Saturated Fatty Acids Induce Post-transcriptional Regulation of HAMP mRNA via AU-rich Element-binding Protein, Human Antigen R (HuR)*

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Background: Expression of HAMP, the key iron-regulatory gene, is modulated in fatty liver disease, but the mechanisms are unknown.

Results: Palmitic acid (PA) regulates HAMP mRNA levels via PKC and binding of HuR to AU-rich element in HAMP 3′-UTR.

Conclusion: HuR is required for post-transcriptional HAMP regulation by PA.

Significance: Post-transcriptional HAMP regulation by lipids could play a role in fatty liver disease.

Iron is implicated in fatty liver disease pathogenesis. The human hepcidin gene, HAMP, is the master switch of iron metabolism. The aim of this study is to investigate the regulation of HAMP expression by fatty acids in HepG2 cells. For these studies, both saturated fatty acids (palmitic acid (PA) and stearic acid (SA)) and unsaturated fatty acid (oleic acid (OA)) were used. PA and, to a lesser extent, SA, but not OA, up-regulated HAMP mRNA levels, as determined by real-time PCR. To understand whether PA regulates HAMP mRNA at the transcriptional or post-transcriptional level, the transcriptional and translational inhibitor actinomycin D was employed. PA-mediated induction of HAMP mRNA expression was not blocked by actinomycin D. Furthermore, PA activated HAMP 3′-UTR, but not promoter, activity, as shown by reporter assays. HAMP 3′-UTR harbors a single AU-rich element (ARE). Mutation of this ARE abolished the effect of PA, suggesting the involvement of ARE-binding proteins. The ARE-binding protein human antigen R (HuR) stabilizes mRNA through direct interaction with AREs on 3′-UTR. HuR is regulated by phosphorylation-mediated nucleo-cytoplasmic shuttling. PA activated this process. The binding of HuR to HAMP mRNA was also induced by PA in HepG2 cells. Silencing of HuR by siRNA abolished PA-mediated up-regulation of HAMP mRNA levels. PKC is known to phosphorylate HuR. Staurosporine, a broad-spectrum PKC inhibitor, inhibited both PA-mediated translocation of HuR and induction of HAMP expression. Similarly, rottlerin, a novel class PKC inhibitor, abrogated PA-mediated up-regulation of HAMP expression. In conclusion, lipids mediate post-transcriptional regulation of HAMP through PKC- and HuR-dependent mechanisms.

Due to the increasing prevalence of obesity worldwide, metabolic syndrome, characterized by visceral adiposity, dyslipidemia, and insulin resistance, is becoming a major public health problem. Non-alcoholic fatty liver disease (NAFLD) 2 is the hepatic manifestation of metabolic syndrome. The accumulation of fat in the liver, which occurs in the absence of significant alcohol consumption, is a key feature of NAFLD (1). Increased lipolysis as a result of insulin resistance and excess lipid uptake from the diet both contribute to the elevated levels of free fatty acids in the circulation. The increase in the uptake of free fatty acids by hepatocytes and de novo fatty acid synthesis lead to fat accumulation in the livers of NAFLD patients (2).

Although lipid accumulation alone may be benign, more aggressive forms of NAFLD can develop. Iron has been shown to be one of the risk factors in the pathogenesis of NAFLD (3). Clinical studies with NAFLD patients have shown a clear correlation between hepatic iron deposition and more advanced disease stages with liver fibrosis (4–6). Iron acts as a risk factor by inducing oxidative stress and mitochondrial dysfunction (3). Hepcidin is the key iron-regulatory protein, which is primarily synthesized in the liver (7). Humans express a single hepcidin gene, HAMP. It controls iron absorption from the duodenum and iron release from macrophages by inhibiting the iron exporter, ferroportin (8). Besides being an iron-regulatory protein, hepcidin also acts as an acute phase protein. In NAFLD patients, HAMP expression has been suggested to be regulated by both systemic iron levels (6, 9) and inflammation (10). A role for lipids in the regulation of HAMP expression has not been established. A microarray study, however, reported that the saturated fatty acid, palmitic acid (PA), modulates HAMP expression in HepG2 cells, but the underlying mechanisms are unknown (11).

Previous studies on hepcidin expression have mainly focused on the transcriptional regulation of HAMP. Inflammatory cyto-

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‡The abbreviations used are: NAFLD, non-alcoholic fatty liver disease; HuR, human antigen R; ARE, AU-rich element; ARE-BP, AU-rich element-binding protein; PA, palmitic acid; SA, stearic acid; OA, oleic acid; rRNA, ribosomal RNA; miRNA, microRNA; IRP, iron-regulatory RNA-binding protein; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; IP, immunoprecipitation; qPCR, quantitative real-time PCR.
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Kines, serum transferrin, erythropoiesis, and hepatic iron stores have all been shown to stimulate the promoter activities of both human and mouse hepcidin genes via the activation of specific transcription factors (12–15). It should, however, be noted that other iron-regulatory proteins, transferrin receptor 1 and ferritin, are regulated at the post-transcriptional and translational level, respectively, by iron-regulatory RNA-binding proteins (IRPs), IRP1 and IRP2 (16). Although no IRP recognition sequences have been identified in HAMP mRNA, the involvement of post-transcriptional mechanisms warrants further investigation. The limited studies conducted up to now examined mainly the role of microRNAs in this process. An indirect role for miR-122 and miR-130a has been reported in the regulation of mouse and human hepcidin gene expression. Namely, miR-122 and miR-130a modulated HAMP expression by altering the mRNA stability of transcriptional activators, which are known to activate HAMP transcription (17, 18). Other mechanisms besides microRNAs might be involved in direct targeting of HAMP 3'–UTR and post-transcriptional regulation of hepcidin expression.

HAMP 3'–UTR harbors a single AU-rich element (ARE), but its functional role in the post-transcriptional regulation of HAMP expression is unknown. AU-rich element-binding proteins (ARE-BPs) participate in the regulation of mRNA stability by recognizing the AU-rich element (i.e. AUUUA pentamer) embedded in a U-rich context (19). ARE-BPs include human antigen R (HuR), tristetraprolin, butyrate response factor 1, ARE/poly(U)-binding/degradation factor 1, and KH-type splice- ing regulatory protein (19). Unlike other ARE-BPs, which mostly act as negative regulators and induce mRNA decay, HuR has been shown to exert a stabilization effect on the target mRNA (19). Phosphorylation is important for HuR function. HuR harbors several phosphorylation sites for protein kinase C (PKC) and has been shown to be phosphorylated by diverse isoforms of PKC (20). Following phosphorylation, HuR translocates from the nucleus to the cytoplasm (19, 20). The phosphorylation-mediated nucleo-cytoplasmic shuttling of HuR is essential for its mRNA stabilization function (20).

Saturated fatty acids have been shown to activate various signaling pathways in hepatoma cells, including PKCs (21–25). However, the role of HuR or PKCs in HAMP regulation has not been investigated. The objective of this study is to understand the mechanisms by which hepatic HAMP mRNA expression is regulated by saturated fatty acids. We have identified a functional role for ARE in HAMP 3'-UTR and the ARE-BP, HuR, in post-transcriptional regulation of HAMP expression by lipids in human hepatoma cells. These findings will help us to further understand the role of hepcidin and iron in obesity and fatty liver disease pathogenesis.

**Experimental Procedures**

**Reagents**—Palmitic acid, stearic acid, and oleic acid were purchased from Sigma-Aldrich and dissolved in isopropyl alcohol as 80 mM stock solutions. Staurosporine was purchased from Adipogen Corp. mirVana microRNA mimic and negative controls were purchased from Life Technologies, Inc. siGENOME Human HuR siRNA SMART pool and control siRNA were obtained from Dharmacon. Rottlerin and Go6976 were kindly provided by Dr. Todd Wyatt (University of Nebraska Medical Center).

**Cell Culture**—HepG2 human hepatoma cells were maintained in high glucose Dulbecco’s modified Eagle’s medium supplemented with glutamine and 10% fetal calf serum (Atlantic Biologicals). 12–24 h prior to experiments, 1.3 × 10^6 or 3.9 × 10^6 cells were seeded in 25- or 75-cm^2 flasks, respectively. Cells were treated with fatty acids or solvent (0.375% isopropyl alcohol) as control for 8 h.

**Transfections**—Negative control microRNA (miRNA) or miR-214 mimic was introduced into HepG2 cells with Nucleofector (Lonza). Plasmids and siRNA were transfected by using Lipofectamine 3000 or Lipofectamine RNAiMax (Life Technologies), respectively, according to the instructions of the manufacturer. For siRNA experiments, two consecutive transfections were performed.

**Lipid Droplet Staining**—Following treatment, HepG2 cells were washed twice with PBS and fixed with 4% paraformaldehyde (Sigma) at room temperature for 15 min. The fixed cells were stained with 0.2 mg/ml Nile Red (ACROS Organics) for 5 min at room temperature. The nuclei were counterstained with Hoechst 33342 (Invitrogen). Fluorescent images were obtained with a Nikon Eclipse E400 fluorescence microscope using a CC-12 digital camera and analySIS software (Soft Imaging System).

**DNA Isolation, cDNA Synthesis, and Real-time Quantitative PCR Analysis**—DNA isolation, cDNA synthesis, and quantitative real-time PCR (qPCR) were performed as described previously (26). A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene probe was used as the endogenous control. The Taqman microRNA assay kit (Life Technologies) was employed to determine the expression of microRNAs by qPCR, according to the manufacturer’s instructions.

**mRNA Half-life Measurements**—HepG2 cells were treated with PA or solvent in the presence of actinomycin D for various time periods as indicated. The level of HAMP mRNA expression, as measured by qPCR, was expressed as -fold change relative to that in control cells, which were treated with solvent in the presence of DMSO, at corresponding time points. The rate constant for RNA decay (k_{decay}) was determined with linear regression (least-square) analysis. RNA half-life was calculated with the equation, t_{1/2} = ln 2/k_{decay} as described (27).

**Plasmid DNA Constructs and Dual-Luciferase Reporter Assay**—The 1.5-kbp HAMP promoter region (+36 to −1544) was amplified by PCR using Phusion High-Fidelity DNA polymerase (Thermo Scientific) and specific primers containing restriction sites (forward primer with MluI site, 5'-GGACCGGCTCTGCGCTCTGGTGTCTG-3'; reverse primer with XhoI site, 5'-GACTCGAGTGAGCTTGCTCTGGTGTTGTCGCTG-3'). Following restriction digestions, PCR product was inserted into pGL3-Basic luciferase reporter vector (Promega). The ligated plasmid was electroporated into competent DH5α bacterial cells with the MicroPulse electroporator system (Bio-Rad). The cloned plasmid was verified by DNA sequencing. The 101-bp full-length HAMP 3'-UTR region was amplified by PCR using specific primers harboring a 5'-phosphate group (forward, 5'-AACCTACCTGCGCTCCTGGCCCTGCGCTCTG-3'; reverse, 5'-TTTGGGAAACAAAGATGGCAGTCGCTG-3'). PCR product was amplified by Phusion PCR Master Mix (Thermo Scientific) and specific primers containing restriction sites (forward primer with BstXI site, 5'-ACCTTTGAGTAATACGACTCACTATAGGG-3'; reverse primer with XhoI site, 5'-GACTCGAGTGAGCTTGCTCTGGTGTTGTCGCTG-3'). Following restriction digestions, PCR product was inserted into pGL3-Basic luciferase reporter vector (Promega). The ligated plasmid was electroporated into competent DH5α bacterial cells with the MicroPulse electroporator system (Bio-Rad). The cloned plasmid was verified by DNA sequencing.
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inserted into the Pmel site of the pMIR-REPORT reporter vector (Life Technologies) by blunt end cloning. DNA sequencing was employed to identify the clone harboring 3′-UTR in the “forward” direction.

Plasmids were purified with a plasmid maxikit (Qiagen). HepG2 cells were transfected with either empty (as control) or recombinant reporter vector using Lipofectamine 3000. pRL-SV40 plasmid encoding Renilla luciferase was co-transfected to standardize transfection efficiency. Dual-Luciferase reporter assays were performed according to the manufacturer’s instructions (Promega).

**Mutagenesis**—The ARE sequence in HAMP 3′-UTR was deleted using a commercial mutagenesis kit (QuickChange II Site-Directed Mutagenesis; Agilent Technologies) and specific primers (forward, 5′-CTGGGCGACGGAATAGGAGAGGGAGG-3′; reverse, 5′-GCCCTCCCTTAAATTCTGCTGCCCAAG-3′), according to the manufacturer’s instructions.

**Cell Lysis and Western Blotting**—To prepare whole cell lysates, HepG2 cells were incubated for 15 min in lysis buffer (10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM EDTA, and 10% glycerol) supplemented with 1 mM PMSF, 0.5% Triton X-100, protease inhibitor mixture, pepstatin A (Sigma-Aldrich), and activated sodium orthovandanate (Acros Organics). The lysates were sonicated (50% amplitude, three 5-s cycles) with ultrasonic dismembrator 150T (Fisher) to facilitate cell lysis. Following centrifugation (3000 × g for 5 min) at 4 °C, supernatants were collected and used for Western blotting.

To isolate cytosolic and nuclear fractions, HepG2 cells were trypsinized and washed twice with PBS. The cell pellets were subsequently resuspended in a hypotonic buffer (10 mM Tris-HCl, 10 mM NaCl, 2.5 mM MgCl2, 1% Igepal, 0.5% deoxycholate) and sonicated (30% amplitude, three 5-s cycles). Sonicated lysates were centrifuged at 2800 × g for 10 min to remove any debris. The supernatants were utilized as nuclear fractions.

Western blotting was performed as described (26) using commercial primary antibodies: hepcidin (Abcam, ab30760), HuR (Santa Cruz Biotechnology, Inc., sc-5261), GAPDH (Millipore, MAB374), histone H3 C-terminal (Active Motif, 39163), and secondary anti-mouse or -rabbit antibodies (Cell Signaling). Immunoreactive bands were detected by ImmunoStar-AP secondary anti-mouse or -rabbit antibodies (Cell Signaling).

**Hepcidin Measurements**—Determination of hepcidin in conditioned media was performed as described by Costantino et al. (29) with minor modifications. Briefly, HepG2 cells were seeded onto poly-L-lysine-coated coverslips in 24-well plates. After treatments, cells were washed twice with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Fixed cells were permeabilized with 0.5% Triton X-100 for 15 min prior to blocking with 5% BSA for 1 h. After washes with 1× PBS, cells were incubated with an anti-HuR (1:50 dilution; Santa Cruz Biotechnology, sc-5261) primary antibody at 4 °C rotating overnight. Control cells were incubated with normal mouse IgG (Santa Cruz Biotechnology) in parallel. The next day, cells were washed three times with TBS buffer containing 1% Tween 20 (Sigma) (TBST) and incubated with a secondary Texas Red-conjugated horse anti-mouse IgG (1:300 dilution; Vector Laboratories) for 1 h at room temperature. Subsequently, coverslips were washed with TBST three times and mounted with VECTASHIELD® Hard-Set Mounting Medium with DAPI (Vector Laboratories).

**Statistical Analysis**—The significance of difference between groups was determined by Student’s t test or one-way analysis of variance with Tukey’s honest significant difference post hoc test by using SPSS software. A value of p < 0.05 was accepted as statistically significant.

**Results**

**Regulation of HAMP mRNA Expression by Fatty Acids in HepG2 Cells**—Fat-laden hepatocytes contribute to NAFLD pathogenesis (34). HepG2 hepatoma cells were chosen as an experimental model because they are commonly used as human hepatocytes and express adequate levels of HAMP (35). Hepatocytes take up free fatty acids through diffusion and specific transporters, such as CD36 and fatty acid transport peptide (36). In order to validate that fatty acids were taken up by HepG2 cells under our experimental conditions, the intracellular lipid content was examined by Nile Red staining as described under “Experimental Procedures” (Fig. 1). A significant increase in lipid content was observed in cells treated with 0.3 mM saturated fatty acids (PA and stearic acid (SA)) or unsat-
urate fatty acid (oleic acid (OA)), as compared with control cells treated with solvent (isopropyl alcohol) (Fig. 1). In contrast to PA- or SA-treated cells, OA-treated cells exhibited punctate lipid droplet-like structures (Fig. 1). This is consistent with previous reports showing that unsaturated fatty acids are more readily esterified to produce triglycerides (37, 38). To investigate the role of fatty acids in the regulation of HAMP expression, HepG2 cells were treated with different concentrations of PA, SA, or OA for 8 h. Control cells were treated with solvent as described under “Experimental Procedures.”

HAMP mRNA expression was determined by qPCR. Cells treated with PA displayed a concentration-dependent increase in HAMP expression compared with solvent-treated control cells (Fig. 2A). Similar concentrations of SA exerted a less prominent induction than PA. In contrast to PA and SA, OA treatment did not significantly alter HAMP expression compared with control cells treated with solvent, as determined by Western blotting (Fig. 2A, inset). Accordingly, PA treatment elevated the level of hepcidin protein expression compared with control cells treated with solvent, as described by Western blotting (Fig. 2A, inset). Earlier studies concentrated on the regulation of HAMP expression at the transcriptional level (12–15). The effect of fatty acids on HAMP transcription was examined by luciferase reporter assays in HepG2 cells transfected with pGL-3 Basic vector harboring the 1.5-kbp HAMP promoter (HAMP Prom-Luc). HepG2 cells transfected with the recombinant vector displayed higher luciferase activity compared with cells transfected with the empty vector (Fig. 2B). However, the levels of HAMP promoter activity were similar in HepG2 cells treated with PA or solvent control (Fig. 2B). To further investigate the mechanisms by which saturated fatty acids up-regulate HAMP mRNA expression, we used the transcription inhibitor, actinomycin D. Actinomycin D by itself significantly inhibited basal HAMP mRNA expression in HepG2 cells (Fig. 2C). On the other hand, HepG2 cells treated with PA in the presence of actinomycin D exhibited a significant increase in HAMP mRNA expression compared with cells treated with actinomycin D and solvent control (Fig. 2D). To investigate HAMP mRNA stability, time course experiments (0–10 h) were performed with HepG2 cells treated with PA or solvent in the presence of actinomycin D. HAMP mRNA levels in HepG2 cells were plotted against time in a semi-log plot (Fig. 2E). The exponential trend lines indicated that HAMP mRNA decayed at a slower rate in PA-treated cells compared with solvent-treated controls. The half-lives of HAMP mRNA, calculated as described under “Experimental Procedures,” were 2.70 ± 0.08 and 4.44 ± 0.01 h in solvent- and PA-treated cells, respectively (Fig. 2F). Our findings strongly suggest that HAMP mRNA expression is regulated through post-transcriptional mechanisms in HepG2 cells treated with the saturated fatty acid, PA.
Palmitic Acid and HAMP 3′-UTR Regulation—3′-UTR regions of mRNA are important in the regulation of mRNA stability by various post-transcriptional pathways (19). We therefore investigated the effect of PA on the 3′-UTR of HAMP by reporter assays. For these studies, the 101-bp 3′-UTR of the HAMP gene was inserted downstream of the luciferase gene in pMIR reporter vector as described under “Experimental Procedures.” To perform Dual-Luciferase reporter assays, HepG2 cells, transfected with either the empty pMIR vector (control) or recombinant pMIR vector harboring HAMP 3′-UTR (HAMP 3′-UTR), were co-transfected with the reference plasmid, pRL-SV40. Treatment with PA significantly stimulated luciferase activity in cells transfected with the HAMP 3′-UTR but not with empty vector (Fig. 3A). Our reporter assay experiments strongly suggest the involvement of 3′-UTR in the regulation of HAMP mRNA expression by PA. ARE-BPs regulate mRNA stability through the recognition of AREs in 3′-UTR (19). The examination of HAMP 3′-UTR revealed a single ARE (Fig. 3B). We performed mutagenesis to delete the ARE sequence. Recombinant pMIR vector harboring mutant HAMP 3′-UTR (3′-UTR ΔAU) was employed in luciferase reporter assays. Unlike in cells transfected with wild type 3′-UTR, PA...
with normal mouse IgG as negative control did not display any specific signal. In solvent-treated control cells, HuR protein was mainly located in the nucleus (Fig. 4A). Upon PA treatment, HuR shuttled from nucleus to the cytosol and exhibited a more diffused distribution (Fig. 4A). In contrast, OA did not induce the translocation of HuR to the cytosol (Fig. 4A). The percentage of HuR localized in the cytosol was quantified as described under “Experimental Procedures.” In HepG2 cells treated with solvent, PA, or OA, 8.01 ± 2.56, 35.33 ± 3.89, and 10.07 ± 3.16% of HuR resided in the cytosol, respectively (Fig. 4B). The PA-induced nucleo-cytoplasmic shuttling of HuR was further supported by Western blotting performed with whole cell lysates and nuclear and cytosolic fractions isolated from solvent- or PA-treated HepG2 cells. PA treatment did not affect the basal protein expression levels of HuR (Fig. 4C). PA-treated cells displayed a significant increase in the level of cytosolic HuR protein compared with that in control cells (Fig. 4C). Accordingly, the expression level of HuR protein in the nucleus was decreased following PA treatment (Fig. 4C). Both immunofluorescent staining and Western blotting experiments indicate that nucleo-cytoplasmic shuttling of HuR protein is induced by PA treatment.

Phosphorylation by protein kinases, including PKC, is important for the shuttling and activation of HuR (20). Independently, PA has also been shown to induce the activation of multiple isoforms of PKC (21–25). We therefore examined the role of PKC in post-transcriptional regulation of HAMP by PA. For these studies, HepG2 cells were first treated with staurosporine, a potent broad-spectrum protein kinase C inhibitor (39, 40). The level of HuR shuttling was determined in HepG2 cells treated with PA in the presence of 0.2 μM staurosporine or DMSO as control. PA-induced nucleo-cytoplasmic shuttling of HuR was abolished in cells treated with staurosporine, but not with DMSO, as confirmed by immunofluorescent staining and further quantification (Fig. 5, A and B). Cells treated with PA in the presence of DMSO as control displayed a significant increase (from 7.51 ± 2.67 to 38.55 ± 5.18%) in the level of cytosolic HuR (Fig. 5B). In contrast, HepG2 cells treated with PA and staurosporine did not exhibit a significant change (from 9.30 ± 4.83 to 11.76 ± 6.48%) in HuR translocation compared with cells treated with solvent and staurosporine (Fig. 5B). Contrary to DMSO, staurosporine treatment significantly blocked PA-mediated induction of HAMP mRNA expression in HepG2 cells (Fig. 6, A and B). Both classical and novel classes of PKC isoforms have been suggested to phosphorylate HuR (20). We therefore employed PKC inhibitors, Go6976 and rottlerin, which have been shown to block classical or novel class of PKC isoforms, respectively (24, 41). Rottlerin (10 μM), but not Go6976 (1 μM), treatment abolished PA-induced HAMP mRNA increase in HepG2 cells (Fig. 6, C and D).

To determine the direct role of ARE and HuR in HAMP mRNA regulation by PA, we employed a commercial human HuR siRNA pool, composed of four different specific siRNAs, as described under “Experimental Procedures,” to suppress HuR expression. In parallel, HepG2 cells were transfected with

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**FIGURE 3.** The role of PA in the regulation of HAMP 3′-UTR. A, HepG2 cells, transfected with pMIR vector harboring the 3′-UTR of the HAMP gene (3′-UTR) or empty pMIR vector, were treated with PA or solvent (Solv.). pRL-SV40 vector was co-transfected as a reference for transfection efficiency. Dual-Luciferase assays were performed as described under “Experimental Procedures.” The relative luciferase levels were expressed as -fold change relative to that in solvent-treated cells transfected with empty vector. B, the HAMP 3′-UTR region harboring a single ARE is shown as a schematic diagram. The position of ARE sequence (AUUUA) is underlined. C, mutagenesis of the AU-rich element in the HAMP 3′-UTR region was performed by deleting the UUU base pairs by site-directed mutagenesis as described under “Experimental Procedures.” HepG2 cells were transfected with pMIR vector harboring wild type 3′-UTR or 3′-UTR with a mutated AU-rich element (3′-UTR ∆AU) to perform Dual-Luciferase assays as described above. Error bars, S.D.

failed to up-regulate luciferase activity in cells transfected with mutant HAMP 3′-UTR (3′-UTR ∆AU) (Fig. 3C). Mutagenesis experiments strongly suggest a functional role for HAMP 3′-UTR ARE in PA-mediated up-regulation of HAMP RNA expression.

HAMP 3′-UTR Regulation by ARE-BP—The ARE-BP HuR is well known for its role in enhancing mRNA stability (19). The activation of HuR is achieved via phosphorylation and subsequent nucleo-cytoplasmic shuttling (20). The subcellular distribution of HuR in HepG2 cells treated with either PA or solvent was examined by immunofluorescent staining. Cells stained

3 S. Lu and D. Harrison-Findik, unpublished observations.
control siRNA (Fig. 7, A and B). The expression levels of HuR mRNA and protein in cells transfected with control or HuR siRNA were determined by qPCR and Western blotting (Fig. 7A). Significant inhibition of HuR was achieved in HuR siRNA-transfected cells compared with control cells under our experimental conditions. Subsequently, transfected HepG2 cells were treated with 0.3 mM PA or solvent, and HAMP mRNA expression was determined by qPCR. In solvent-treated HepG2 cells, the basal expression level of HAMP mRNA was down-regulated by HuR siRNA compared with control siRNA (Fig. 7B). The PA-induced increase in HAMP mRNA levels was significantly abrogated in HuR siRNA-transfected cells compared with cells transfected with control siRNA (Fig. 7B). Our siRNA experiments clearly show that HuR is required for PA-mediated up-regulation of HAMP mRNA expression.

To confirm the direct interaction of HuR with HAMP mRNA, ribonucleoprotein IP assays were performed using cytosolic fractions isolated from HepG2 cells as described under “Experimental Procedures.” Specific immunoprecipitation of HuR proteins by our anti-HuR antibody was confirmed by Western blotting (Fig. 8A, inset). The amount of HAMP mRNA in HuR or control IgG co-precipitated total RNA was quantified by qPCR. The level of HAMP mRNA present in control IgG immunoprecipitates was not different between PA and solvent-treated HepG2 cells (Fig. 8A). In contrast, 8.58 ± 0.51-fold more HAMP mRNA was detected in HuR immunoprecipi-
tates of PA-treated HepG2 cells compared with those from solvent-treated cells (Fig. 8A). To confirm specificity, the presence of 18S rRNA in HuR immunoprecipitates was determined, as a negative control. PA treatment did not significantly alter the level of 18S rRNA, validating our ribonucleoprotein IP assays (Fig. 8B).

The Role of MicroRNAs in PA-induced HAMP Up-regulation—miRNAs are negative regulators of mRNA stability and translation (42, 43). We therefore determined whether PA-mediated up-regulation of HAMP mRNA involves the suppression of miRNAs, which might potentially target HAMP 3’-UTR. Online algorithms (e.g. TargetScan, miRGen, and RNA22) for in silico identification of miRNA targets were employed to scan HAMP 3’-UTR sequence. The results obtained from different databases commonly identified miR-214. Previous studies have also shown that miR-122 indirectly regulates HAMP gene expression by targeting 3’-UTR of other upstream iron-regulatory genes, such as Hfe and Hjv (17). We therefore included these two microRNAs in our investigations and determined the expression levels in PA- or solvent-treated HepG2 cells by qPCR as described under “Experimental Procedures.” PA elevated the expression of miR-214 but not miR-122 (Fig. 9, A and B). Contrary to our hypothesis, PA treatment did not suppress but rather induced the expression of miR-214. Nevertheless, we further investigated the potential role of miR-214 in HAMP regulation by transfecting HepG2 cells with miR-214 mimic or negative control miRNA. HAMP mRNA expression was determined 24 h after transfections. In parallel, the mRNA expression level of MEK3 (mitogen-activated protein kinase kinase 3), which is a validated miR-214 target (44), was also determined by qPCR. Compared with negative control miRNA, miR-214 mimic significantly elevated HAMP mRNA.

FIGURE 5. PA-mediated shuttling of HuR was abolished by staurosporine. A, immunofluorescent staining was performed with HepG2 cells treated with solvent (Solv.) or 0.3 mM PA in the presence of 0.2 μM staurosporine (STAU) or DMSO as control as described. Representative fluorescent images are shown (magnification, ×40). B, quantification of HuR nuclear localization was performed as described under “Experimental Procedures.” Error bars, S.D.
expression (Fig. 9C). However, the expression of MEK3 mRNA was significantly suppressed by miR-214 mimic, confirming its specificity under our experimental conditions (Fig. 9D). These findings in concert with the defined role of miRNAs as mRNA destabilizers strongly suggest that miR-214, despite being regulated by PA, is not directly involved in the induction of HAMP mRNA expression in PA-treated HepG2 cells.

Discussion

Hepatic iron overload in NAFLD patients has been shown to be associated with disease severity (3–5). Clinical studies have also reported changes in the expression levels of hepcidin, the key iron regulator, in the liver and sera of NAFLD patients (6, 9). However, the underlying mechanisms are unclear. Hepcidin expression has been suggested to respond to changes in iron levels or obesity-mediated inflammatory cytokines (6, 9, 10). Obesity and NAFLD are associated with dysregulated lipid metabolism. This study investigated the regulation of human hepcidin gene, HAMP, by fatty acids in hepatoma cells and identified a novel signaling pathway and regulatory mechanism for HAMP expression.

Our findings have not only clearly shown that saturated fatty acids, particularly PA, stimulate hepcidin expression in human liver cells but also revealed that HAMP expression can be regulated at the post-transcriptional level. This post-transcriptional regulation utilizes a single ARE in the HAMP 3′-UTR. Furthermore, we have identified an additional role for the ARE-
binding protein HuR in the regulation of iron homeostasis. Interestingly, our findings also suggested an association of novel class PKC isoforms with HAMP and iron metabolism. Collectively, we have shown unique mechanisms that connect iron homeostasis with lipid metabolism in hepatoma cells and have implications for the pathogenesis of fatty liver disease and obesity.

Previous studies have particularly focused on the transcriptional regulation of HAMP. Unlike other iron-regulatory proteins, such as ferritin and transferrin receptor 1, which are regulated by RNA-binding proteins, IRP1 and IRP2, our knowledge on the post-transcriptional regulation of HAMP is very limited. An indirect role for microRNAs has been suggested in the regulation of mouse and human hepcidin genes via the targeting of transcriptional activators (17, 18). In agreement, our findings in this study also indicated an indirect role for miR-214 in HAMP regulation by saturated fatty acids. In fact, miR-214 mimic up-regulated HAMP expression contrary to the widely accepted miRNA function of destabilizing mRNA (43, 45). It is possible that miR-214 may have inhibited an as yet unidentified suppressor of HAMP. Nevertheless, our studies and others clearly indicate that miRNAs do not directly regulate human or mouse hepcidin gene expression. Accordingly, in this study, we have shown other post-transcriptional mechanisms that directly target and regulate 3′-UTR of HAMP.

The ARE-BP HuR has been shown to be involved in the post-transcriptional regulation of genes mediating inflammatory reactions and tumorigenesis by targeting AU-rich elements (19, 20). Nucleo-cytoplasmic shuttling of HuR, regulated by phosphorylation, is crucial for its function (20). Both classical (PKCα) and novel (PKCβ) PKC isoforms have been shown to phosphorylate HuR at serine residues within or adjacent to the HuR-nucleo-cytoplasmic shuttling sequence and thereby activate its translocation to the cytoplasm (46, 47). Numerous studies have clearly shown that PA treatment activates both the classical and novel class isoforms of PKC (22–24, 48), but the effect of PA on HuR activation is unknown. We have established a connection between PA and HuR and validated the importance of HuR in PA-induced HAMP mRNA stabilization. Inhibitor studies have also suggested that PA induces HAMP mRNA expression through the activation of novel class of PKC isoforms. Further studies are required to confirm that the direct phosphorylation of HuR by PKC is essential for PA-mediated up-regulation of HAMP mRNA levels. Although HuR binding is sufficient for HAMP induction, the possibility of interaction of other post-transcriptional regulators with HuR on HAMP 3′-UTR cannot be excluded. Interestingly, we detected the presence of miR-214 in immune complexes pulled down with our anti-HuR antibody in HepG2 cells. The biological significance of ARE-BP and microRNA interaction in the regulation of HAMP mRNA stability will be investigated as a part of future studies.

In summary, we have identified a novel regulatory mechanism for human hepcidin gene expression, which occurs at the post-transcriptional level through AU-rich element RNA-binding protein HuR and protein kinase C signaling. We have included a model describing the molecular mechanisms of PA-induced HAMP mRNA stabilization (Fig. 10). These findings have implications for understanding the role of iron metabolism in the pathogenesis of fatty liver disease and obesity. Because this ARE sequence is also present in the 3′-UTR of mouse hepcidin genes, mouse models of NAFLD can be used.
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for further studies. Targeting of AU-rich element RNA-binding proteins might also provide novel therapeutic approaches for the treatment of diseases associated with metabolic syndrome.

Author Contributions—S. L. and D. D. H. F. designed the study. S. L. performed the experiments and drafted the manuscript. D. D. H. F. critically revised the manuscript. J. L. M. provided expertise with post-transcriptional studies and critically revised the final version of the manuscript.

References

1. Ratziu, V., Bellentani, S., Cortez-Pinto, H., Day, C., and Marchesini, G. (2010) A position statement on NAFLD/NASH based on the EASL 2009 special conference. J. Hepatol. 53, 372–384
2. Donnelly, K. L., Smith, C. I., Schwarzenberg, S. J., Jessurun, J., Boldt, M. D., and Parks, E. J. (2005) Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. J. Clin. Invest. 115, 1343–1351
3. Nelson, J. E., Wilson, L., Brunet, E. M., Yeh, M. M., Kleiner, D. E., Unalp-Arida, A., and Kowdley, K. V., and Nonalcoholic Steatohepatitis Clinical Research Network (2011) Relationship between the pattern of hepatic iron deposition and histological severity in nonalcoholic fatty liver disease. Hepatology 53, 448–457
4. Nelson, J. E., Brunet, E. M., Kowdley, K. V., and Nonalcoholic Steatohepatitis Clinical Research Network (2012) Lower serum hepcidin and greater parenchymal iron in nonalcoholic fatty liver disease patients with C282Y HFE mutations. Hepatology 56, 1730–1740
5. Courcelaud, B., Pigeon, C., Inoue, Y., Inoue, J., Gonzalez, F. J., Leroyer, P., Vann, E., Canavesi, E., Latuada, E., Roviaro, G., Marchesini, G., and Fargion, S. (2010) HFE genotype, parenchymal iron accumulation, and liver fibrosis in patients with nonalcoholic fatty liver disease. Gastroenterology 138, 905–912
6. Nelson, J. E., Wilson, L., Brunet, E. M., Yeh, M. M., Kleiner, D. E., Unalp-Arida, A., and Kowdley, K. V., and Nonalcoholic Steatohepatitis Clinical Research Network (2011) Relationship between the pattern of hepatic iron deposition and histological severity in nonalcoholic fatty liver disease. Hepatology 53, 448–457
7. Aigner, E., Theurl, I., Theurl, M., Lederer, D., Haufe, H., Dietze, O., Haufe, H., Dietze, O., Dietze, O., Haufe, H., Dietze, O., Dietze, O., Theurl, I., Dietze, O., Haufe, H., Dietze, O., Dietze, O., and Babitt, J. L. (2014) MicroRNA-130a is up-regulated in mouse liver by iron deficiency and targets the bone morphogenetic protein (BMP) receptor ALK2 to attenuate BMP signaling and hepatic transcription. J. Biol. Chem. 289, 23796–23808
8. Schoenengraber, D. B., and Maquat, L. E. (2012) Regulation of cytoplasmic mRNA decay. Nat. Rev. Genet. 13, 246–259
9. Döller, A., Pfeilschifter, J., and Eberhardt, W. (2008) Signalling pathways regulating nucleo-cytoplasmic shuttling of the mRNA-binding protein HuR. Cell. Signal. 20, 2165–2173
10. Lee, J. Y., Cho, H.-K., and Kwon, Y. H. (2010) Palmitate induces insulin resistance without significant intracellular triglyceride accumulation in HepG2 cells. Metabolism 59, 927–934
11. Greene, M. W., Burginton, C. M., Ruhoff, M. S., Johnson, A. K., Chongkrairatanakul, T., and Kangwanpornsiri, A. (2010) PKCδ is activated in a dietary model of steatohepatitis and regulates endoplasmic reticulum stress and cell death. J. Biol. Chem. 285, 42115–42129
12. Dasgupta, S., Bhattacharya, S., Maitra, S., Pal, D., Majumdar, S. S., Datta, A., and Bhattacharya, S. (2011) Mechanism of lipid induced insulin resistance: activated PKCe is a key regulator. Biochim. Biophys. Acta 1812, 495–506
13. Tan, S. H., Shui, G., Zhou, J., Li, J. J., Bay, B.-H., Wenk, M. R., and Shen, H.-M. (2012) Induction of autophagy by palmitic acid via protein kinase C-mediated signaling pathway independent of mTOR (mammalian target of rapamycin). J. Biol. Chem. 287, 14364–14376
14. Greene, M. W., Burginton, C. M., Lynch, D. T., Davenport, S. K., Johnson, A. K., Horsman, M. J., Chowdhry, S., Zhang, J., Sparks, J. D., and Tirrell, P. C. (2014) Lipid metabolism, oxidative stress and cell death are regulated by PKC δ in a dietary model of nonalcoholic steatohepatitis. PLoS One 9, e85848
15. Harrison-Findik, D. D., Schafer, D., Klein, E., Timchenko, N. A., Kukazsk, H., Clemens, D., Fein, E., Andriopoulos, B., Pantopoulos, K., and Gollan, J. (2006) Alcohol metabolism-mediated oxidative stress down-regulates hepcidin transcription and leads to increased duodenal iron transporter expression. J. Biol. Chem. 281, 22974–22982
16. Chen, C.-Y. A., Ezzeddine, N., and Shyu, A.-B. (2008) Messenger RNA half-life measurements in mammalian cells. Methods Enzymol. 448, 335–357
17. Schägger, H., and von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. 166, 368–379
18. Costantino, C. L., Witkiewicz, A. K., Kuwano, Y., Cozzitorto, J. A., Kennedy, E. P., Dasgupta, A., Keen, J. C., Yeo, C. J., Gores, M., and Brody, J. R. (2009) The role of HuR in gemcitabine efficacy in pancreatic cancer: HuR up-regulates the expression of the gemcitabine metabolizing enzyme deoxycytidine kinase. Cancer Res. 69, 4567–4572
19. Rizk, A., Paul, G., Incardona, P., Bugarski, M., Mansouri, M., Niemann, A., Ziegler, U., Berger, P., and Salazarini, I. F. (2014) Segmentation and quantification of subcellular structures in fluorescence microscopy images using Squash. Nat. Protoc. 9, 586–596
20. Lal, S., Burkhardt, R. A., Beehary, N., Bhattacharjee, V., Londin, E. R., Cozzitorto, J. A., Romeo, C., Jimbo, M., Norris, Z. A., Yeo, C. J., Sawicki, J. A., Winter, J. M., Rigoutsos, I., Yen, T. J., and Brody, J. R. (2014) HuR posttranscriptionally regulates WEE1: implications for the DNA damage response in pancreatic cancer cells. Cancer Res. 74, 1128–1140
21. Lal, A., Mazan-Mamczarz, K., Kawai, T., Yang, X., Martinodale, J. L., and Gores, M. (2004) Concurrent versus individual binding of HuR and...
AUF1 to common labile target mRNAs. *EMBO J.* 23, 3092–3102
33. García-Domínguez, D. J., Morello, D., Cisneros, E., Kontoyiannis, D. L., and Frade, J. M. (2011) Stabilization of Dll1 mRNA by Elavl1/HuR in neuroepithelial cells undergoing mitosis. *Mol. Biol. Cell* 22, 1227–1239
34. Tiniakos, D. G., Vos, M. B., and Brunt, E. M. (2010) Nonalcoholic fatty liver disease: pathophysiology. *Annu. Rev. Pathol. Mech. Dis.* 5, 145–171
35. Vecchi, C., Montosi, G., and Pietrangelo, A. (2010) Huh-7: A human “hemochromatotic” cell line. *Hepatology* 51, 654–659
36. Bradbury, M. W. (2006) Lipid metabolism and liver inflammation. I. Hepatic fatty acid uptake: possible role in steatosis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 290, G194–G198
37. Listengarten, L. L., Han, X., Lewis, S. E., Cases, S., Farese, R. V., Jr., Ory, D. S., and Schaffer, J. E. (2003) Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *Proc. Natl. Acad. Sci.* 100, 3077–3082
38. Ricchi, M., Odoardi, M. R., Carulli, L., Anzivino, C., Ballestri, S., Pinetti, A., Fantoni, I. I., Marra, F., Bertolotti, M., Banni, S., Lonardo, A., Carulli, N., and Loria, P. (2009) Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. *J. Gastroenterol. Hepatol.* 24, 830–840
39. Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M., and Tomita, F. (1986) Staurosporine, a potent inhibitor of phospholipid Ca2+/H11001-dependent protein kinase. *Biochem. Biophys. Res. Commun.* 135, 397–402
40. Martiny-Baron, G., Kazanietz, M. G., Mischak, H., Blumberg, P. M., Kochs, G., Hug, H., Marmé, D., and Schächtele, C. (1993) Selective inhibition of protein kinase C isozymes by the indolocarbazole Gö 6976. *J. Biol. Chem.* 268, 9194–9197
41. Kubitz, R., Saha, N., Kühlkamp, T., Dutta, S., vom Dahl, S., Wettstein, M., and Häussinger, D. (2004) Ca2+–dependent protein kinase C isozymes induce cholestasis in rat liver. *J. Biol. Chem.* 279, 10323–10330
42. Valencia-Sanchez, M. A., Liu, J., Hannon, G. J., and Parker, R. (2006) Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes Dev.* 20, 515–524
43. Bartel, D. P. (2009) MicroRNAs: target recognition and regulatory functions. *Cell* 136, 215–233
44. Yang, Z., Chen, S., Luan, X., Li, Y., Liu, M., Li, X., Liu, T., and Tang, H. (2009) MicroRNA-214 is aberrantly expressed in cervical cancers and inhibits the growth of HeLa cells. *IUBMB Life* 61, 1075–1082
45. Rottiers, V., and Näää, A. M. (2012) MicroRNAs in metabolism and metabolic disorders. *Nat. Rev. Mol. Cell Biol.* 13, 239–250
46. Doller, A., Huwiler, A., Müller, R., Radeke, H. H., Pfleischfeder, J., and Eberhardt, W. (2007) Protein kinase Cα-dependent phosphorylation of the mRNA-stabilizing factor HuR: implications for posttranscriptional regulation of cyclooxygenase-2. *Mol. Biol. Cell* 18, 2137–2148
47. Doller, A., Akool-el-S., Huwiler, A., Müller, R., Radeke, H. H., Pfleischfeder, J., and Eberhardt, W. (2008) Posttranslational modification of the AU-rich element binding protein HuR by protein kinase Cδ elicits angiotensin II-induced stabilization and nuclear export of cyclooxygenase 2 mRNA. *Mol. Cell Biol.* 28, 2608–2625
48. Eitel, K., Staiger, H., Rieger, J., Mischak, H., Brandhorst, H., Brendel, M. D., Bretzel, R. G., Häring, H.-U., and Kellerer, M. (2003) Protein kinase Cδ activation and translocation to the nucleus are required for fatty acid-induced apoptosis of insulin-secreting cells. *Diabetes* 52, 991–997