Regulation of the enzymes of hepatic microsomal triacylglycerol lipolysis and re-esterification by the glucocorticoid dexamethasone

Vernon W. DOLINSKY*, Donna N. DOUGLAS*, Richard LEHNER†‡ and Dennis E. VANCE*[1]

*Department of Biochemistry and CIHR Group on the Molecular and Cell Biology of Lipids, University of Alberta, Edmonton, Alberta, Canada T6G 2S2, †Department of Pediatrics and CIHR Group on the Molecular and Cell Biology of Lipids, University of Alberta, Edmonton, Alberta, Canada T6G 2S2, and ‡Department of Cell Biology and CIHR Group on the Molecular and Cell Biology of Lipids, University of Alberta, Edmonton, Alberta, Canada T6G 2S2

Hepatic VLDL (very-low-density lipoprotein) assembly is a complex process that is largely regulated by the provision of lipid for apolipoprotein B assembly. Intracellular stored TAG (triacylglycerol) undergoes an initial lipolysis followed by re-esterification of the lipolytic products to form TAG prior to their incorporation into a VLDL particle. TGH (TAG hydrolase) is a lipase that hydrolyses intracellular TAG within the hepatocyte. We have utilized both dexamethasone-injected mouse and primary hepatocyte models to address whether stimulation of TAG biosynthesis by the synthetic glucocorticoid, dexamethasone, altered hepatic lipolysis and re-esterification and the provision of stored TAG for lipoprotein secretion. Dexamethasone treatment resulted in decreased TGH expression, primarily due to a dexamethasone-induced decrease in TGH mRNA stability. The expression and activities of diacylglycerol acyltransferases 1 and 2 were stimulated by dexamethasone. The combination of reduced intracellular TAG lipolysis and increased TAG biosynthesis contributed to the accumulation of TAG within the livers of dexamethasone-injected mice. The rate of hepatic TAG secretion in dexamethasone-treated mice was maintained at similar levels as in control mice. Our data demonstrate that stimulation of *de novo* TAG synthesis by dexamethasone increased the proportion of secreted TAG that was derived from *de novo* sources, while the utilization of stored TAG for secretion was reduced. The results show that, during markedly increased TAG synthesis, some TAGs are diverted from the cytosolic storage pool and are utilized directly for VLDL assembly within the endoplasmic reticulum lumen.

Key words: apolipoprotein B, diacylglycerol acyltransferase, triacylglycerol hydrolase, very-low-density lipoprotein (VLDL).

INTRODUCTION

The liver secretes TAGs (triacylglycerols) as VLDL (very-low-density lipoprotein). The majority of TAG secreted by primary hepatocytes is derived from a cytosolic storage pool [1,2]. It has been demonstrated that the stored TAG undergoes an initial lipolysis followed by re-esterification of the lipolytic products to TAG prior to incorporation into a VLDL particle [3–5]. TGH (TAG hydrolase), a lipase that is a member of the carboxylesterase gene family, has been implicated in this process [6]. TGH is highly expressed in the liver [7,8] and it has been estimated that, in the rat, TGH accounts for approx. 70 % of hepatic microsomal alkaline lipase activity [9]. TGH has been immunolocalized to regions of the ER (endoplasmic reticulum) in close contact with the lipid droplet [8]. TGH is absent from hepatoma cell lines that secrete poorly lipidated VLDL [10]. Transfection of McArdle RH7777 hepatoma cells with rat TGH cDNA resulted in an increased mobilization of intracellular TAG and lipidation of apoB100 (apolipoprotein B100) [10]. Furthermore, chemical inhibition of TGH reduced TAG and apoB100 secretion by primary rat hepatocytes [9]. Collectively, these data demonstrated that TGH is a lipase involved in the mobilization of stored TAG, some of which is secreted by the liver.

Following the lipolysis of stored TAG, lipolytic products are re-synthesized to form TAG at the ER, where the TAG may be utilized for lipoprotein assembly [11,12]. The final stage of TAG synthesis is catalysed by DGAT (diacylglycerol acyltransferase). To date, two cDNAs encoding proteins with DGAT activity have been identified. DGAT1 is expressed in a wide variety of tissues, but targeted deletion of the murine DGAT1 gene failed to affect the levels of circulating TAGs [13]. DGAT2 is most highly expressed in liver and adipose tissues [14].

The mechanism of VLDL assembly is complex, and potential for its regulation exists at a variety of levels. Since the rate of VLDL assembly and secretion depends upon the efficiency of apoB lipidation [15], hepatic TAG lipolysis and re-esterification within the ER represents a potential regulatory stage that exerts control over the lipidation of apoB100 [8,9]. At present, little is known regarding the regulation of the lipases and acyltransferases that catalyse the lipolysis and re-synthesis of TAG in the liver. We have recently cloned the murine TGH promoter and characterized the regulation of TGH at the time of weaning [16]. The Sp1 transcription factor was identified as a positive regulatory factor that stimulated TGH expression at the time of weaning, a stage when the liver secretes larger quantities of TAG [16].

The experiments presented herein address whether stimulation of TAG biosynthesis alters hepatic lipolysis and re-esterification and the provision of stored TAG for lipoprotein secretion. Glucocorticoids are steroid hormones secreted from the adrenal glands that have dramatic effects on lipid synthesis. For example, excess glucocorticoids are associated with increased *de novo* hepatic fatty acid and TAG synthesis and increased circulating TAG levels [17–21]. Removal of glucocorticoids by adrenalectomy attenuated the development of obesity in a variety of genetic and dietary models of obesity [20,22]. Therefore we investigated the regulation of TGH, DGAT and intracellular hepatic TAG turnover in response to a synthetic glucocorticoid, dex (dexamethasone).

Abbreviations used: apoB, apolipoprotein B; ARE, AU-rich element; dex, dexamethasone; DGAT, diacylglycerol acyltransferase; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; MUH, 4-methylumbelliferyl heptanoate; PDI, protein disulphide isomerase; RT-PCR, reverse transcription–PCR; SV40, simian virus 40; TAG, triacylglycerol; TGH, triacylglycerol hydrolase; UTR, un translated region; VLDL, very-low-density lipoprotein.

[1] To whom correspondence should be addressed (e-mail dennis.vance@ualberta.ca).

MATERIALS AND METHODS

Reagents

Restriction endonucleases, modifying enzymes, random primer labelling kit, DMEM (Dulbecco's modified Eagle's medium), sodium pyruvate, penicillin/streptomycin, fetal bovine serum and horse serum were obtained from Life Technologies Inc. Radioisotopes ([α-^{32}P]dCTP, [γ-^{32}P]dATP, [^{3}H]glycerol and [^{14}C]oleate) were from Amersham-Pharmacia. Western blotting reagents were obtained from Amersham-Pharmacia. Silica gel H TLC plates were from Merck. The Topo-TA cloning kit was from Invitrogen. Essentially fatty acid-free BSA and all other chemicals were purchased from Sigma.

PCR primer sequences

All primers were synthesized at the DNA core facility, University of Alberta, using a 394 DNA/RNA synthesizer (Applied Biosystems). Sequences were as follows: CYC1A, 5′-TCCAAAGACAGCAGAAAACTTTCG-3′; CYC2B, 5′-TCTTCTTGCTGGTCTTGCCATTCC-3′; DGAT1F, 5′-ATTCACGGATCATTGAGCG-3′; DGAT1R, 5′-CTGCCATGTCTGAGCATAGG-3′; DGAT2A, 5′-CTACGTTGGCTGGTAACTTCC-3′; DGAT2B, 5′-AACCAGATCAGCTCCATGG-3′; DGAT2F, 5′-TTCAGCATGAAGACCCTCATC-3′; DGAT2R, 5′-TGGCACAACAATCTCATGG-3′; Ex6F, 5′-CACTGCTGCTCTGATTACAACAG-3′; Ex10R, 5′-GCCTTCAGCGAGTGGATAGC-3′; P-TGHII, 5′-GAGCAAAGTTGGCCCAGTATTTCATCACCATTTTGCTGAG-3′.

Reverse transcription of RNA, cloning of murine DGAT2 cDNA and quantification of mRNA expression by real-time PCR

Total RNA was reverse transcribed using an oligo(dT)$_{20}$ primer and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. PCR amplification of the DGAT2 cDNA was done as follows: DGAT2 (GenBank Accession No. NM026384) primers DGAT2F/DGAT2R; 95 °C for 30 s, 57 °C for 1 min and 72 °C for 1 min (number of cycles indicated in the Figure legends). The DGAT2 cDNA was cloned into pCR2.1-Topo (Invitrogen), following the manufacturer's protocol, sequenced and confirmed by comparison with the sequences available in the GenBank database. TGH (primers EX6F/EX10R), DGAT1 (primers DGAT1F/DGAT1R), DGAT2 (primers DGAT2A/DGAT2B) and cyclophilin (primers CYC1A/CYC2B) transcripts were detected by real-time PCR using a LightCycler (Roche Diagnostics) instrument. Reaction mixtures contained 0.5 mM dNTPs, 3 mM $MgCl_2$, 2.5 μM of each primer, 1 × SYBR Green I (Molecular Probes) in DMSO and 3 units of *Taq* polymerase in a total volume of 20 μl. Amplification was performed as follows: 95 °C for 10 s, 57 °C for 10 s and 72 °C for 15 s (number of cycles indicated in the Figure legends). Data analysis was performed using LightCycler Software version 3.5 (Roche Diagnostics).

Animals

Male C57BL6 mice (6 weeks of age) were housed in a thermostatically controlled room with artificial lighting (12 h light/12 h dark). Animals had unrestricted access to water and a commercial pelleted diet (22 % protein/22 % fat/56 % carbohydrate). Mice were injected intraperitoneally with 40 mg/kg body weight dex, delivered in 200 μl of corn oil, for 4 consecutive days and killed 24 h after the final injection, as described in a previous study [23]. Control mice received corn oil only.

RNA isolation and Northern analysis

Total murine tissue RNA was isolated using Trizol Reagent (Life Technologies) according to the manufacturer's instructions. To perform Northern blot analysis, total RNA was separated in a 1 % (w/v) agarose/2.2 M formaldehyde gel and transferred to a Hybond-N^{+} nylon membrane (Amersham) using a vacuum blotter (Bio-Rad) according to the manufacturer's instructions. Prehybridization and hybridization steps were done at 55 °C in 0.144 M dibasic sodium phosphate, 7 % SDS, 2 μM tetrasodium pyrophosphate, 2 μM EDTA, 100 μg/ml sheared salmon testes DNA (Sigma) and 1.5 × 10^{6} c.p.m./ml labelled probe. For detection of TGH, a ^{32}P-labelled 40 bp oligonucleotide, P-TGHII, was utilized. Full-length ^{32}P-labelled DGAT1 (a gift from Dr R. V. Farese, Jr, Gladstone Institute, San Francisco, CA, U.S.A.), DGAT2 and PDI (protein disulphide isomerase; a gift from Dr M. Michalak, University of Alberta) cDNAs were utilized for their respective detection by Northern analysis.

Isolation of crude microsomal fractions and protein determination

Crude microsomal fractions were prepared for assay of enzyme activities and immunoblotting. Murine livers were excised and rinsed in ice-cold PBS. Subsequently, a 10 % (w/v) homogenate was prepared by disrupting the liver (Polytron; 20 s burst) in buffer containing 10 mM Tris/HCl, 0.25 M sucrose and 2 mM EDTA, pH 7.4. The homogenate was centrifuged at 1000 ***g*** for 20 min. The resulting pellet was discarded and the supernatant was centrifuged at 12 000 ***g*** for 10 min. The resulting pellet was resuspended in 5 ml of homogenizing buffer by 15 strokes of a loose-fitting hand-held Dounce tissue homogenizer and again centrifuged at 12 000 ***g*** for 10 min. The resulting pellets were discarded (the mitochondrial fraction) and the combined post-mitochondrial supernatants were centrifuged for 60 min at 100 000 ***g***. The resulting pellet was resuspended in 0.5 ml of homogenizing buffer by 15 strokes of a tight-fitting hand-held Dounce tissue homogenizer and used as the source of the microsomal fraction. The microsomal fraction was aliquoted into 1.5 ml microcentrifuge tubes and frozen at − 70 °C. The protein concentration of the crude microsomal fraction was determined by a bicinchoninic acid protein assay (Pierce).

Immunoblot analysis

Murine hepatic microsomes (25 μg of protein) were electrophoresed on a SDS/10 % (w/v) polyacrylamide gel and transferred to a nitrocellulose membrane, and the expression of TGH was analysed by blotting with anti-TGH polyclonal antibody as described previously [8]. The expression of PDI was analysed by blotting with anti-PDI polyclonal antibody (Santa Cruz) according to the manufacturer's instructions.

Lipase assay

Lipolytic activity in murine hepatic microsomes was assessed using the fluorogenic substrate MUH (4-methylumbelliferyl heptanoate). A 1 mM stock of MUH was prepared in tetrahydrofuran. The enzymic reaction was initiated by the injection of 2.5 μM MUH in 20 mM Tris/HCl, pH 8.0, 1 mM EDTA and 300 μM taurodeoxycholate to 1 μg of microsomal protein in a 96-well plate (total volume 100 μl). The plate was agitated at room temperature and fluorescence was read with a Fluoroskan Ascent FL Type 374 (Thermo Labsystems) in a kinetic fashion up to 10 min (excitation/emission wavelengths of 355/460 nm). Data were analysed using Ascent software version 2.4.2.

DGAT assay

The synthesis of TAG from 1,2(2,3)[^{3}H]dioleoyl-*sn*-glycerol was measured using crude hepatic microsomes as described previously [24]. The assay mixture contained 20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 4 mM $MgCl_2$, 20 mM NaF, 1 mM dithiothreitol and 0.1 % CHAPS. A substrate suspension of 25 μM dioleoylglycerol (50 000 d.p.m./assay) and 50 μM oleoyl-CoA was prepared by sonication in the assay mixture. The reaction was initiated by the addition of 50 μg of microsomes to the assay mixture (final volume 200 μl). The reaction proceeded for 10 min at 37 °C and was terminated by the addition of 4 ml of chloroform/methanol (2:1, v/v) and 750 μl of water. Samples were centrifuged at 1000 ***g*** for 10 min to separate organic and aqueous phases. The aqueous phase was removed and the organic phase dried under nitrogen. Lipids were resuspended in 50 μl of chloroform/methanol (2:1, v/v) and spotted on a TLC plate. Lipids were separated in hexane/diethyl ether/acetic acid (80:20:1, by vol.) and visualized by staining in iodine vapour. Radioactive TAG was determined by scintillation counting.

GC determination of TAG mass

In order to analyse TAG mass by GC, 200 μl samples of plasma, cell media and cells/tissues were digested with 2 units of phospholipase C from *Clostridium welchii* (Sigma) to hydrolyse phospholipids to the corresponding diacylglycerol and ceramide, which were converted together with TAG to trimethylsilyl ether/ester derivatives [5]. Samples were separated using an Agilent 6890 Series gas chromatograph (Agilent Technologies) with an HP-5 column. Data analysis was performed using GC Chemstation software (Agilent Technologies).

Hepatic TAG secretion rate

Mice were injected intraperitoneally daily for 4 days with 40 mg/kg dex (controls received vehicle only), as described previously [23]. At 24 h after the final injection, and after an overnight fast, a blood sample was removed (baseline) and then mice were injected in the tail vein with 20 mg of Triton WR 1339 in a total volume of 100 μl of PBS [25]. Subsequently, 50 μl blood samples were removed from the leg vein 1 h and 2 h after the Triton injection. Plasma TAG concentrations were determined by enzymic methods using the GPO-Trinder kit (Sigma-Aldrich).

Preparation of primary hepatocyte cultures from mice

Hepatocytes were prepared under sterile conditions from mice (17–24 g body weight) at 10:00 h (approx. 6 h into the light phase of the cycle), by collagenase perfusion of the liver, as described previously [26]. Following cell attachment, the medium was removed and cells were washed twice with PBS and further cultured in DMEM containing the above antibiotics in the absence of serum.

Pulse–chase experiments with primary murine hepatocytes and lipid analysis

Cells were prepared as described above from either a control mouse or a mouse that had received 40 mg/kg body weight dex. Following cell attachment, the medium was replaced by DMEM containing [^{3}H]glycerol (0.25 mM, 10 μCi), [^{14}C]oleate (0.75 mM, 1 μCi) and 0.5 % BSA in order to pre-label the cellular TAG stores. After 4 h, medium was removed and cells were washed twice with PBS. Some dishes were harvested (pulse), whereas others were cultured in DMEM in the absence of exogenous glycerol and oleate for a further 18 h (chase). After 18 h, cells and media were collected. A 50 μl aliquot of disrupted cells was removed for determination of protein. Addition of 6 ml of chloroform/methanol (2:1, v/v) and 1 ml of 0.73 % NaCl to the tube facilitated separation of organic and aqueous phases. Tubes were centrifuged at 1000 ***g*** for 10 min and the upper (aqueous) phase was removed. The remaining organic phase was washed with 5 ml of theoretical upper phase (chloroform/methanol/0.9 % NaCl, 3:48:47, by vol.). Tubes were again centrifuged at 1000 ***g*** for 10 min and the upper phase was removed. The lower (organic) phase was dried under nitrogen. The lipid sample was redissolved in 50 μl of chloroform/methanol (2:1, v/v) and applied to a silica gel H plate. Lipids were separated using the chloroform/methanol/acetic acid/water (75:45:12:6, by vol.) solvent system, allowing the solvent to proceed up one-third of the plate. The plate was dried and further separation of neutral lipids was achieved in the heptane/isopropyl ether/acetic acid (90:60:6, by vol.) solvent system, allowing the solvent to proceed up the entire height of the plate. Lipids were visualized by iodine vapour and radioactive TAG was determined by scintillation counting.

Calculation of intracellular TAG turnover

Double labelling of the hepatocyte TAG storage pool with [^{3}H]glycerol and [^{14}C]oleate gives rise to a relative decline in the specific radioactivity of the ^{3}H label compared with that of the ^{14}C label [27]. Hepatocytes do not synthesize much fatty acid over a 24 h culture period [3]. Consequently, in the absence of exogenously supplied fatty acid, stored TAG is the major source of fatty acids for TAG secretion. During the chase period, the glycerol and fatty acid moieties of the prelabelled TAG pools appeared to undergo differential metabolism. The extent of the dilution of the TAG glycerol pool reflects the degree of TAG lipolysis and re-esterification as the [^{3}H]glycerol pool becomes diluted with unlabelled glycerol, resulting in a lower specific radioactivity, while in the absence of exogenously supplied fatty acids, the released [^{14}C]oleate is re-synthesized into the TAG pool. Therefore we define the initial (pulse) ^{3}H/^{14}C specific radioactivity ratio of the cellular TAG as X and the final specific radioactivity ratio of TAG as Y. If $X = Y$ then no excess glycerol label has entered the TAG pool and lipolysis is zero. If $X > Y$ then excess unlabelled glycerol has entered the pool. This may be represented as the fractional turnover of the cellular TAG pool ($X/Y - 1$). Because the total (cellular and media) TAG pool size at the end of the culture period is known, the absolute TAG turnover may be calculated as the product of the fractional turnover multiplied by the TAG pool size.

PCR-based nuclear run-on assay

This assay was performed essentially as described in [16]. Highly purified and transcriptionally active nuclei were prepared from murine liver according to the methods of Marzluff and Huang [28]. Freshly prepared or frozen/thawed nuclei (200 μl) were divided into two aliquots and incubated for 30 min at 30 °C in 20 % (v/v) glycerol, 30 mM Tris/HCl (pH 8.0), 2.5 mM $MgCl_2$, 150 mM KCl, 1 mM dithiothreitol and 40 units of RNasin (Promega) with or without 0.5 mM (each) ribonucleoside triphosphates. After 30 min, nuclei were lysed by the addition of 200 μl of 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 4.0), 0.5 % sarcosyl and 0.1 M 2-mercaptoethanol. Yeast tRNA (20 μg) was added and RNA was extracted by the acid guanidinium thiocyanate method, then resuspended in water treated with diethyl pyrocarbonate, and RT-PCR (reverse transcription–PCR) was performed.

Table 1 Lipid levels and weight parameters of mice used in the study

Mice were treated as described in the Materials and methods section and, after an overnight fast, were killed. TAG quantity was determined by GC.

Parameter	Control	Dex	*P* (Student's *t* test)
Body weight (g) ($n = 6$)	20.57 ± 1.68	18.86 ± 1.18	0.051
Liver weight (g) ($n = 6$)	0.28 ± 0.03	0.32 ± 0.05	0.053
$10^2 \times$ Liver/body weight ratio ($n = 6$)	1.38 ± 0.074	1.79 ± 0.19	0.0007
Liver TAG (μg/mg of protein) ($n = 6$)	212 ± 97	1095 ± 291	0.0007
Fasting plasma TAG (mg/dl) ($n = 9$)	30.5 ± 12.8	67.8 ± 26.4	0.0003
Hepatic TAG secretion rate (mg/h per kg body weight) ($n = 10$)	83.9 ± 8.4	91.8 ± 16.8	0.190

Transfections and reporter assays

Rat McArdle RH7777 cells (CRL-1601; A. T. C. C.) were plated and transiently transfected with 5 μg of TGH promoter–luciferase reporter constructs or pGL3-basic and pGL3-control plasmids (Promega) using a cationic liposome technique. All plates received 2 μg of pSV-βgal (Promega) as a control for transfection efficiency. Deletion mutants of the murine TGH promoter were created as described previously [16]. The complete 3′-UTR (untranslated region) of TGH was isolated previously [7]. Creation of the pGL3-TGH plasmid was achieved through a *Bam*HI/*Xba*I restriction digest of the pGL3-control plasmid (Promega) in order to excise the SV40 (simian virus 40) late poly(A) signal and replace it with the TGH 3′-UTR sequence. Reporter assays using cell lysates were performed as recommended (Promega), and luminometric measurements used a Fluoroskan Ascent FL Type 374 (Thermo Labsystems). Luciferase activity was normalized to β-galactosidase activity (Promega).

RESULTS

Dex treatment increases hepatic and plasma TAG levels, but not the hepatic TAG secretion rate, in mice

Excess glucocorticoids are associated with reduced body weight gain and increased plasma and hepatic TAG levels [29,30]. We injected mice with 40 mg/kg body weight dex for 4 days. Excess glucocorticoids are associated with an increased liver/body weight ratio. We also observed a higher liver/body weight ratio in the mice that were injected with dex compared with controls (Table 1). Hepatic TAG and plasma TAG accumulation in hypercortisolaemic humans and rodents has been observed [19–21]. In the dex-injected mice, we observed a 5-fold ($P < 0.001$) increase in hepatic TAGs (Table 1). In addition, the plasma TAG concentration, measured after an overnight fast (therefore excluding TAGs in chylomicrons), was also observed to be 2.2-fold ($P < 0.001$) higher in the dex-injected mice than the control mice (Table 1). The non-ionic detergent Triton WR 1339 was used to determine rates of hepatic TAG entry into the circulation [25]. We observed no statistically significant differences in the rate of TAG secretion between control and dex-injected mice (Table 1). Furthermore, when hepatocytes were cultured under the same conditions as the cells in the TAG turnover study, no significant differences were observed between the mass of TAG secreted into the medium by murine hepatocytes derived from dex-injected mice compared with those from controls during an 18 h period (Table 2). These data demonstrate the utility of the dex-injected mouse as a model for TAG metabolic alterations associated with excess glucocorticoids.

Table 2 Dex suppresses intracellular TAG turnover

Hepatocytes were cultured as described in the Materials and methods section. After 4 h in the presence of [^{14}C]oleate (0.75 mM) and [^{3}H]glycerol (0.25 mM), cells from some dishes were harvested and the ^{3}H and ^{14}C specific radioactivity of the cellular TAG was determined. The remaining dishes were cultured for an additional 18 h in the absence of oleate and glycerol. At the end of this period, the ^{3}H and ^{14}C specific radioactivities of TAG was calculated as described in the Materials and methods section. Values represent the means ± S.D. of triplicate assays from three independent hepatocyte preparations.

Parameter	Control	Dex	*P* (Student's *t* test)
Total hepatocyte cell TAG (μg/mg of protein)	280.5 ± 58.5	871.3 ± 42.51	0.016
Total hepatocyte medium TAG (μg/18 h per mg of protein)	1.18 ± 0.02	1.16 ± 0.03	0.866
TAG turnover (μg/h per mg of protein)	14.0 ± 0.02	7.25 ± 0.03	0.01

Dex decreases TGH expression and increases DGAT expression in murine liver

The mRNA expression of several genes in the livers of control and dex-injected mice was examined (Figure 1A). The expression of DGAT1 was increased ~1.6-fold and that of DGAT2 was increased ~1.7-fold relative to PDI in the livers of dex-injected mice, compared with controls. By contrast, a marked decrease in TGH mRNA was observed in dex-treated mice relative to control mice.

Dex increases DGAT activity in murine hepatic microsomes

Although evidence shows that TAG biosynthesis is stimulated by dex, a possible stimulation of hepatic DGAT activity by dex has not been reported. Since DGAT1 mRNA and protein expression do not necessarily correlate with DGAT activity [31,32], microsomal DGAT activity was measured *in vitro* using [^{3}H]dioleoylglycerol as the substrate. DGAT activity was increased 1.2-fold ($P < 0.05$) in murine hepatic microsomes from dex-injected mice (Table 3). Since DGAT-isoform-specific antibodies are not available, we could not investigate whether the changes in mRNA and activity were correlated with microsomal protein content. However, our assay conditions (low [Mg^{2+}]) ensure the simultaneous measurement of both DGAT1 and DGAT2 activities.

Dex decreases microsomal esterase activity in murine hepatic microsomes

Hydrolysis of the fluorogenic substrate MUH was measured using crude microsomal preparations. Microsomal esterase activity was decreased by approx. 40 % ($P < 0.005$) in microsomes from dex-injected mice, compared with controls (Table 3). Since hydrolysis of MUH requires an enzyme that can accommodate substrates with substantial hydrophobic character, this assay is a better reflection of lipolytic activity than those using standard short-chain substrates to assay carboxylesterase activity. Furthermore, an immunoblot with polyclonal anti-TGH antibodies showed that TGH protein levels had also decreased by approx. 40 % in hepatic microsomes from dex-injected mice compared with mice that had received vehicle alone (Figure 1B). Others have demonstrated regulation of rat hepatic microsomal carboxylesterase expression by dex [23,33]. Since TGH accounts for the majority of microsomal esterase activity in the liver [9], the decreased esterase activity in murine hepatic microsomes from dex-injected mice is primarily the consequence of the reduced expression of TGH in their livers.

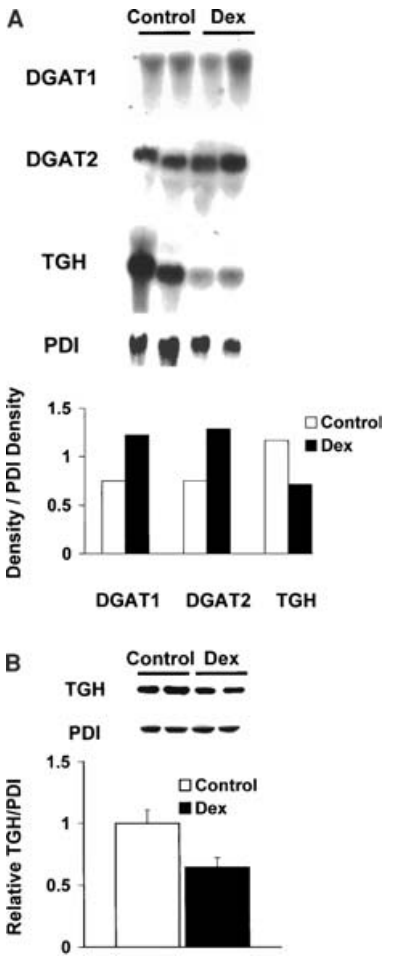

Figure 1 Dex regulates the expression of the enzymes of lipolysis and re-esterification in the liver

(**A**) Northern blotting was performed as described in the Materials and methods section. Murine TGH mRNA was detected by hybridization with a ^{32}P-labelled TGH oligonucleotide probe (pTGHII). Mouse DGAT1, DGAT2 and PDI mRNAs were detected using their respective ^{32}P-labelled cDNA probes. Quantification of TGH, DGAT1 and DGAT2 signals relative to that of PDI is shown. (**B**) Western blotting was performed as described in the Materials and methods section. TGH and PDI proteins were detected using respective polyclonal antibodies. A representative Western blot is shown, along with and quantification (mean ± S.D.) of Western blots from six separate mice, expressed as TGH relative to PDI.

Intracellular TAG turnover in primary murine hepatocytes

Stored TAG within hepatocytes undergoes lipolysis followed by re-esterification prior to secretion. The primary hepatocyte model is a well characterized model for measuring the contribution of the lipolysis and re-esterification pathway to TAG secretion [3,4,27]. A dual-labelling, pulse–chase experiment was designed to calculate the intracellular TAG turnover and secretion by murine hepatocytes during the first 24 h of hepatocyte culture. Cells were harvested and media and cellular lipids were analysed after 24 h in culture, because hepatocytes secrete TAG mass at a constant rate during the first 24 h [34], and because beyond 24 h TGH, DGAT1 and DGAT2 expression declines markedly in concert with the mass of secreted TAG (V. Dolinsky, unpublished work). Table 2 shows that murine hepatocytes derived from the dex-injected mice metabolized (lipolysis followed by re-esterification) 7.25 μg of TAG/h per mg of protein, compared with 14.0 μg of TAG/h per mg of protein by the hepatocytes from control mice. This represents an almost 50% lower rate of turnover by the

Table 3 Esterase and DGAT activities in murine hepatic microsomes from control and dex-injected mice

Data are means ± S.D. of triplicate assays of three independent hepatic microsomal preparations.

Activity	Control	Dex	*P* (Student's *t* test)
Esterase activity (nmol/min per mg of protein)	22.54 ± 3.35	13.95 ± 2.5	0.001
DGAT activity (pmol/min per mg of protein)	246.82 ± 21.97	300.47 ± 8.69	0.017

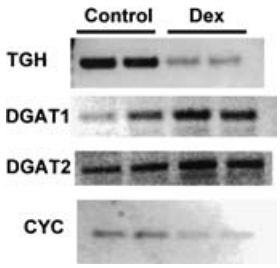

Figure 2 Expression of DGAT1/2 mRNAs is higher and of TGH mRNA is lower after 24 h in hepatocyte cell cultures derived from dex-injected mice compared with controls

Cells were harvested following a 24 h incubation and RNA was extracted. DGAT1, DGAT2 and TGH mRNA expression were determined by RT-PCR as described in the Materials and methods section (22 cycles).

labelled TAG in the storage pool of hepatocytes from dex-treated mice during the 18 h chase period compared with hepatocytes from control mice. As observed with murine liver, DGAT1 and DGAT2 mRNA expression were higher in hepatocytes prepared from dex-injected mice following 24 h of incubation, whereas TGH mRNA expression was reduced when compared with that in control cells (Figure 2), indicating that the changes induced by dex in TGH as well as DGAT1 and DGAT2 expression observed in primary mouse hepatocytes parallel those observed in the whole murine liver. In addition, the expression of other closely related carboxylesterase genes, *Es22* and *EsX*, were not significantly altered by dex (results not shown). Therefore these results provide insight into the mechanism by which dex promotes hepatic TAG storage.

Dex does not regulate the transcriptional activity of the *Tgh* gene

Both nuclear run-on assays and TGH promoter–reporter gene transfection assays were performed to determine whether dex regulates *Tgh* gene expression at the transcriptional level. Transcriptionally active nuclei were isolated from livers of dex-injected and control mice. As shown in Figure 3, dex treatment had no effect on the run-on activity of the *Tgh* gene compared with the cyclophilin gene. To verify further this observation, transient transfection assays were performed on dex-treated and control McArdle RH7777 rat hepatoma cells with murine *Tgh* promoter–reporter gene plasmids [16]. The difference in activity between dex-treated and untreated cells was not significant (results not shown). These results suggested that transcriptional regulation of TGH expression was not involved in the dex-mediated gene suppression.

Dex regulates TGH expression at the level of mRNA stability

Using actinomycin D to block new transcription, the half-life of TGH mRNA in primary murine hepatocytes revealed a complex

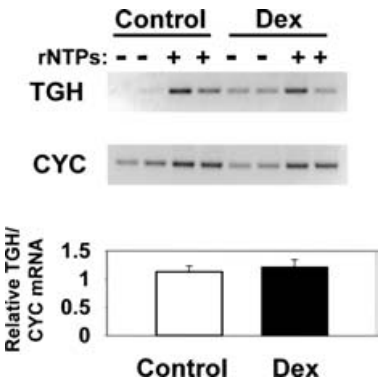

Figure 3 Nuclear run-on assay of TGH transcription in liver nuclei isolated from dex-injected and control mice

Nuclei were isolated and used for nuclear run-on assays as described in the Materials and methods section. RT-PCR was performed as described (25 cycles). Using cyclophilin (CYC) as a control, dex had no effect on TGH transcription. The gel is representative of two independent experiments. The bar graph gives results from four different preparations of nuclei (means ± S.D.).

mRNA decay pattern. A 50% decrease in TGH mRNA levels was observed approx. 4 h after the inhibition of new transcription (Figure 4, left panel). We established that a concentration of 1 μM dex was sufficient to achieve a maximal reduction of TGH mRNA levels in primary murine hepatocytes during a 24 h culture period (results not shown). As shown in Figure 4 (right panel), treatment of hepatocytes with dex caused TGH mRNA to decrease by 50% within 2 h of dex treatment, suggesting that dex decreases the stability of TGH transcripts in hepatocyte cultures.

The murine TGH 3′-UTR is only 187 bp and contains several polyadenylation signals [7]. In addition, the TGH 3′-UTR contains three AREs (AU-rich elements) in the 3′-UTR sequence. AREs are potent destabilizing elements that regulate the post-transcriptional stability of several mRNAs [35]. To determine whether the 3′-UTR of the murine TGH mRNA has a role in the regulation of the TGH transcript in response to dex, a chimaeric gene that bears the luciferase reporter gene upstream of the entire 3′-UTR of the murine TGH gene was constructed (pGL3-TGH). The pGL3-control plasmid containing the 3′-UTR of the SV40 early gene was used as the reference gene. Therefore these constructs shared common SV40 promoters and 5′-UTR sequences and differed only in the portion that encoded the 3′-UTR of their respective sequences. These plasmids were transiently expressed in McArdle RH7777 cells. The luciferase activity of cells transfected with pGL3-TGH was 20.5 ± 3.7% of that of cells transfected with pGL3-control. Incubation of pGL3-TGH-transfected cells in the presence of 1 μM dex for 24 h decreased luciferase activity to 9.7 ± 2.4% of that in the pGL3-control transfected cells, approx. 50% lower ($P < 0.001$) than that in pGL3-TGH cells incubated with DMSO for 24 h. Incubation of pGL3-control-transfected cells with 1 μM dex did not affect luciferase activity. Thus the decreased level of TGH expression in dex-treated cells transfected with pGL3-TGH was attributable to elements located in the murine TGH 3′-UTR.

DISCUSSION

Glucocorticoids cause increases in circulating TAG levels and hepatic TAG synthesis and storage [18,19,22,29]. Dex is a potent analogue of glucocorticoids (cortisol and corticosterone), and induces effects similar to those of natural glucocorticoids [19]. The dex-injected mouse exhibits the physiological and metabolic alterations in lipid metabolism associated with excess glucocorticoid. These include an increased liver/body weight ratio and increased hepatic and plasma TAGs. The liver is central to lipid homoeostasis; it has a great capacity to store TAG and to secrete TAG as apoB-containing lipoprotein particles. The rate of VLDL-TAG secretion is determined by the efficiency of apoB lipidation [15]. Since dex does not affect the expression of apoB [19] or the microsomal triglyceride (TAG) transfer protein [36], we examined whether the changes in TAG levels in the dex-injected mice could be associated with changes in the expression and activities of enzymes that catalyse hepatic TAG lipolysis and re-esterification. We found that DGAT1 and DGAT2 expression was increased in livers from mice that had received dex injections compared with controls, whereas TGH mRNA and protein expression was decreased. In agreement, hepatic microsomal DGAT activity was increased and hepatic microsomal esterase activity, primarily a reflection of TGH activity [9], was decreased. Next, we showed that, in primary hepatocytes

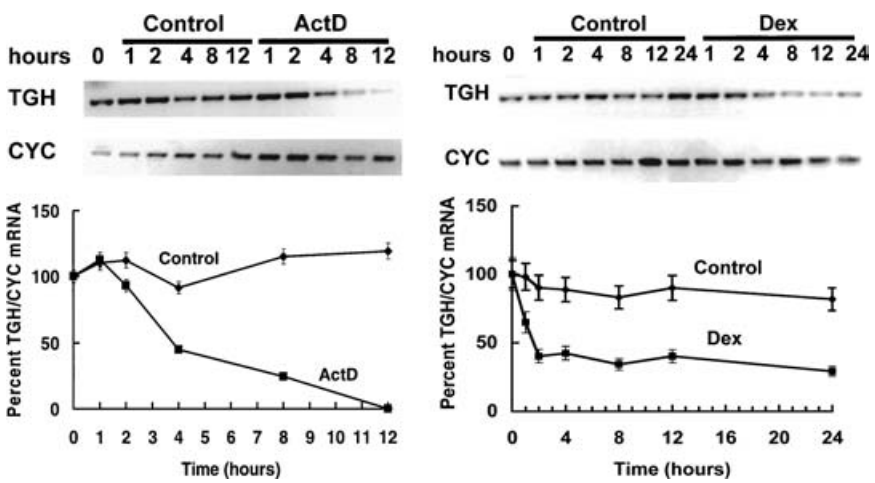

Figure 4 Dex regulates the stability of TGH mRNA in primary murine hepatocytes

Left panel: cells were plated and incubated in the presence or absence of 10 μg/ml actinomycin D (ActD) for the indicated times, and TGH and cyclophilin (CYC) expression was determined by RT-PCR (28 cycles). Right panel: cells were plated and incubated for the indicated times in the presence or absence of 1 μM dex, and TGH and CYC expression was determined by RT-PCR (20 cycles). Representative gels of four independent hepatocyte preparations are shown. Fold changes in TGH mRNA expression are calculated relative to CYC, and are expressed as mean ± S.D.

derived from dex-injected mice, there was a clear suppression of intracellular TAG turnover, consistent with the proposed role of TGH in the lipolysis of intracellular stored TAG.

Glucocorticoids regulate hepatic TAG metabolism

It is likely that dex regulation of enzymes involved in hepatic TAG lipolysis and re-esterification had affected the balance between TAG synthesis and lipolysis in such a way that accumulation of intracellular TAG occurred. TAG accumulation within the livers of dex-injected animals has been observed [19,29]. Dex administration stimulates the synthesis of TAG and increases the activity of several enzymes in the pathway for the *de novo* synthesis of TAG. Dex is an activator of rat fatty acid synthase [17,37] and rat liver phosphatidate phosphohydrolase [38]. It is possible that inhibition of β-oxidation may contribute to the accumulation of hepatic TAG. Dex has not been demonstrated to affect mitochondrial β-oxidation, except in an instance where the concentrations of dex used exceeded the concentration used in the present study [39]. Therefore these previous experiments and our present results suggest that a combination of high TAG synthesis and decreased TAG lipolysis results in hepatic lipid accumulation.

A 2-fold higher plasma TAG concentration was observed in the dex-injected mice when compared with controls. Some groups have suggested that excess glucocorticoids stimulate TAG secretion by the liver [40,41]. Since TGH has been implicated in the provision of stored TAG for VLDL secretion [9,10], the higher plasma TAG was not consistent with reduced TGH expression and activity. Nevertheless, when the hepatic TAG secretion rate was measured in fasting mice following Triton WR 1339 injection, we did not observe any differences in the hepatic TAG secretion rate of control and dex-injected mice. Our observation is consistent with the results of several groups that have reported no significant changes in hepatic TAG secretion rate in response to dex or cortisol [29,42,43]. Furthermore, primary murine hepatocytes from dex-injected and control mice secreted similar quantities of TAG mass into the media, consistent with other studies [34,44]. Therefore decreased clearance of circulating TAG in the mouse probably resulted in the increased circulating TAG concentration. Extracellular lipolysis is controlled primarily by lipoprotein lipase. Lipoprotein lipase activity is greatest in adipose tissue, and several studies have demonstrated that endogenous [45,46] or exogenous [29,42] glucocorticoid excess increase plasma TAG concentration and decrease adipose tissue lipoprotein lipase activity. Therefore decreased adipose tissue lipoprotein lipase activity, and not increased hepatic secretion, is the likely cause of the elevated plasma TAG concentration in our dex-injected mice.

It has been shown that the pool of stored TAG in the hepatocyte is not in simple equilibrium with VLDL-TAG [3–5]. The bulk addition of TAG to the apoB100 particle represents the second stage of VLDL assembly that may be associated with the lipolytic mobilization of stored TAG [15]. In the dex-injected mouse, the turnover of the stored TAG was suppressed. Since the rate of hepatic TAG secretion by dex-injected animals was at a similar level as in controls, it appeared that the major effect of dex was to increase *de novo* TAG synthesis to such an extent that the normal regulation of VLDL secretion was overpowered. Consequently, the balance between the utilization of stored TAG and *de novo* TAG synthesis to supply TAG for secretion was altered, such that more TAG from the *de novo* pathway was available directly for TAG secretion. At the same time, the rate of TAG synthesis by dex-injected mice exceeded the rate of intracellular hepatic TAG lipolysis as well as secretion, and TAG accumulated within the liver.

Expression of TGH is modulated by the stability of its mRNA

Regulated mRNA stability is achieved through fluctuations in transcript half-lives in response to nutrient levels and hormones [35]. For the first time, we observed that TGH expression could be regulated in murine hepatocytes at the level of mRNA stability. Dex treatment of mice caused the levels of TGH mRNA to be reduced. The murine TGH promoter has putative glucocorticoid response element motifs, although the involvement of these sites in dex-mediated transcription is not evident, as judged by nuclear run-on and deletion mutant promoter–reporter analyses. The half-life of the TGH mRNA was only 2 h in hepatocytes treated with dex. Proteins that bind to RNAs containing AREs can target susceptible mRNAs for cytosolic degradation through the exosome pathway [47]. In particular, dex has been shown to decrease the stability of several mRNAs that have AREs [48–50]. The 3′-UTR of the murine TGH transcript contains three AREs [7]. The expression of the luciferase reporter gene was substantially reduced in the presence of dex by the addition of the TGH 3′-UTR. Together, these results suggest the murine TGH 3′-UTR contains elements that mediate the dex-induced destruction of TGH mRNA.

Conclusions

We have utilized the dex-injected mouse and primary hepatocyte models to study the regulation of the enzymes involved in hepatic microsomal TAG lipolysis and re-esterification. TGH expression and activity were decreased by dex, with reduced intracellular TAG lipolysis, while expression of DGAT was stimulated by dex and contributed to increased hepatic TAG biosynthesis. During markedly stimulated TAG biosynthesis, an increased proportion of newly synthesized TAG was secreted and the proportion of secreted TAG derived from the intracellular storage pool was reduced. Thus concordant regulation of TGH mRNA degradation and increased TAG biosynthesis by dex or the circadian fluctuations in circulating glucocorticoids [51,52] may allow the liver to adjust to the different metabolic demands during the post-prandial and post-absorptive states [2,53].

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