA is short-lived or absent in THP-1 cells under normal conditions, suggesting rapid degradation in the cytoplasm. It is not clear whether these events take place at specific cytoplasmic organelles such as P-bodies, which play key roles in mRNA metabolism (40–42). To test the role of P-bodies, HEK 293 cells were transfected with a vector expressing GFP-tagged human mRNA decapping enzyme DCP-1a (GFP-hDCP1a), a known marker for P-bodies. Transfected cells were analyzed by in situ hybridization using fluorescently labeled oligonucleotides complementary to the COX-2 coding region. Nuclei were stained with DAPI. Fluorescence microscopic analysis showed clear colocalization (Fig. 8A, panel a) of GFP-hDCP1a (green, panel a) and COX-2 mRNA (red, panel b) and merged (panel c), suggesting that COX-2 mRNA is entrapped in P-bodies in untreated cells. We next tested whether S100b can release mRNAs entrapped in P-bodies for further stabilization or translation. THP-1 cells were transfected with GFP-hDCP-1a and treated with or without S100b for 2 h. Integrtity of P-bodies was visualized by GFP-hDCP1a fluorescence. As shown in Fig. 8B, untreated cells displayed distinct spots of intact P-bodies (Fig. 8B, panel a). In contrast, only a diffuse pattern of GFP fluorescence was seen in the cytoplasm of S100b-treated cells (Fig. 8B, panel d), suggesting that S100b treatment can destroy the integrity of P-bodies.

Next, we examined whether S100b treatment can relieve P-body-entrapped COX-2 mRNA. Cell lysates of THP-1 cells co-transfected with pGFP-hDCP1a and pZeo/Luc-COX-2–1477 plasmids were immunoprecipitated with GFP antibody, and Luc mRNA levels in the GFP immunoprecipitates were determined by RT-PCR (upper panel). Luc mRNA levels in total lysates were also analyzed by RT-PCR (lower panel). The bar graph shows Luc mRNA levels in GFP-immunoprecipitates (shown in panel A) quantified by RT-PCR (mean ± S.E., *, p < 0.0118, n = 3). COX-2 mRNA was specific only to the subcellular P-body compartment. Luc mRNA associated with GFP-hDCP1a immunoprecipitates was significantly reduced in S100b-treated cells relative to untreated cells (Fig. 9A, upper panel, and B). On the other hand, RT-PCR analysis of total mRNA from these cells showed that S100b responses were normal and that the reduction in Luc mRNA was specific only to the subcellular P-body compartment. These results demonstrate for the first time that diabetic stimuli such as S100b can release mRNAs entrapped in P-bodies.

FIGURE 8. Co-localization of COX-2 mRNA with P-body markers. A, colocalization of COX-2 mRNA with the P-body marker Dcp1a. Images of HEK293 cells transfected with GFP-hDcp-1a expression vector followed by in situ hybridization with an Alexa Fluor 555-labeled 41-mer oligonucleotide probe complementary to COX-2 mRNA are shown. B, S100b-induced disruption of P bodies in monocytes. Fluorescence microscopic analysis of GFP-hDcp1a expressing THP-1 cells treated without (NG) or with S100b is shown. DAPI indicates nuclear staining.

FIGURE 9. A, S100b-induced release of COX-2 mRNA entrapped in P-bodies. Cell lysates from THP-1 cells co-transfected with pZeo/Luc-COX-2–1477 vector and GFP-Dcp1a expression vector were immunoprecipitated with GFP antibody, and Luc mRNA levels in the GFP immunoprecipitates were determined by RT-PCR (upper panel). Luc mRNA levels in total lysates were also analyzed by RT-PCR (lower panel). B, the bar graph shows Luc mRNA levels in GFP-immunoprecipitates (shown in panel A) quantified by RT-PCR (mean ± S.E., *, p < 0.0118, n = 3). C, hnRNPK does not interact with P bodies. Green and red, fluorescence microscopic analysis of THP-1 cells transfected with GFP-Dcp1a (green) and immunostained using hnRNPK antibody (anti-hnRNPK) followed by Texas Red-conjugated secondary antibodies (red). The data shown are representative of 12 out of 23 cells in the field. D, increased miR-16 binding to the COX-2 3′-UTR in hnRNPK knockdown cells. Levels of miR-16 associated with COX-2 3′-UTR in cell lysates derived from control (Ctrl) and hnRNPK knockdown (hnRNPK k/d) THP-1 cells are shown. In vitro binding assays were performed by incubating 32P-labeled miR-16 with biotinylated COX-2 3′-UTR (420 bp) in the presence of cell lysates from Ctrl and hnRNPK k/d THP-1 cells. Antisense 32P-labeled miR-16 (miR-16AS) was used as negative control for binding. COX-2 3′-UTR and miR-16 complexes were captured on avidin-coated magnetic beads, and radioactivity due to bound miR-16 was determined by liquid scintillation counting (mean ± S.E., *, p < 0.01, versus Ctrl, n = 3).
Next, we examined whether hnRNPK can interact with P-bodies and accelerate the disassembly of P-bodies to release entrapped COX-2 mRNA in S100b-treated cells. THP-1 cells transfected with GFP-hDcp1a were immunostained with hnRNPK antibody followed by secondary antibody conjugated to Texas Red. Results showed that S100b could induce cytosolic translocation of endogenous hnRNPK (Fig. 9C, red, versus panel c) and reduced the number of P-bodies (Fig. 9C, panel e versus panel a). However, the merged images did not show co-localization of hnRNPK with GFP-hDcp-1a in P-bodies (Fig. 9C, panel h). Thus, hnRNPK does not interact directly with P-bodies in monocytes.

**Inhibition of miR-16 Binding to COX-2 3’-UTR by hnRNPK**

Our results up to now suggest that S100b enhances COX-2 mRNA stabilization in monocytes both by promoting hnRNPK nuclear export and by down-regulating miR-16. To further explore a potential cross-talk between hnRNPK and miR-16 actions, we next examined whether hnRNPK can modulate miR-16 binding to COX-2 3’-UTR. We performed *in vitro* binding assays by incubating 32P-labeled miR-16 with biotinylated COX-2 3’-UTR in the presence of CE from Ctrl and hnRNPK knockdown (hnRNPK k/d) THP-1 cells. As negative control for binding, 32P-labeled antisense miR-16 (miR-16AS) was used. The biotin-COX-2 UTR and [32P]miR-16 complexes were captured with avidin beads, and the radioactivity due to bound 32P-labeled miR-16 was determined. Results showed that binding of miR-16 to COX-2 3’-UTR was significantly increased (p < 0.01) with CE from hnRNPK k/d cells when compared with Ctrl (Fig. 9D, middle bar versus left bar). The negative control miR-16AS probe did not bind to COX-2 3’-UTR, showing binding specificity (Fig. 9D, bar 3). These results demonstrate that miR-16 binding to COX-2 3’-UTR is increased in the absence of hnRNPK, further supporting a cross-talk between them.

**DISCUSSION**

Several studies have demonstrated that diabetic stimuli such as high glucose, AGEs, and RAGE ligands increase the expression of inflammatory genes in monocytes by transcriptional mechanisms. However, much less is known regarding post-transcriptional mechanisms. We recently showed for the first time that the interferon γ-inducible protein-10 (IP-10) is induced by S100b via increased mRNA stability (31). In the current report, we investigated the mechanisms by which S100b can induce COX-2 mRNA stability in monocytes at early time periods. We noted that hnRNPK binding to the distal promoter was clearly attenuated in S100b-treated cells relative to control cells, suggesting that hnRNPK might act as a repressor of COX-2 promoter transcription at 2 h. Upon S100b treatment, hnRNPK occupancy was reduced to relieve this repression, allowing binding of the S100b-specific transcription factor complex to the promoter and promote transcription at later time points. Such repressor activity of hnRNPK has been reported for the osteocalcin promoter (43).

Because hnRNPK is a versatile DNA-RNA-binding protein (34), we investigated whether hnRNPK can also regulate COX-2 mRNA stability. Our data with COX-2 3’-UTR reporter constructs confirmed the involvement of COX-2 3’-UTR sequence in RNA stability. RNA-IPs demonstrated that hnRNPK interacted with the COX-2 3’-UTR in S100b-treated cells, suggesting that hnRNPK is an S100b-specific factor regulating COX-2 mRNA stability. This was further supported by data showing that siRNAs targeting hnRNPK increased COX-2 mRNA degradation and that this could be reversed by overexpressing hnRNPK. Thus, hnRNPK can act as a docking platform for COX-2 promoter regulation as well as an mRNA-stabilizing transacting factor.

If hnRNPK regulates both COX-2 promoter and mRNA stability, the question was whether these were mediated by separate cytoplasmic and nuclear pools of hnRNPK. To address this, we performed a cellular surveillance of fluorescent GFP-hRNPK in THP-1 cells and PBMC, which revealed that S100b treatment could facilitate the translocation of hnRNPK from the nuclear to cytoplasmic compartments. These data confirmed that, in response to S100b, hnRNPK has to be released from the COX-2 promoter and then exported to the cytoplasm, where it can bind to the COX-2 3’-UTR to stabilize COX-2 mRNA.

We also observed for the first time that S100b could significantly decrease miR-16 levels in monocytes. These data, coupled with results obtained using miR-16 inhibitor or mimics, suggested that miR-16 can promote rapid degradation of COX-2 mRNA in normal cells and that this degradation was blocked by diabetic stimuli like S100b by inhibiting miR-16 expression. It is not clear from the current study whether S100b can inhibit miR-16 transcription or increase its degradation. More importantly, we also uncovered a novel cross-talk

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**FIGURE 10. Proposed mechanisms involving opposing actions of hnRNPK and miR-16 in the regulation of COX-2 mRNA stability under diabetic conditions.**
between hnRNPK and miR-16 because hnRNPK gene silencing could increase the binding of miR-16 to COX-2 3’-UTR, while also decreasing COX-2 mRNA stability. Thus, in response to S100b treatment, hnRNPK might move to the 3’-UTR and displace or prevent miR-16 binding and thereby stabilize COX-2 mRNA.

Because our data suggested that under normal conditions, COX-2 mRNA is rapidly degraded, we evaluated whether this could be taking place at specific cytoplasmic sites such as P-bodies. Our in situ hybridization data suggested that under normal conditions, COX-2 mRNA was present in P-bodies where it was being rapidly degraded and that treatment with S100b released COX-2 mRNA entrapped in P-bodies. Taken together, our data support a novel mechanism for the stabilization of inflammatory genes such as COX-2 under diabetic conditions (Fig. 10). Thus, under non-diabetic conditions, COX-2 promoter in the nucleus is occupied by hnRNPK and remains in the repressed state. In the cytoplasm, miR-16 binds to 3’-UTR and continuously degrades COX-2 mRNA entrapped in P-bodies. Exposure to diabetic stimuli such as S100b can displace nuclear hnRNPK from the distal COX-2 promoter NF-κB binding region. This displaced nuclear hnRNPK translocates to the cytoplasm where hnRNPK interacts with COX-2 3’-UTR prevents miR-16 binding to its target COX-2 3’-UTR, whereas miR-16 degradation is also triggered by S100b. S100b treatment also relieves P-body-entrapped COX-2 mRNA. In summary, we not only report a new role for hnRNPK and miR-16 in COX-2 gene regulation by S100b but also report how a novel intracellular cross-talk between RNA-binding proteins and miRs can promote inflammatory gene expression under diabetic conditions.

Evidence shows that postprandial as well as oscillating “hyperglycemic spikes” in diabetic patients may be a major risk factor for vascular complications (44, 45). Such fluctuations in glucose levels may result in spikes of acute inflammation that can be propagated to a chronic inflammatory state over longer time periods of diabetes. Our current data might represent one of the mechanisms involved in such acute regulation of inflammatory genes by diabetogenic agents such as high glucose, AGEs, and S100b. Further studies are needed to verify the role of these mechanisms in human diabetic complications.

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