Oleate-Mediated Stimulation of Microsomal Triglyceride Transfer Protein (MTP) Gene Promoter: Implications for Hepatic MTP Overexpression in Insulin Resistance†

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ABSTRACT: Hepatic lipoprotein overproduction in a fructose-fed hamster model of insulin resistance was previously shown to be associated with a significant elevation of intracellular mass of microsomal triglyceride transfer protein (MTP) and elevated plasma levels of free fatty acids (FFA). Here, we further establish that fructose feeding and development of an insulin resistant state result in higher levels of MTP mRNA, protein mass, and lipid transfer activity. MTP protein mass was increased in fructose-fed hamster hepatocytes to 161 ± 35.8% of control ($p < 0.05$), while MTP mRNA levels and MTP lipid transfer activity were increased to 147.5 ± 30.8% ($p < 0.05$) and 177.5 ± 14.5% ($p < 0.05$) of control levels, respectively. To identify underlying mechanisms, we also investigated the potential link between enhanced FFA flux and hepatic MTP gene expression. Direct modulation of MTP gene transcription by fatty acids was investigated by transfecting HepG2 cells with a reporter (luciferase) construct containing various base pair regions of the human MTP promoter including pMTP124 (with the sterol response element (SRE)), pMTP116, pMTP109 and pMTP100 (no SRE), and pMTP124SREKO (SRE sequences mutated). Treatment of HepG2 cells with oleic acid (360 μM) significantly increased luciferase activities in cells transfected with pMTP124 (136.6 ± 11.0%, $p < 0.05$) and pMTP124SREKO (153.9 ± 11.1%, $p < 0.01$) compared with control. Luciferase activity was also increased in a time and dose-dependent manner in the presence of oleic acid when transfected with pMTP124SREKO but not pMTP109 and pMTP100. Furthermore, long-term oleic acid treatment of HepG2 cells (10 days) induced higher levels of MTP mRNA ($p < 0.05$) confirming transcriptional stimulation of the MTP gene by oleic acid. In contrast, palmitate, arachidonic acid or linoleic acid did not significantly stimulate pMTP124 or pMTP124SREKO luciferase activity ($p > 0.05$). These data demonstrate that (1) MTP gene transcription may be directly up-regulated by oleic acid; (2) up-regulation of MTP gene transcription by oleic acid is SRE sequence independent; and (3) the sequence −116 to −109 in the MTP promoter region is essential for oleic acid-mediated stimulation. Stimulation of MTP gene expression may be a novel mechanism by which certain FFAs can induce hepatic lipoprotein secretion in insulin resistant states.

Microsomal triglyceride transfer protein (MTP),[1] a key factor involved in VLDL-apoB synthesis and secretion (*1*, *2*), is a heterodimeric protein consisting of a 97 kDa catalytic subunit and the 58 kDa enzyme, protein disulfide isomerase (PDI) (*3*). MTP is located in the ER lumen of apoB secreting cells: hepatocytes, enterocytes and cardiac myocytes (4−6), and acts as a neutral lipid transfer protein. MTP is thought to transfer lipids to apoB while the apoB polypeptide chain is being translated and translocated into the lumen of ER, allowing apoB to fold properly and assemble into a spherical lipoprotein with a core of neutral lipids (*7*). The larger catalytic subunit of MTP is essential for lipid transfer activity and apoB-containing lipoprotein secretion. In the absence of MTP, newly synthesized apoB is rapidly degraded by the proteasomal pathway (*8*) and essentially none of it is secreted. Patients with abetalipoproteinemia, an autosomal recessive disease in which MTP activity is virtually absent, exhibit an almost complete absence of apoB-containing lipoproteins in their plasma (*9*, reviewed *10*). This has been confirmed in both cell and animal studies where apoB secretion is decreased in a dose-dependent manner upon treatment with specific MTP inhibitors (*11*−*16*). In addition, apoB production and secretion in nonhepatic and nonintestinal cells requires coexpression of apoB with MTP (*17*−*19*).

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[1] Abbreviations: apoB, apolipoprotein B-100; ER, endoplasmic reticulum; FF, fructose-fed; FFA, free fatty acids; LDL, low-density lipoproteins; MTP, microsomal triglyceride transfer protein; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PDI, protein disulfide isomerase; PCR, polymerase chain reaction; PMSF, phenylmethyl-sulfonylfluoride; SDS, sodium dodecyl sulfate; SRE, sterol response element; SREBP, sterol regulatory element binding protein; VLDL, very low-density lipoproteins.

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MTP gene expression is transcriptionally regulated by dietary fat and carbohydrate (*20*), insulin (*21*), and ethanol intake (*22*). Studies of the gene promoter revealed that the 5′ ends of human and hamster MTP genes share similar structural features (*23*). The promoter sequences are well conserved and consist of similar functional elements. Transient transfection of an MTP promoter-luciferase reporter revealed that the promoter is active in liver and intestinal cells (*23*). The −123 to −85 bp region of the human promoter is critical for MTP expression and contains the consensus recognition sequences for hepatocyte-specific factors. The human promoter activity is positively regulated by cholesterol and negatively regulated by insulin (*23*). Sato et al. (*24*) used a series of promoter-luciferase reporter constructs and showed that the region at −124 to +33 base pairs of the human promoter contains the elements required for the suppression of transcription by sterol depletion. Enforced expression of an active form of sterol regulatory element-binding protein (SREBP)-1 (amino acids 1−487) or -2 (amino acids 1−481), both of which are activated under sterol-depleted conditions, is able to mimic sterol-mediated down-regulation. Either further truncation of the promoter region or mutation of the putative SRE sequence (5′-GCAGCCCAC-3′, −124 to −116 base pairs) abolished the sterol- and SREBP-dependent transcriptional regulation (*24*).

A fructose-fed hamster model of insulin resistance has been recently reported by our laboratory that exhibits elevated MTP protein mass and hepatic overproduction of VLDL associated with increased plasma levels of fatty acids (FFA) (*4*). Evidence suggests that the high FFA flux to the liver observed in the insulin resistant state contributes to overproduction of VLDL by providing core lipid substrates required for lipoprotein assembly and/or facilitates translocation of newly synthesized apoB as well as reducing cotranslational apoB degradation (*25*−*27*). The purpose of the present study was to test the hypothesis that overproduction of VLDL might be mediated via FFA-stimulated upregulation of MTP gene transcription. Our results suggest that oleic acid can stimulate MTP gene transcription in an SRE-independent manner, providing a possible additional mechanism by which fatty acids enhance the assembly and secretion of apoB-containing lipoproteins.

EXPERIMANTAL PROCEDURES

Animal Protocol and Primary Hamster Hepatocytes. Male Syrian golden hamsters (*Mesocricetus auratus*) weighing 100−120 g were purchased from Charles River (Montréal, QC), and liver perfusion medium, hepatocyte wash medium, liver digest medium, hepatocyte attachment medium, and Williams' medium E were obtained from Life Technologies (Grand Island, NY). All surgical disposable materials were obtained from Johnson & Johnson Medical Inc., Arlington, TX. Fructose-enriched diet (hamster diet with 60% fructose, pelleted) was from Dyets Inc., Bethlehem, PA. At the end of the 3-week feeding period, primary heptocytes were prepared from both chow fed and fructose-fed hamsters as described (*4*, *28*). Liver was perfused as described (*28*) with small modifications which included using commercial liver perfusion and liver digest media to achieve partial liver tissue digestion. Cell viability was measured by exclusion of 0.2% trypan blue, and viable cell suspensions were either used directly for MTP activity assay or seeded in 35 mm dishes, previously coated with collagen Type I (100 μg/dish, ICN), with 1.5 million viable cells for MTP protein mass and mRNA measurements. Dishes were incubated for 4 h to allow attachment of viable cells. Nonviable cells were removed and cells were incubated with Williams' medium E supplemented with 5% fetal bovine serum and 1.5 ng/mL insulin. Primary hamster hepatocytes were used in experiments after either 4 h or overnight incubation and additional washing.

Immunoblot Analysis. Primary hepatocytes isolated from control and fructose-fed hamsters were lysed in solubilizing buffer containing protease inhibitors (PBS containing 1% Nonidet P-40, 1% deoxycholate, 5 mM EDTA, 1 mM EGTA, 2 mM PMSF, 0.1 mM leupeptin, 100 KIU/mL Trasylol, 0.5 μM ALLN) and equal amount of cell lysates resolved on 8% SDS PAGE, transferred onto nitrocellulose membrane, and subjected to chemiluminescent immunoblotting for the 97 kDa subunit of MTP. The membranes were blocked with a 5% solution of fat free dry milk powder, incubated with a goat anti-bovine MTP antiserum provided by Dr. David Gordon (Bristol-Myers Squibb), washed, and then incubated with a rabbit anti-goat secondary antibody conjugated to peroxidase. Membranes were then incubated in an enhanced chemiluminescence detection reagent (Amersham Pharmacia Biotech) for 1 min and exposed to Kodak Hyperfilm. Films were developed and quantitative analysis was performed using an imaging densitometer.

RNase Protection Solution Hybridization Assay for Hamster MTP mRNA. Hamster MTP cDNA was kindly provided by Dr. David Gordon (Bristol-Myers Squibb, Princeton, NJ). Two primers GCGCCTCGAGGCCTTCATTCAGCACCTC (*Xho*I site underlined) and GCGCAAGCTTCCAGCCTCAGCATACTTC (*Hin*dIII site underlined) were used to amplify a 517 bp fragment of hamster MTP cDNA. After digestion with *Xho*I and *Hin*dIII, this fragment was ligated with *Xho*I/*Hin*dIII digested pGEM-7Zf vector (Promega). These constructs served as templates to synthesize antisense RNA probes and standard cRNA. Unlabeled cRNA corresponding to the sense DNA strand was prepared for use as hybridization standard. Total RNA from liver tissue was isolated using Trizol reagent (Gibco BRL). RNase protection analyses were performed as described by Azrolan and Breslow (*29*). Briefly, riboprobe and either sample or standard cRNA were hybridized overnight in 40 μL of hybridization buffer (80% (v/v) formamide; 40 mM HEPES, pH 6.7; 0.4 M NaCl; 1 mM EDTA) at 63 °C. RNase A and RNase T1 in digestion buffer (0.3 mM NaCl; 10 mM Tris-HCl, pH7.4; 5mM EDTA) were added to each sample and incubated at 34 °C for 1 h. After incubation, 20% trichloroacetic acid (TCA) and 100 μg of salmon sperm DNA were added and incubated for 15 min to precipitate protected RNA, and each sample was filtered using glass fiber filters (Whatman). Filters were washed with 10% TCA and counted with scintillation fluid in a β counter.

MTP Activity Assay. The MTP activity assay was carried out using a commercially available kit (Roar Biomedical, Inc., New York, NY) according to the manufacturer's recommendations. The MTP activity assay is based on MTP mediated transfer of a proprietary fluorescent neutral lipid entrapped in donor vesicles (self-quenched state) to the acceptor (fluorescent state). The MTP mediated transfer is observed by the increase in fluorescence intensity as the

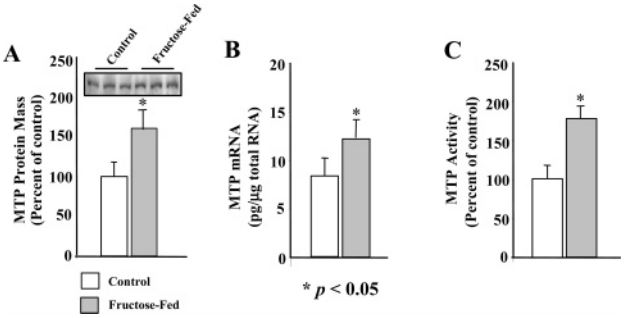

FIGURE 1: Elevated hepatic MTP mRNA, protein mass, and lipid transfer activity in fructose-fed hamsters, an animal model of insulin resistance. (A) Hepatocytes isolated from chow-fed and fructose-fed hamsters were solubilized, equal amounts of cell protein were resolved by SDS−PAGE (10% (v/v) resolving gel), and proteins were then transferred onto nitrocellulose membranes. Immunoblotting was performed to detect the 97 kDa MTP subunit with a goat anti-bovine MTP antiserum (see inset), the MTP bands were quantified by densitometric scanning. The mass of the 97 kDa MTP subunit detected was expressed as a percentage of the MTP mass detected in control cells ($n = 6$, (*) $p < 0.05$). (B) MTP mRNA levels were analyzed by RNase protection/solution hybridization assays as described in the Experimental Procedures section. The results are expressed as pg of MTP mRNA per μg of total RNA and normalized to control β-actin mRNA. Values are given as the mean ± SD from three experiments. (*) $p < 0.05$ ($n = 3$) vs control. (C) MTP lipid transfer activity was measured using a commercially available MTP activity kit from Roar Biomedical, Inc., as described in the Experimental Procedures. MTP activity was measured on triplicate liver samples from groups of 3 control and 3 fructose-fed hamsters, and is shown as percent of the activity in control samples, (*) $p < 0.05$.

fluorescent neutral lipid is transferred from the self-quenched donor to the acceptor. The primary hamster hepatocytes isolated from control and fructose-fed hamsters were suspended in a homogenization buffer (150 mM NaCl, 1 mM EDTA, 0.5 mM PMSF, 2 mg of leupeptin, in 100 mL of 10 mM tris pH 7.4). The suspensions were then sonicated on ice with 5-s bursts in a solicitor fitted with a microtip on power setting 4. One hundred micrograms of homogenate protein of each group was used in the assay. The MTP source (0.5 mL total volume) was combined with 10 μL of donor and 10 μL of acceptor and then incubated for 12 h at 37 °C. The assay was read in a fluorescence spectrometer at an excitation wavelength of 465 nm and an emission wavelength of 535 nm.

Cell Culture and MTP Promoter-Luciferase Reporter Gene Constructs. HepG2 cells were from American Type Culture Collection and were maintained at 37 °C in an atmosphere with 5% CO_2 in α-MEM medium (GibcoBRL) containing 10% fetal calf serum (FCS, certified grade), penicillin (100 unit/mL), and streptomycin (100 μL/mL) (GibcoBRL).

The MTP promoter-luciferase reporter gene constructs pMTP100, pMTP109, pMTP124, and pMTP124SREKO were previously reported by Dr. Ryuichiro Sato (*24*) and are listed in Figure 2A or Figure 4A. The promoter sequences $^{-124}$GCAGCCCAC^{-116} were mutated to $^{-124}$GCGAATTCC^{-116} in the pMTP124SREKO construct in order to mutate the SRE sequences (*20*). The pMTP116 luciferase reporter plasmid was constructed by cloning the *Bgl*II-*Hin*dIII PCR fragment coding the −116 to +33 region of the human MTP gene into the same restriction sites of a pGL3 basic vector (Figure 4A).

Transient Transfection and Luciferase Assay. HepG2 cells (5×10^5) were seeded in a 6-well plate 18 h before the experiments. After washing the cells once with 2 mL of sterile PBS, 1 μg of DNA of each MTP promoter-luciferase reporter gene construct was transfected into HepG2 cells using 15 μL of Lipofectamine (Life Technologies, Grand Island, NY) according to the manufacturer's protocol. Transfection efficiency was monitored by cotransfecting cells with 0.5 μg of the PRL-TK vector (Promega). This vector contains the renilla luciferase gene and the HSV-thymidine kinase promoter. This weak promoter is suitable to use as a control as it provides neutral constitutive expression of the renilla luciferase. Forty-eight hours post-transfection, the samples were washed twice with 1 X PBS and then treated in the presence or absence of 360 μM oleic acid, palmitate, arachidonic acid, or linoleic acid in serum free α-MEM culture medium. The luciferase assay was carried out by using Dual-Luciferase Reporter Assay System (Promega). A ratio of MTP promoter luciferase:renilla luciferase activity was calculated for each sample to normalize differences in cell number and transfection efficiency.

RT-PCR for Determination of MTP and β-Actin mRNAs in HepG2 Cells. Following oleic acid treatment, total RNA was extracted from HepG2 cells using a commercially available kit (RNeasy, Qiagen). First-strand cDNA was synthesized from 5 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen). The resulting cDNA was subjected to 28 cycles of PCR amplification (denaturation at 95 °C for 30 s; annealing at 55 °C for 60 s; extension at 72 °C for 90 s) using primers specific for either MTP or β-actin and 2 units of Taq polymerase (recombinant, Invitrogen). The primer pairs for MTP were 5′-TGAAAAGC-CAGAGAAAAAGGAG-3′ and 5′-CCACTCATCAGTTT-CAAAACCA-3′ which amplified a 1319 bp MTP fragment, and for β-actin were 5′-CCTTTTCCAGCCTTCCTTC-3′ and 5′-TACTCCTGCTTGCTGATCC-3′ which amplified a 305 bp β-actin fragment. The PCR products were visualized following electrophoresis on 1% (MTP) or 1.5% (β-actin) agarose gels.

RESULTS

Evidence for Elevated mRNA Levels and Increased MTP Activity in Hepatocytes from Fructose-Fed Hamsters. We have previously reported that chronic fructose feeding of hamster (2−3 weeks) leads to a significant increase in plasma FFA as well as hepatic MTP mass (*4*). Here, we further investigated the modulation of hepatic MTP at the protein, mRNA, and activity levels. Hamsters were fed a fructose-enriched diet for a period of 3 weeks, and the development of an insulin resistant state was confirmed as previously reported (*4*). A specific anti-MTP antibody was used to estimate the protein mass of MTP in control and fructose-fed hamsters. Equal protein quantities of total cell lysate were loaded in each lane and subjected to immunoblotting with the anti-bovine MTP antibody. Figure 1A shows that cellular protein mass of MTP (see inset) in hepatocytes from fructose-fed hamsters increased to 161 ± 35.8% ($n = 6$, $p < 0.05$) of that in control hepatocytes. This confirmed our previously reported observation that hepatic MTP protein mass is significantly increased in fructose-fed hamsters (*4*). To investigate whether the increased MTP protein level in fructose-fed hamsters was due to up-regulation of MTP gene transcription, MTP mRNA was measured using an RNase protection assay. MTP mRNA levels were significantly

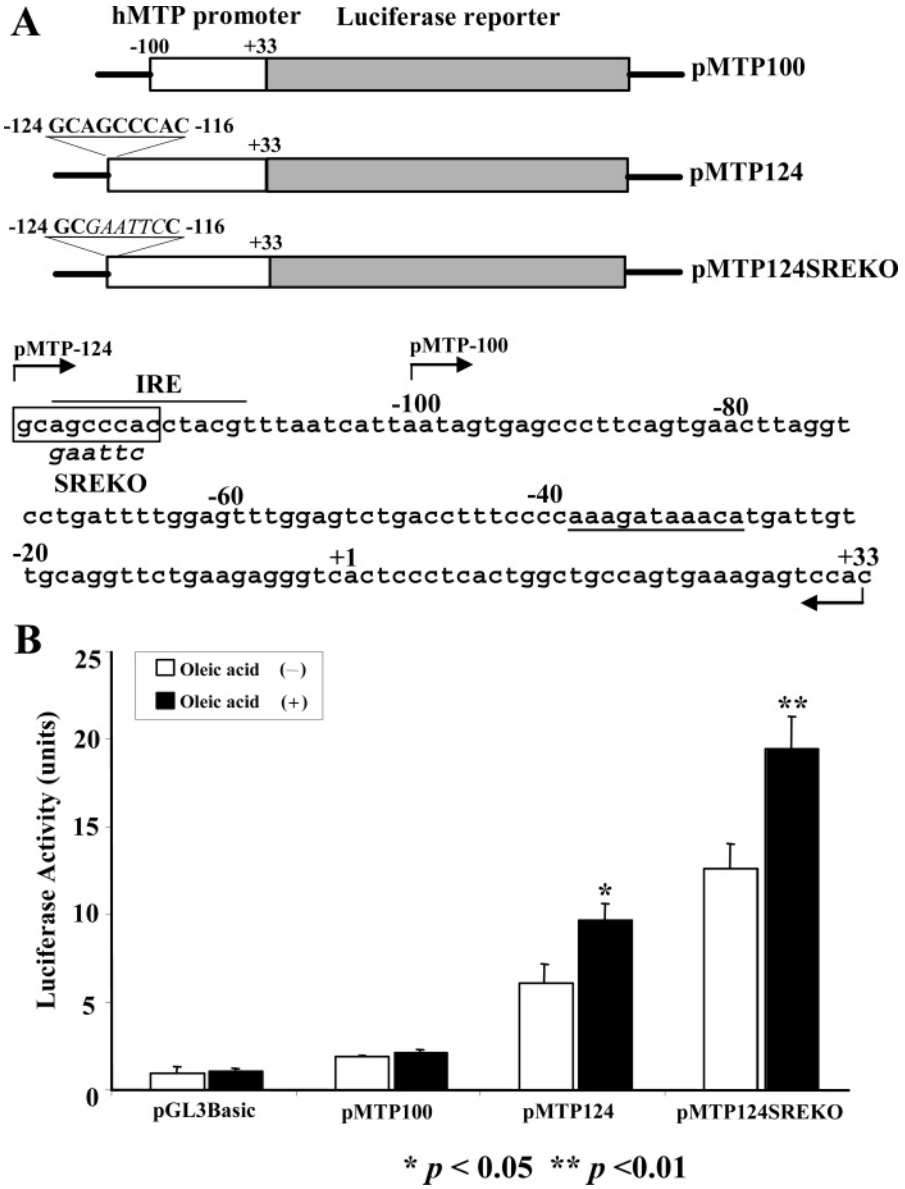

FIGURE 2: Oleic acid treatment stimulates expression of MTP gene promoter-luciferase reporter constructs in HepG2 cells. (A, upper panel) Schematic diagrams showing the MTP promoter-luciferase reporter constructs: pMTP100, pMTP124, and pMTP124SREKO. The open box represents the MTP promoter region and italic capital letters represent mutated DNA sequences in the pMTP124SREKO. The shaded box shows the luciferase reporter region. (A, lower panel) Human MTP promoter sequence (*20*). The transcription start site is at +1. The TATA-like sequence is underlined. The putative insulin-responsive element (IRE) is overlined. The SRE site is boxed. The mutant sequence in the SRE sites is shown by italic letters under the individual original sequence. Arrows indicate the sites used for preparation of truncated reported gene constructs. (B) HepG2 cells (5×10^5) were transiently transfected with 1 μg of each MTP promoter-luciferase reporter construct and 0.5 μg of renilla luciferase control vector. Thirty hours after transfection, the culture medium was replaced with serum-free α-MEM and the incubation was continued. Forty-eight hours after transfection, the cell cultures were treated with 360 μM oleic acid at 37 °C for 1 h. The luciferase activities of MTP promoter-luciferase reporter constructs were determined by using the dual-luciferase reporter assay system. The ratio of firefly luciferase activity (from the MTP promoter construct) to renilla luciferase activity provides a measure of MTP promoter activity normalized for transfection efficiency. The values given are the average of triplicate samples and representative of two independent experiments expressed as mean ± SD, (*) $p < 0.05$, (**) $p < 0.01$.

increased in insulin resistant hepatocytes to 147.5 ± 30.8% ($n = 3$, $p < 0.05$) of that in the control cells (Figure 1B). To evaluate the impact of elevated MTP protein mass and mRNA on its functionality, MTP lipid transfer activity was measured in hepatocytes isolated from control and fructose-fed hamsters. As depicted in Figure 1C, MTP activity in hepatocytes isolated from fructose-fed hamsters was also significantly higher than that in control hepatocytes (177.5 ± 14.5%, $n = 3$, $p < 0.05$), suggesting that MTP lipid transfer activity was enhanced in fructose-fed hamsters.

Stimulation of MTP Promoter-Luciferase Reporter Gene by Oleic Acid in HepG2 Cells. To explore whether MTP gene transcription may be up-regulated by the enhanced plasma FFA (commonly observed in insulin resistant states, including the fructose-fed hamster model (*4*)), initially, three MTP promoter-luciferase reporter constructs, pMTP100, pMTP124, and pMTP124SREKO were tested for their activities in HepG2 cells in the presence or absence of oleic acid. Figure 2A shows the plasmid constructs of the MTP promoter region and luciferase gene. Transient transfection of HepG2 cells with each of the three MTP promoter-luciferase reporter genes was performed followed by treatment with 360 μM oleic acid at 37 °C for 1 h. There were significantly increased luciferase activities in pMTP124

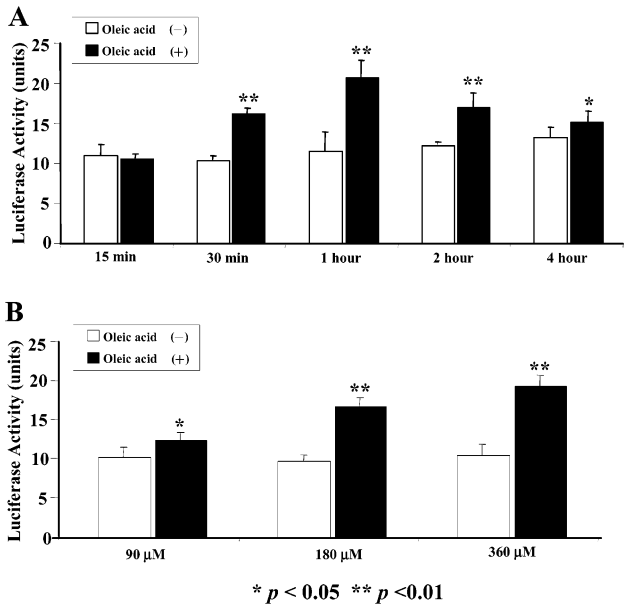

FIGURE 3: Oleic acid-stimulated up-regulation of the promoter activity of pMTP124SREKO by oleic acid is time and dose dependent. Lipofectamine-mediated transient transfection was carried out in HepG2 cells (5×10^5) with 1 μg of MTP promoter-luciferase report construct, pMTP124SREKO, and 0.5 μg of renilla luciferase control vector. Thirty hours after transfection, the culture medium was replaced with serum-free α-MEM and the incubation continued. Forty-eight hours after transfection the cell cultures were treated with 360 μM oleic acid for 15 min to 4 h (A) or with 90−360 μM oleic acid for 1 h (B). The activities of MTP promoter-reporter constructs were determined by using the dual-luciferase reporter assay system. The ratio of firefly luciferase activity (from the MTP promoter construct) to renilla luciferase activity provides a measure of MTP promoter activity normalized for transfection efficiency. The values given are the average of triplicate samples and represent three experiments expressed as mean ± SD, (*) $p < 0.05$, (**) $p < 0.01$.

(136.6 ± 11.0%, $p < 0.05$) and pMTP124SREKO (153.9 ± 11.1%, $p < 0.01$) in the presence of oleic acid (Figure 2B). By contrast, there was no significant luciferase activity change in HepG2 cells transfected with pMTP100 in the presence of oleic acid (Figure 2B). We also observed that the luciferase activity was significantly reduced in pMTP100 compared to pMTP124 (28.6 ± 3.0%, $p < 0.01$) in the absence of oleic acid (Figure 2B). These data suggest that the MTP promoter-luciferase gene transcription may be directly up-regulated by oleic acid, that up-regulation of MTP gene transcription by oleic acid is SRE sequence independent, and the sequences −124 to −100 within the promoter region are essential for oleic acid-mediated stimulation.

Up-Regulation of Luciferase Activity of pMTP124SREKO by Oleic Acid Is Time and Dose Dependent. To confirm our observation that up-regulation of MTP gene transcription by oleic acid is SRE sequence independent, the MTP promoter-luciferase reporter gene construct, pMTP124SREKO, was transiently transfected into HepG2 cells. Figure 3A shows oleic acid stimulation of luciferase activity in a time dependent manner. In the presence of 360 μM oleic acid, the luciferase activities of pMTP124SREKO were significantly increased to 157 ± 5.89% ($p < 0.01$) at 30 min, 180 ± 19.1% ($p < 0.01$) at 1 h, 140 ± 4.1% ($p < 0.01$) at 2 h, and 120 ± 8.3% ($p < 0.05$) at 4 h in comparison with control, respectively. As shown in Figure 3B, oleic acid enhanced luciferase activity in a dose dependent manner. After 1 h stimulation with oleic acid at concentrations of 90, 180, and 360 μM, the luciferase activities of pMTP124SREKO were significantly increased to 122 ± 12.4% ($p < 0.05$), 172 ± 8.4% ($p < 0.01$), and 185 ± 14.1% ($p < 0.01$), respectively, compared to untreated controls.

The Sequence −116 to −109 in the MTP Promoter Region Mediates Oleic Acid Stimulation of MTP Gene Promoter. To narrow down the promoter region involved in oleate-mediated stimulation of the MTP promoter, we prepared a new plasmid reporter construct, pMTP116, containing the sequences −116 to +33 of human MTP promoter region. Another construct, pMTP109, containing 7 fewer base pairs of the 5′ end of the MTP promoter region compared with pMTP116 (Figure 4A), was also employed. The activities of the pMTP116 and pMTP109 constructs were compared with those of pMTP100 and pMTP124 in HepG2 cells with or without oleic acid treatment. As shown in Figure 4B, while oleic acid induced higher luciferase expression in cells transfected with pMTP124 (184.1 ± 18.7%, $p < 0.01$) and pMTP116 (164.7± 11.8%, $p < 0.01$), no effect was observed in cells transfected with pMTP109. These data support the notion that the upstream sequences between −116 to −109 of MTP promoter may be responsible for oleate-mediated stimulation of MTP gene transcription. We also observed that the luciferase activity was significantly reduced in pMTP100 compared to pMTP109 in either the absence (26.9 ± 2.6%, $p < 0.01$) or the presence (29.6 ± 0.8%, $p < 0.01$) of oleic acid (Figure 4B). Thus, the sequence between −109 and −100 in the MTP promoter that contains the activator protein 1 (AP-1) motif is essential for basal MTP gene transcription in HepG2 cells (*24*).

Stimulation of the MTP Promoter Region Is Oleic Acid Specific. To test whether other free fatty acids have a stimulatory effect on MTP promoter activity, the effects of oleate and palmitate, arachidonic acid and linoleic acid were compared on two MTP promoter-luciferase reporter constructs, pMTP124 and pMTP124SREKO. After transient transfection of HepG2 cells with each of the two MTP promoter-luciferase reporter cDNAs, cells were treated with 360 μM oleic acid, palmitate, arachidonic acid, or linoleic acid. While oleate treatment significantly increased luciferase activity in cells transfected with either pMTP124 (1.58 ± 0.14-fold, $p < 0.01$) or pMTP124SREKO (1.68 ± 0.08-fold, $p < 0.01$) (Figure 5), palmitate, arachidonic acid, or linoleic acid treatment at the same concentration (360 μM) had no significant effect on MTP promoter activity ($p > 0.05$) with either of the two constructs (Figure 5). The data appears to suggest that stimulation of MTP promoter is oleic acid specific.

Oleic Acid Treatment Stimulates MTP mRNA and MTP Protein Levels in HepG2 Cells. Finally, we examined the effect of oleic acid treatment (24 h or 10 day) of HepG2 cells on the levels of MTP mRNA and MTP protein mass to assess whether MTP gene expression can be induced in normal, untransfected HepG2 cells. Following treatment of HepG2 cells with 360 μM oleic acid for 24 h or 10 days, total cellular RNA was isolated and first-strand cDNA was synthesized. PCR amplifications of MTP and the control β-actin cDNAs were conducted using specific primers. A ratio of MTP:β-actin PCR product was calculated for each sample to normalize differences in cell number. As shown in Figure 6, mRNA levels changed slightly following 24 h

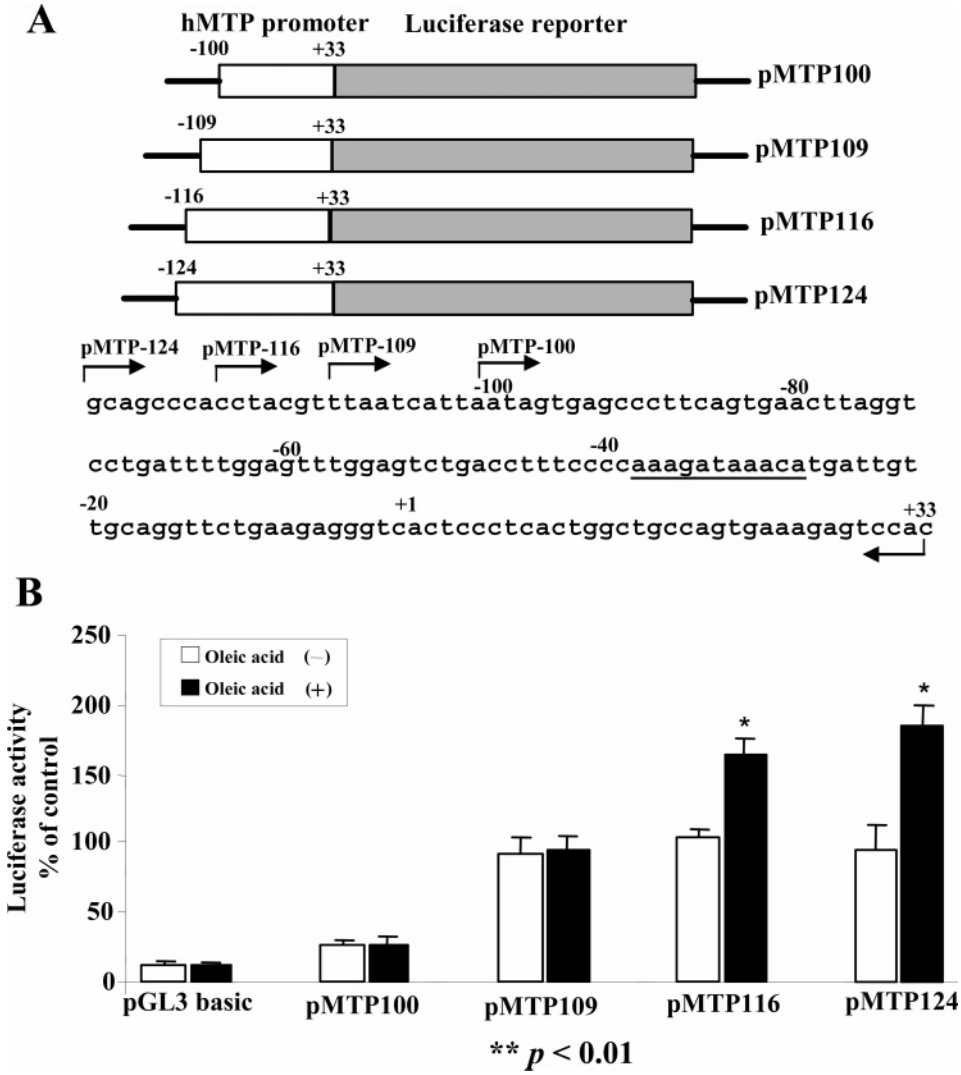

FIGURE 4: The sequences −116 to −109 in the MTP promoter region mediate oleic acid stimulation of MTP gene promoter. (A, upper panel) Schematic diagrams showing the MTP promoter-luciferase reporter constructs: pMTP100, pMTP109, pMTP116, and pMTP124. The open box represents the MTP promoter region, and the shaded box shows the luciferase reporter region. (A, lower panel) Human MTP promoter sequence (*20*). The putative transcription start site is at +1. The TATA-like sequence is underlined. Arrows indicate the sites used for preparation of truncated reported gene constructs. (B) Transient transfection was carried out in HepG2 cells (5×10^5) with 1 μg of MTP promoter-luciferase reporter constructs, pMTP100, pMTP109, pMTP116, and pMTP124, and 0.5 μg of renilla luciferase vector. Thirty hours after transfection, the culture medium was replaced with serum-free α-MEM and the incubation continued for an additional 18 h. Forty-eight hours after transfection, the cell cultures were treated with 360 μM oleic acid for 1 h at 37 °C. The luciferase activities of MTP promoter-luciferase reporter constructs were determined by using the dual-luciferase reporter assay system. Shown are ratios of firefly luciferase activity to renilla luciferase activity to normalize for transfection efficiency. The values given are the average of triplicate samples and representative of two independent experiments expressed as mean ± SD, (**) $p < 0.01$.

oleate treatment (not statistically significant) (panel A), but significantly increased to 135.0 ± 14.9% ($n = 3$, $p < 0.05$) in cells treated with oleic acid for 10 days (panel B). After treatment of HepG2 cells with 360 μM oleic acid for 10 days, total cell lysates were also prepared and probed with anti-MTP antibody or anti-albumin antibody, respectively. As depicted in Figure 6C, MTP protein level increased to 156.1 ± 14.0% ($n = 3$, $p < 0.05$) in cells treated with oleic acid compared to untreated cells. No change in albumin protein mass could be detected.

DISCUSSION

The role of FFA in apoB maturation and lipoprotein secretion has been well established; however, it remains unclear how FFA enhances secretion of apoB-containing lipoproteins. Increased FFA flux to the liver is likely to increase synthesis of core lipoprotein lipids, particularly triglyceride, which can in turn promote hepatic lipoprotein secretion (*4*, *25*, *26*, *28*). Here, we hypothesized that elevated FFA levels may also directly or indirectly stimulate MTP gene expression and thus hepatic lipoprotein production. FFA has been previously shown to interfere with cellular insulin signaling and induce insulin resistance (*26*). Since MTP has an insulin response element and is known to be regulated by insulin (*23*), it is plausible that enhanced hepatic FFA flux can indirectly affect MTP gene expression and activity by interfering with hepatic insulin signaling. In the present study, using an MTP promoter-luciferase reporter gene expression system, we have demonstrated direct oleate mediated stimulation of MTP gene promoter activity via a mechanism involving binding of factors to the MTP promoter region and activation of specific sequences. Up-regulation

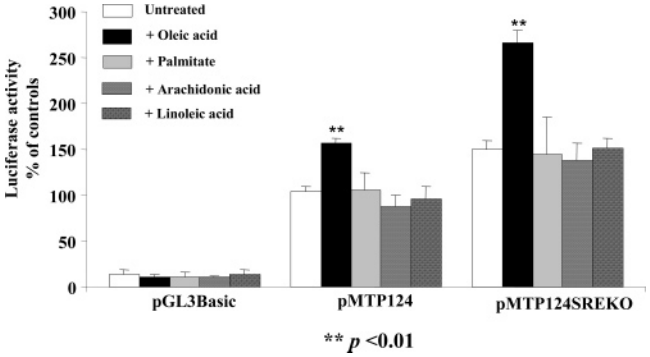

FIGURE 5: Stimulation of the MTP promoter region is oleic acid specific. Transient transfection was carried out in HepG2 cells with MTP promoter-luciferase reporter constructs, pMTP124 and pMTP124SREKO. Forty-eight hours after transfection, cells were treated with or without 360 μM oleic acid, palmitate, arachidonic acid, or linoleic acid for 1 h at 37 °C, respectively. Cell lysates were prepared and luciferase activity was determined using the dual-luciferase reporter assay system. The ratio of firefly luciferase activity (from the MTP promoter construct) to renilla luciferase activity provides a measure of MTP promoter activity normalized for transfection efficiency. The values given are the average of triplicate samples and representative of two independent experiments expressed as mean ± SD, (**) $p < 0.01$.

of MTP may in turn increase assembly and secretion of apoB-containing lipoproteins. Interestingly, however, palmitate, arachidonic acid, and linoleic acid had no significant stimulatory effect on the MTP promoter activity.

MTP is essential for the assembly and secretion of apoB-containing lipoproteins (*30*, *31*). It has been shown by several groups that overexpression of the catalytic subunit of MTP can up-regulate VLDL-apoB. Liao et al. (*32*) reported that adenovirus-mediated overexpression of MTP stimulated apoB secretion in HepG2 cells. Using adenovirus-mediated overexpression of MTP in vivo, Tietge et al. (*33*) showed that, 4 days after virus injection, hepatic VLDL-apoB secretion in the adenovirus-injected group was 74% higher than in the control group. There is also direct evidence that MTP inhibitors inhibit both MTP activity and newly synthesized apoB (*12*). Recently, in vivo studies by Shiomi et al. (*34*) have confirmed that MTP inhibition decreases VLDL secretion by 80% in LDL-deficient Watanabe rabbits. MTP gene knockout mice provided further evidence. In liver specific knockout mice, Raabe et al. (*35*) demonstrated that inactivation of the MTP gene can reduce apoB100 levels in the plasma by 95%. VLDL-sized lipid-stained particles were not observed in the ER nor in the Golgi apparatus of MTP-deficient hepatocytes.

Overproduction of apoB-containing lipoprotein associated with elevated hepatic MTP protein mass was observed in our own laboratory in an animal model of diet-induced insulin resistance, the fructose-fed Syrian golden hamster (*4*). Studies in the fructose-fed Syrian golden hamster model revealed that hepatic overproduction of VLDL is associated with a significant increase in cellular synthesis and secretion of total triglyceride (TG) and a striking elevation of MTP in the liver (*4*) and in the intestine (*5*). We hypothesized that VLDL overproduction was partially due to MTP gene up-regulation in the fructose-fed hamster model. To further test this hypothesis here, we have measured MTP protein mass, mRNA levels, and lipid transfer activity in primary hepatocytes isolated from control and fructose-fed hamsters. Increased levels of MTP mass, mRNA, and activity were found in fructose-fed hamsters compared to controls. Our data is supported by other laboratories that have shown that hepatic MTP mRNA can be up-regulated by a high-fat diet in the hamster (*36*). Lin et al. (*20*) also reported that MTP protein level was increased in the intestine of the fat-fed hamster. They confirmed enhanced hepatic MTP mRNA levels in high sucrose-diet-fed hamsters. Hyperlipidemia in an animal model of type 2 diabetes with visceral fat obesity, the Otsuka Long-Evans Toskushima fatty rat, is also associated with elevated hepatic MTP mRNA (*37*).

The MTP gene appears to be transcriptionally regulated. Recently, experimental evidence showed that no MTP protein is expressed in rat liver L35 cell line due to inactive MTP gene transcription (*38*). It appears that a conserved DR1 element within the 135-bp proximal MTP promoter is responsible for differential expression by L35 cells. Transfection of retinoid X receptor (RXRalpha), a protein factor that can bind to the conserved DR1 element, increased MTP

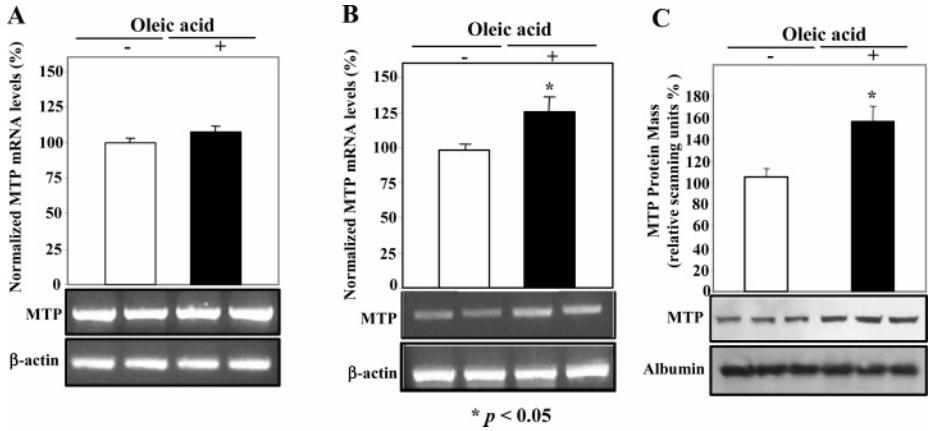

FIGURE 6: Determination of MTP mRNAs and MTP protein mass in HepG2 cells treated with or without oleate. HepG2 cells treated with oleic acid for 24 h (A) or 10 days (B) in culture were used to isolate total RNA, which was then analyzed by RT-PCR assays to measure MTP mRNA levels. Cell lysates were also prepared and probed with an MTP antibody by Western blotting (C) to measure MTP protein mass as described in the Experimental Procedures section. A and B, lower panels, show the amplified RT-PCR products of MTP or β-actin. The amplified MTP and β-actin products were quantified by densitometric scanning and the ratio of MTP:β-actin calculated as shown in the upper panels. Panel C shows the Western blot analysis of MTP protein as well as the control protein, albumin. Values shown are the mean ± SD of three experiments; (*) $p < 0.05$ vs control.

promoter activity by 6-fold in L35 cells (*38*). Modulation of MTP expression could potentially regulate the assembly and secretion of apoB-containing lipoproteins. Insulin ($\geq 10^{-9}$ M) and high concentrations of glucose (>30 mM) were found to decrease MTP large subunit mRNA levels in HepG2 cells (*21*). Insulin treatment was also reported to decease VLDL secretion from rat hepatocytes (*39*). By contrast, sterols positively regulate MTP gene transcription (*24*). In this report, we provide evidence showing that oleic acid can stimulate MTP-promoter luciferase reporter gene expression and increase MTP mRNA levels in HepG2 cells. The luciferase activity was significantly increased in a time dependent (30 min to 4 h) and dose dependent (90−360 μM) manner in the presence of oleic acid in HepG2 cells transiently transfected with the MTP-promoter luciferase reporter, pMTP124SREKO in which SRE sequences were mutated. Our observations suggest that stimulation of the MTP promoter by oleic acid is specific and independent from that of sterols and the sequence −116 to −109 in the MTP promoter region is essential for oleic acid-mediated stimulation. Interestingly, we noted that the sequence between −109 and −100 in the MTP promoter containing the activator protein 1 (AP-1) binding motif is essential for MTP gene transcription in HepG2 cells as its deletion resulted in a substantially lower level of MTP promoter activity. This compares with observations reported by Navasa et al. (*40*), who observed a significant reduction in basal promoter activity of MTP following mutations in the consensus AP-1 binding motif. In contrast to our observations, however, Lin et al. (*21*) reported that MTP mRNA levels did not significantly change in HepG2 cells when treated with 0.8 mM oleate for 24 h. One possible explanation is that the measurement of luciferase activity of expressed MTP promoter-reporter constructs may be more sensitive than the measurement of MTP mRNA levels. We also observed significant changes in MTP mRNA and protein following long term (10 day) treatment with oleate suggesting that chronic treatment may be needed in vivo to allow physiologically appreciable changes in MTP gene expression.

Recently, Roglans et al. (*41*) reported that fructose-feeding in rats could induce hepatic lipogenesis and reduce PPARα (peroxisome proliferator activated receptor α), a nuclear hormone receptor whose ligand-activated transcription factors have been implicated in such diverse pathways as lipid and glucose homeostasis, control of cellular proliferation, and differentiation (*42*). We have also recently found down-regulation of PPARα and PPARγ mRNA in hepatocytes isolated from fructose-fed hamsters (manuscript in preparation). In vivo studies of Rosiglitazone, a PPARγ agonist, in fructose-fed hamsters have also shown a strong link between PPAR activation, hepatic insulin sensitivity, and hepatic VLDL secretion (*43*). It thus appears that overproduction of VLDL-apoB in the liver and intestine in fructose-fed hamsters involves not only up-regulation of the MTP gene but also down-regulation of PPARα and PPARγ. Although down-regulation of PPAR alpha (leading to enhanced hepatic lipogenesis) may not be sufficient to increase VLDL production, in combination with an increased MTP expression, it can significantly augment the stimulation of hepatic VLDL production. Moreover, Au et al. (*44*) recently showed that insulin decreased the MTP mRNA level mainly through transcriptional regulation in HepG2 cells. It appears that insulin inhibits MTP gene transcription through the $MAPK^{erk}$ cascade but not through the PI-3 kinase pathway. Cellular $MAPK^{erk}$ and $MAPK^{p38}$ activities play a counterbalancing role in regulating the MTP gene transcription. Oleate may inhibit MAPK activity (ERK) leading to increased expression of MTP mRNA.

In conclusion, stimulation of MTP gene transcription by oleic acid may partially explain the postulated link between high FFA flux to the liver and overproduction of VLDL in insulin resistance. Further work is needed to identify *cis*-acting sequence elements in the MTP promoter and *trans*-acting factors mediating fatty acid-induced stimulation of MTP gene expression.

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