Apolipoprotein A-IV is regulated by nutritional and metabolic stress: involvement of glucocorticoids, HNF-4α, and PGC-1α[S]

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Abstract Apolipoprotein A-IV (apoA-IV) is a 46 kDa glycoprotein that associates with triglyceride-rich and high density lipoproteins. Blood levels of apoA-IV generally correlate with triglyceride levels and are increased in diabetic patients. This study investigated the mechanisms regulating the in vivo expression of apoA-IV in the liver and intestine of mice in response to changes in nutritional status. Fasting markedly increased liver and ileal apoA-IV mRNA and plasma protein concentrations. This induction was associated with increased serum glucocorticoid levels and was abolished by adrenalectomy. Treatment with dexamethasone increased apoA-IV expression in adrenalectomized mice. Marked increases of apoA-IV expression were also observed in two murine models of diabetes. Reporter gene analysis of the murine and human apoA-IV/C-III promoters revealed a conserved cooperative activation by the hepatic nuclear factor-4α (HNF-4α) and the peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) but no evidence of a direct regulatory role for the glucocorticoid receptor. Consistent with these in vitro data, induction of apoA-IV in response to fasting was accompanied by increases in HNF-4α and PGC-1α expression and was abolished in liver-specific HNF-4α-deficient mice. Together, these results indicate that the induction of apoA-IV expression in fasting and diabetes likely involves PGC-1α-mediated coactivation of HNF-4α in addition to glucocorticoid-dependent actions.— Hanniman, E. A., G. Lambert, Y. Inoue, F. J. Gonzalez, and C. J. Sinal. **Apolipoprotein A-IV is regulated by nutritional and metabolic stress: involvement of glucocorticoids, HNF-4α, and PGC-1α. *J. Lipid Res.* 2006.** 47: **2503–2514.**

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Apolipoprotein A-IV (apoA-IV) is a 46 kDa glycoprotein found primarily in triglyceride-rich chylomicrons and HDLs (1). Synthesis of apoA-IV from the small intestine is proposed to occur in response to the ingestion of fat (2). In humans, the majority of circulating plasma apoA-IV is found associated with HDL, some of which is thought to transfer from chylomicrons and VLDLs (3, 4). In rodents, apoA-IV is present in plasma in HDL particles, with 59% of the apoA-IV being synthesized in the intestine and the remainder by the liver (5, 6). Although the physiological functions of apoA-IV remain to be fully elucidated, a number of functions have been proposed. These include acting as a satiety signal, aiding in apoC-II transfer to triglyceride-rich lipoproteins, increasing lipoprotein lipase activity, and stimulating lecithin:cholesterol acyltransferase activity (7–10). Studies of an apoA-IV knockout mouse demonstrated no abnormalities in lipid absorption or feeding behavior, suggesting that although apoA-IV is synthesized in the intestine, the major function may lie elsewhere (11). Importantly, apoA-IV knockout mice exhibited a marked decrease in HDL cholesterol attributable to an increased catabolism of these particles. Consistent with this, overexpression of liver apoA-IV in mouse models of atherosclerosis led to increased HDL cholesterol and protection from atherosclerotic disease (12). In further support of a protective role for apoA-IV in atherosclerosis, studies have demonstrated an inverse relationship of apoA-IV levels and the risk of coronary heart disease in human subjects (13, 14).

Few studies have attempted to elucidate the regulation of apoA-IV at the promoter level. One such study has

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Abbreviations: apoA-IV, apolipoprotein A-IV; Cyp, cytochrome P450; GR, glucocorticoid receptor; HNF, hepatic nuclear factor; HRE, hormone response element; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; PPAR, peroxisome proliferator-activated receptor; QPCR, real-time quantitative polymerase chain reaction; TBS-T, Tris-buffered saline plus Tween-20.

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[S] The online version of this article (available at http://www.jlr.org) contains additional two tables.

demonstrated that basal apoA-IV levels in the intestine depend on the orphan nuclear receptor estrogen-related receptor α in combination with peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) (15). In addition, the orphan nuclear receptors hepatic nuclear factor-4α and -4γ (HNF-4α and HNF-4γ, respectively) have been shown to contribute to apoA-IV expression in the intestinal villi (16). Another study has demonstrated an induction of apoA-IV in the liver (but not the intestine) of mice treated with a liver X receptor ligand (17). Additional insight into a possible regulatory mechanism of apoA-IV comes from recent work that has indicated a modulation of apoA-IV levels by glycemic status. For instance, insulin-dependent diabetic patients had higher apoA-IV levels (independent of triglyceride or HDL levels) that were closely related to the control of glycemia (18). In addition, mRNA levels of apoA-IV increased in the livers of rats treated with insulin (19).

Two prevalent pathologies associated with diabetes are unchecked gluconeogenesis (primarily in the liver) and hyperlipidemia. Therapeutic targeting of adipose and liver glucocorticoid receptor (GR) improves these conditions, suggesting a key regulatory role for endogenous glucocorticoids and GR (20, 21). Also known to be important for regulating gluconeogenic responses (in both diabetes and fasting) are HNF-4α and the transcriptional coactivator PGC-1α (22). Given that variations in plasma apoA-IV levels occur in response to nutritional and hormonal status, we endeavored to investigate the mechanisms by which apoA-IV synthesis in the liver and intestine is regulated in response to fasting as well as diabetic states. We demonstrate that apoA-IV protein and mRNA levels increase in mouse liver and ileum during fasting and diabetes and that these increases occur in a glucocorticoid-dependent manner. In vitro analyses revealed that human and mouse apoA-IV promoter activity is markedly induced by the interactions of PGC-1α and HNF-4α. Evidence for this interaction was provided by in vivo studies demonstrating a parallel induction of PGC-1α, HNF-4α, and apoA-IV mRNA in fasting and an absence of basal apoA-IV expression and induction in HNF-4α liver-specific knockout mice during fasting.

MATERIALS AND METHODS

Mice and in vivo protocols

All fasting studies began in the late afternoon and continued overnight for a total of 24 h. For the fasting time course, mice were deprived of food at the beginning of the dark cycle and euthanized 6, 12, and 24 h later. In addition, one group was fasted for 24 h, after which free access to food was given for an additional 24 h. Peroxisome proliferator-activated receptor α-deficient (PPARα$^{-/-}$) and liver-specific HNF-4α$^{-/-}$ mice were obtained from our breeding colony and have been described previously (23, 24). Dexamethasone (Sigma-Aldrich, St. Louis, MO) treatment consisted of four daily intraperitoneal injections of 50 mg/kg dexamethasone [concentrated solution dissolved in 65% Cremophor EL (Sigma-Aldrich) and 35% ethanol and subsequently diluted 1:5 in 5% glucose solution]. Streptozocin treatment consisted of two consecutive daily intraperitoneal injections of 250 mg/kg streptozocin (Sigma-Aldrich) in sodium citrate buffer (pH 4.5), after which the diabetic mice (blood glucose levels of ≥15 mmol/l) were housed for 4 weeks. Adrenalectomized and sham-operated mice were obtained from Jackson Laboratories (Bar Harbor, ME) and housed for 1 week (all mice were given 0.9% normal saline to drink) before the commencement of either a 24 h fast or dexamethasone treatment (as described above). ob/ob mice were also obtained from Jackson Laboratories. All mice were of C57BL/6J background and were ~7–8 weeks of age, with the exception of the ob/ob mice and littermate controls (12 weeks of age), and were housed at room temperature under a 12 h light/dark cycle and provided food and water ad libitum. All procedures were conducted at the Carleton Animal Care Facility in accordance with Canadian Council on Animal Care guidelines.

Hepatic and ileal gene expression

Total hepatic and ileal RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the supplier's instructions. Total RNA (2 μg) was reverse-transcribed using Stratascript Reverse Transcriptase (Stratagene, La Jolla, CA) with random hexamers pd(N)$_6$ according to the supplier's instructions. The synthesized cDNA was then amplified by quantitative PCR using a Stratagene MX3000p thermocycler in a total volume of 25 μl with Brilliant SYBR Green QPCR Master Mix. Murine primer sequences are listed in supplementary Table I. Thermal cycling conditions were as described previously (25). Relative threshold cycle values were obtained by the $\Delta\Delta C_T$ method (26) using a threshold of 10 standard deviations above background for the threshold cycle.

Plasma corticosteroid analysis

Blood was collected from adrenalectomized and sham-operated mice (fed or fasted for 24 h) via cardiac puncture using heparanized needles and centrifuged at 6,700 *g* for 5 min. The resulting plasma samples were treated with a steroid displacement reagent, diluted 50-fold, and assayed for corticosteroid levels in a corticosteroid immunoassay according to the manufacturer's instructions (R&D Systems, Inc., Minneapolis, MN).

Western blot analysis

Immunoblot analyses of apoB-100, apoB-48, apoA-I, and apoA-II were performed as described previously (27). For immunoblot analysis of plasma apoA-IV protein, 50 μg of plasma protein was separated on a 10% acrylamide gel, transferred onto a 0.45 μm nitrocellulose membrane (Whatman, Inc., Florham Park, NJ), and blocked for 1 h with 5% milk in Tris-buffered saline plus Tween-20 (TBS-T). The membrane was then probed for 2 h at room temperature with polyclonal anti-mouse apoA-IV-IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a dilution of 1:1,000 in 1% milk in TBS-T. The membrane was then incubated with HRP-conjugated rabbit anti-goat IgG (Sigma-Aldrich) at a dilution of 1:10,000 (in 1% milk in TBS-T) for 1 h at room temperature. Antibody binding was detected using the ECL plus chemiluminescence kit (Amersham Biosciences, Piscataway, NJ) and imaged using a Storm 840 phosphorimager (Molecular Dynamics).

ApoA-IV promoter constructs

The pGL3-heC3A4 construct containing the human apoA-IV promoter fused to the upstream apoC-III enhancer was created using an original construct (eC3A4-CAT) (28) generously donated by Agnès Ribeiro (Institut National de la Santé et de la Recherche Médicale, Université Pierre et Marie Curie, Paris, France) as the template. The primers used for the generation

of this construct are listed in supplementary Table II. Amplification of the promoter region was performed using Elongase PCR (Invitrogen). The PCR product was digested with *Kpn*I/*Mlu*I and ligated into the pGL3-Basic vector (Promega, Madison, WI) previously digested with the same enzymes. For generation of the mouse apoA-IV promoter construct, mouse genomic DNA was isolated from a male C57BL/6J ear tag using phenol-chloroform-isoamyl alcohol organic extraction (25:24:1), precipitated using a 1/10th volume of sodium acetate and a 2.5 volume of ethanol, washed in ethanol, and used as a template for Elongase PCR (Invitrogen) amplification. The mouse apoC-III/apoA-IV intergenic region (approximately −5,808 to +25), which contains both the apoA-IV proximal promoter and the apoC-III enhancer (15), was cloned into pGL3-Basic vector (pGL3-meC3A4). Primers used for amplification of the intergenic region are listed in supplementary Table II. The PCR product was digested with *Mlu*I/*Bgl*II and ligated into the PGL3-Basic vector. All promoter constructs were verified by restriction mapping and sequencing.

Dominant-negative rat HNF-4α-, shRNA-, and nuclear receptor-expressing constructs

The construct expressing a dominant-negative form of rat HNF-4α was generated using primers based on those designed by Ladias and colleagues (29). Sequences of the primers used to generate the dominant-negative rat HNF-4α (DN-rHNF-4α) are listed in supplementary Table II. The PCR product was digested with *Hind*III/*Kpn*I and ligated into pShuttle-CMV (Stratagene) digested previously with the same enzymes. The construct expressing shRNA for human HNF-4α was generated using oligomers designed using Block-iT RNAi Designer Web Software (Invitrogen). Double-stranded oligomers were annealed and ligated into the pENTR/U6 vector using the Block-iT U6 RNAi Entry Vector Kit according to the manufacturer's instructions (Invitrogen). Sequences of the oligonucleotides used to generate the pENTR-sh-hHNF-4α are listed in supplementary Table II. Expression constructs for murine GR and PGC-1α were created by RT-PCR amplification of C57BL/6J mouse liver total RNA using the primers listed in supplementary Table II followed by insertion into the *Bam*HI/*Bgl*II or *Bam*HI sites of pSG5, respectively. The identity of the constructs was verified by restriction digestion and sequencing. The expression construct for rat HNF-4α has been described previously (30).

Cell culture and transfections

Human hepatocellular carcinoma (HepG2) and African green monkey kidney fibroblast (Cos-7) cells (American Type Culture Collection) were maintained in complete medium containing phenol red-free Dulbecco's modified Eagle's medium (Hyclone, Logan, UT) supplemented with 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich), and 10% heat-inactivated charcoal dextran-stripped fetal bovine serum (Gemini Bioproducts, Woodland, CA). Cells were maintained at 37°C in 5% CO_2. For transfections, HepG2 and Cos-7 cells were plated at a density of 150,000 and 120,000 cells/ml, respectively (0.5 ml per well), on 24-well plates and transfected 24 h later.

For transfections of dominant-negative HNF-4α or HNF-4α shRNA-expressing constructs, cells were transfected with 120 ng of luciferase reporter construct, 100 ng of pCMV-Bgal or PRL-TK, and 280 ng of PGC-1α or pSG5. In addition, for shRNA transfections, 75, 150, and 275 ng of pENTR-hHNF-4α or pENTR-lacZ (expresses shRNA for lacZ) was added, and 75, 150, and 275 ng of pShuttle-DN-rHNF-4α or pShuttle-CMV was added for dominant-negative transfections. For all other transfections, cells were transfected with 150 ng of luciferase reporter construct, 50 ng of nuclear receptor expression construct, 125 ng of pCMV-βgal, and 350 ng of PGC-1α or pSG5 to a total of 700 ng. All cells were transfected using 4 μl/μg DNA of TransIT-LT1 (Mirus, Madison, WI). Cells were treated at 24 h after transfection with DMSO as vehicle or 50 μM dexamethasone. DMSO reached a final concentration of 0.25% in all wells. Cells were harvested at 24 h after treatment using Reporter Lysis Buffer (Promega) according to the manufacturer's instructions. For those transfection experiments that did not require treatment, cells were harvested at 36 h after transfection.

Cell lysates were assayed for firefly luciferase using the Luciferase Assay System or renilla luciferase (for shRNA transfections) using the Dual Luciferase Reporter System (Promega). Activity of luciferase(s) was measured using a Luminoskan Ascent (Thermo Labsystems, Franklin, MA). Luciferase activity (in relative light units) was corrected for lysate renilla luciferase activity or β-galactosidase activity that was measured calorimetrically using a Power-WaveX microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT).

Statistics

All comparisons were performed across groups using one-way ANOVA with Tukey-Kramer postanalysis, unless stated otherwise. GraphPad Instat (Instat3 version 3.0a; GraphPad Software, Inc.) was used for all statistical analyses. Values are expressed as means ± SD. Differences between groups were considered statistically significant at $P \leq 0.05$.

RESULTS

To examine apoA-IV expression during changes in nutritional status, wild-type (C57Bl/6J) mice were fasted for 6, 12, and 24 h, and one group was given 24 h access to food after a 24 h fast (refed). After each fasting time point, liver and ileal apoA-IV mRNA expression was measured by real-time quantitative polymerase chain reaction (QPCR). QPCR analysis revealed a time-dependent induction of liver apoA-IV mRNA levels to ~4.5-, 20-, and 15-fold in the fasted mice compared with fed controls (**Fig. 1A**). Refeeding of 24 h fasted mice for an additional 24 h did not reverse this increase in apoA-IV mRNA levels. The genes encoding apoA-I and apoC-III are present in a cluster that also includes the gene for apoA-IV. These three genes have a number of common regulatory sequences present within the intergenic region located between the apoA-IV and apoC-III coding regions (31). Therefore, to determine the specificity of induction of apoA-IV mRNA in fasted liver, it was necessary to also examine apoA-I and apoC-III expression in the livers of these mice. Examination of apoA-I mRNA expression revealed a small, transient increase by 12 h of fasting (3-fold over controls) that was abolished at the 24 h fasting time point (Fig. 1A). ApoC-III mRNA expression in fasted liver was not altered at any of the time points examined (Fig. 1A). Ileal expression of apoA-IV mRNA was also examined in fasted mice and was induced to ~4- and 6-fold over fed controls at the 6 and 24 h time points and was decreased to control levels after 24 h of refeeding (Fig. 1B).

PPARα is a nuclear hormone receptor that mediates many hepatic responses to fasting by regulating the expression of genes with key roles in energy homeostasis (32). Therefore, to determine whether PPARα was involved in the induction of apoA-IV in fasting, PPARα$^{-/-}$

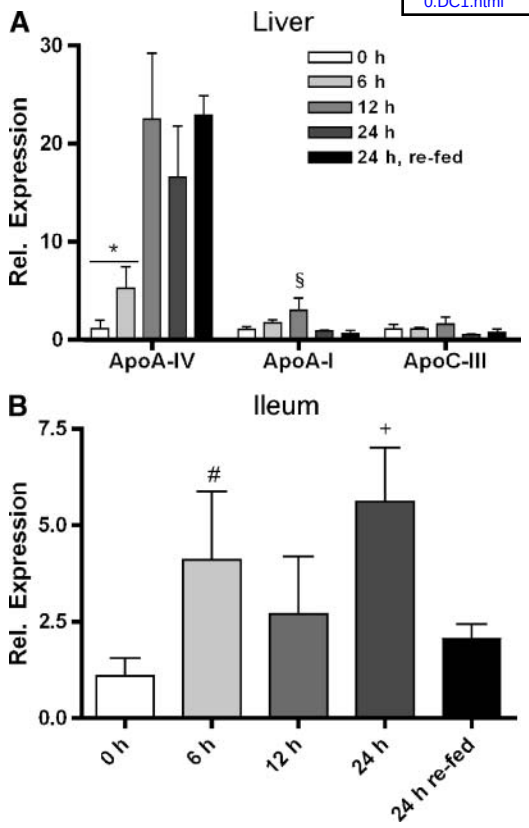

Fig. 1. Hepatic and ileal apolipoprotein mRNA levels are increased in fasting. Real-time quantitative PCR analysis of apolipoprotein A-IV (apoA-IV), apoA-I, and apoC-III mRNA expression in liver (A) and apoA-IV expression in the ileum (B) after 0, 6, 12, and 24 h fasts and 24 h fast followed by a 24 h refeed. All values were normalized to RNA polymerase II mRNA levels and are expressed as the fold difference relative to 0 h fasted controls. * $P < 0.05$ versus 12 and 24 h fasted and 24 h refed. § $P < 0.05$ versus 0 and 24 h fasted and 24 h refed. # $P < 0.05$ versus 0 h fasted. + $P < 0.05$ versus 0 h fasted and 24 h refed. All values are means ± SD (n = 3–4).

mice were fasted for 24 h and examined for changes in expression of both apoA-IV mRNA (liver) and protein (plasma). Consistent with the results of the fasting time course (Fig. 1A), liver apoA-IV mRNA levels were induced by ~19-fold over fed controls by a 24 h fast (**Fig. 2A**). Basal apoA-IV mRNA expression was increased (~3.5-fold over the wild type) in PPARα$^{-/-}$ mouse livers (Fig. 2A). Examination of apoA-IV mRNA levels in fasted PPARα$^{-/-}$ mice revealed a similar magnitude of induction to that seen in the control mice: ~15-fold over wild-type controls (Fig. 2A). When apoA-IV expression in fasted PPARα$^{-/-}$ mice was compared with the high basal expression in PPARα$^{-/-}$ fed mice, the induction level was reduced to ~4-fold (Fig. 2A). QPCR analysis of cytochrome P450 (Cyp) 4a14 mRNA expression, an established PPARα target gene (33), demonstrated a complete lack of expression in both fed and fasted PPARα$^{-/-}$ mice (Fig. 2B). In contrast, fasting induced liver Cyp4a14 mRNA in wild-type mice by ~90-fold over fed controls (Fig. 2B). Protein expression in plasma of fasted and fed PPARα$^{-/-}$ and wild-type mice was determined by Western blot. Consistent with the increases in apoA-IV mRNA, protein expression of apoA-IV was increased in the plasma of both wild-type and PPARα$^{-/-}$ mice fasted for 24 h (Fig. 2C).

Food deprivation is known to increase plasma levels of corticosteroids through the activation of the hypothalamic-pituitary-adrenal axis (32). Therefore, to further characterize the regulatory mechanisms responsible for the induction of apoA-IV expression in fasting, adrenalectomized mice were fasted for 24 h. ELISA was used to determine plasma corticosteroid concentrations in sham-operated and adrenalectomized mice. Plasma corticosteroid levels in sham-operated mice were increased by a 24 h fast (~2.5-fold) over levels seen in fed controls (**Fig. 3A**). Adrenalectomy of mice resulted in a decrease of corticosteroid levels (by ~2.5-fold) compared with the sham-operated controls, and this level was not increased significantly by a 24 h fast (Fig. 3A). Liver mRNA expression of apoA-IV in fasted, adrenalectomized mice was examined by QPCR. Consistent with previous results, fasting sham-operated mice for 24 h resulted in the induction of liver apoA-IV mRNA to ~19-fold over fed, sham-operated controls (Fig. 3B). Adrenalectomized mice that had been fasted for 24 h lacked significant induction of liver apoA-IV mRNA expression (Fig. 3B). Ileal mRNA expression of apoA-IV was also induced by ~24-fold, and this induction was abolished by adrenalectomy (Fig. 3B). Plasma concentrations of apolipoproteins in adrenalectomized and sham-operated mice were examined by Western blot. ApoB-100 and apoB-48 levels were decreased, whereas apoA-IV protein levels were increased, in fasted, sham-operated mice over the levels detected in controls (Fig. 3C). Adrenalectomized mice had decreased plasma apoA-IV protein levels relative to controls that were not increased by a 24 h fast (Fig. 3C). Western blot analysis also revealed that protein levels of apoA-I and apoA-II were not altered in response to either fasting or adrenalectomy (Fig. 3C).

Further investigation of the role of corticosteroids in the in vivo induction of the apoA-IV gene was performed by treatment of wild-type (C57BL/6J) mice with dexamethasone. QPCR analysis of liver apoA-IV mRNA demonstrated induction (~7-fold) over vehicle-treated controls (**Fig. 4A**). Similarly, the mRNA levels of Cyp3a11, an established dexamethasone-responsive gene (34), were increased by ~5-fold (Fig. 4A). Hepatic expression of HNF-4α and PGC-1α was also increased in dexamethasone-treated mice by 1.6- and 4.6-fold, respectively, over vehicle-treated controls (Fig. 4A). Western blot analysis revealed decreases in both apoB-100 and apoB-48 plasma proteins in dexamethasone-treated compared with vehicle-treated controls (Fig. 4B). Consistent with mRNA levels, plasma protein expression of apoA-IV was increased with dexamethasone treatment (Fig. 4B). Plasma protein levels of apoA-I and apoA-II were also increased in dexamethasone-treated mice (Fig. 4B). Fast-performance liquid chromatography separation and subsequent Western blotting of plasma revealed that the increase in apoA-IV protein occurred solely in the HDL fraction (data not shown). To determine whether exogenous glucocorticoid treatment could induce apoA-IV in adrenalectomized mice, mice were treated with dexamethasone. This treatment resulted in a 20-fold induction of apoA-IV mRNA levels (Fig. 4C).

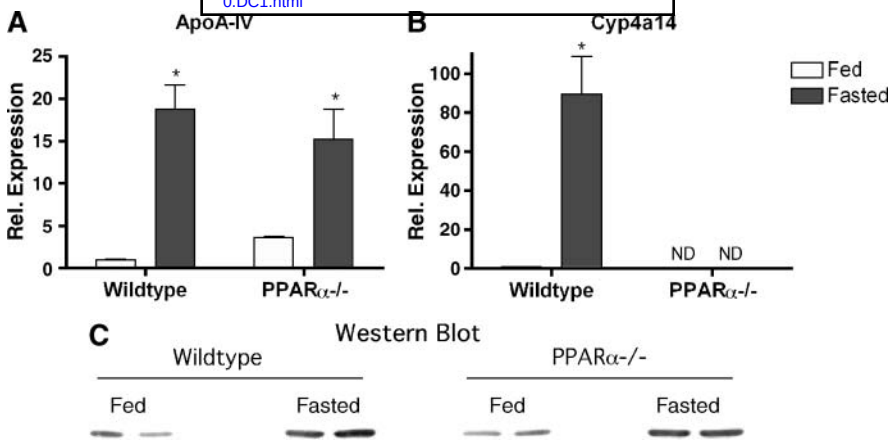

Fig. 2. Fasting apoA-IV induction is not abolished in peroxisome proliferator-activated receptor α-deficient (PPARα$^{-/-}$) mice. A, B: Real-time quantitative PCR analysis of hepatic mRNA expression of apoA-IV (A) and cytochrome P450 (Cyp) 4a14 (B) in 24 h fasted wild-type and PPARα$^{-/-}$ mice. All values were normalized to RNA polymerase II mRNA levels and are expressed as the fold difference relative to wild-type fed controls. C: Western blot analysis of apoA-IV plasma protein levels in wild-type and PPARα$^{-/-}$ mice fasted for 24 h. * $P < 0.05$ versus respective wild-type values. All values are means ± SD (n = 3–4). ND, not detected.

Glucocorticoids are a causative factor in many of the pathologies associated with diabetes, including hyperlipidemia and hyperglycemia (20, 35, 36). The diabetic liver, being unable to properly sense circulating glucose levels, responds similarly to the fasted liver: by increasing gluconeogenesis. Given the importance of glucocorticoids in hepatic responses to fasting and diabetes and the demonstrated importance of corticosteroids in the in vivo induction of apoA-IV in fasting, we endeavored to examine the expression of apoA-IV in two animal models of diabetes. In vivo expression of apoA-IV in diabetes was first investigated by the induction of type I diabetes in C57BL/6J mice by streptozocin injection. High blood glucose (≥15 mmol/l), triglyceride (2-fold), and cholesterol (4-fold) levels were confirmed in streptozocin-injected mice compared with vehicle-treated controls (data not shown). At 4 weeks after injection, liver mRNA expression of apoA-IV was increased by ∼3.5-fold over that in vehicle-treated controls (**Fig. 5A**). Liver expression of the apoA-I and apoC-III genes was also altered in streptozocin-treated mice: a 2-fold decrease and a 4-fold increase in apoA-I and apoC-III mRNA levels were measured, respectively (Fig. 5A). Plasma protein levels of apoA-IV in streptozocin-treated mice were measured by Western blot and found to be increased in diabetic mice compared with vehicle-treated mice (Fig. 5B). The inductive effect of the diabetic state on apoA-IV expression was further examined using ob/ob mice, a model of type II diabetes (37). QPCR analysis of liver mRNA expression in ob/ob mice demonstrated a large induction of apoA-IV expression (∼125-fold) over that measured in control littermates (Fig. 5C). Analysis of mRNA expression of the apoA-I and apoC-III genes revealed no changes in the levels of either transcript in ob/ob mice (Fig. 5C). Consistent with the inductive effect of type I diabetes on plasma protein levels of apoA-IV, the ob/ob model of type II diabetes also demonstrated an increase in the protein compared with wild-type littermate controls (Fig. 5D).

Given the requirement for adrenal corticosteroids for the induction of apoA-IV during fasting and the ability of dexamethasone, a synthetic corticosteroid, to increase the expression of this gene in vivo, the role of the GR in regulating apoA-IV promoter activity was investigated. HNF-4α has been shown to regulate apoA-IV expression in vivo (28), and PGC-1α is known to contribute to a number of the changes in gene expression that occur during fasting. As such, experiments were designed to test the effect of transfection of a mouse promoter reporter construct containing both the apoC-III enhancer region and the apoA-IV promoter region (designated mEC3A4) into HepG2 cells in combination with control (pSG5) vector or GR- or HNF-4α-expressing constructs in the presence or absence of a PGC-1α-expressing construct. Both the human and mouse apoC-III/A-IV intergenic regions contain three conserved hormone response elements (HREs) that bind HNF-4α: one HRE in each of the proximal and distal regions of the apoA-IV promoter and one HRE in the apoC-III enhancer region (16, 28, 38, 39). A scheme representing both the human and mouse apoA-IV promoter constructs used in the transient transfection experiments is provided in **Fig. 6A**. Cotransfection of the PGC-1α construct with pSG5 resulted in an increased activation of mEC3A4 (∼15-fold) and was not further activated by the addition of dexamethasone (Fig. 6B). Transfection of the GR construct alone did not activate mEC3A4, although cotransfection with the PGC-1α construct increased its activity (∼15-fold) and was not further activated by treatment with dexamethasone (Fig. 6B). Transfection of the HNF-4α construct alone caused an induction of mEC3A4 (5-fold), and this induction was synergistically increased by cotransfection of the PGC-1α construct, inducing activity by ∼65-fold (Fig. 6B). Treatment with dexamethasone did not affect the activity of mEC3A4 either in the presence of the HNF-4α construct alone or in combination with the PGC-1α construct

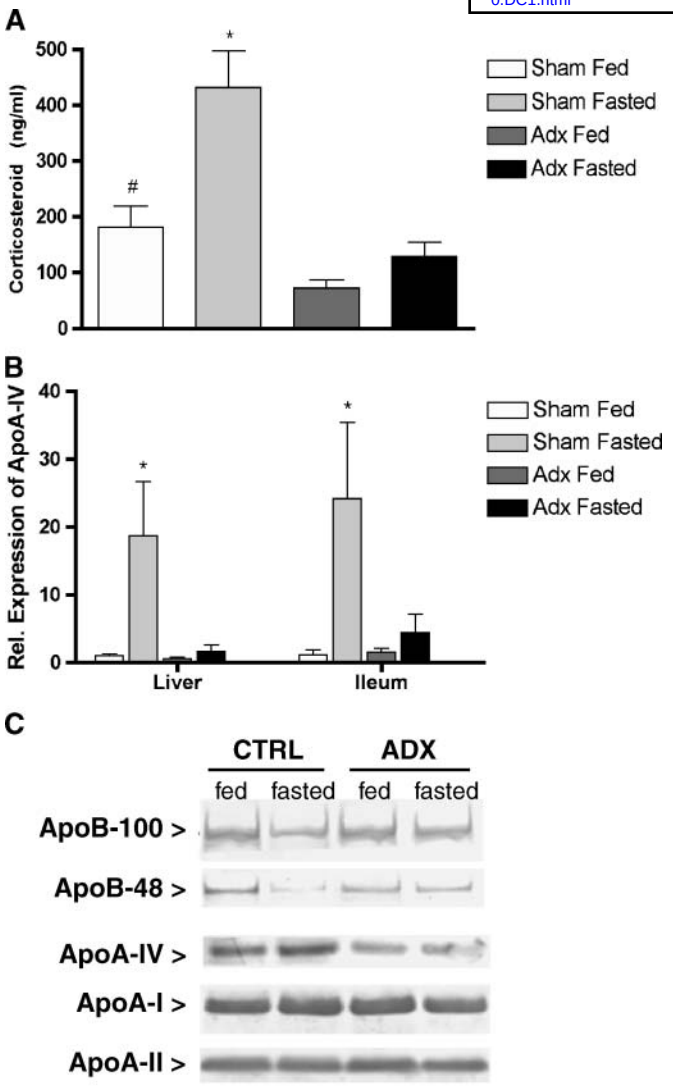

Fig. 3. ApoA-IV induction is abolished in adrenalectomized mice. A: ELISA detection of plasma corticosteroid levels in adrenalectomized (Adx) and sham-operated mice (fed or fasted for 24 h). B: Real-time quantitative PCR analysis of hepatic and ileal apoA-IV mRNA expression. All values were normalized to RNA polymerase II mRNA expression and are expressed as the fold difference relative to sham-operated mice (fed). C: Western blot analysis of plasma lipoproteins from the adrenalectomized and sham-operated mice (fed or fasted for 24 h). * $P < 0.05$ versus all other groups. # $P < 0.05$ versus adrenalectomized fed mice. All values are means ± SD (n = 5).

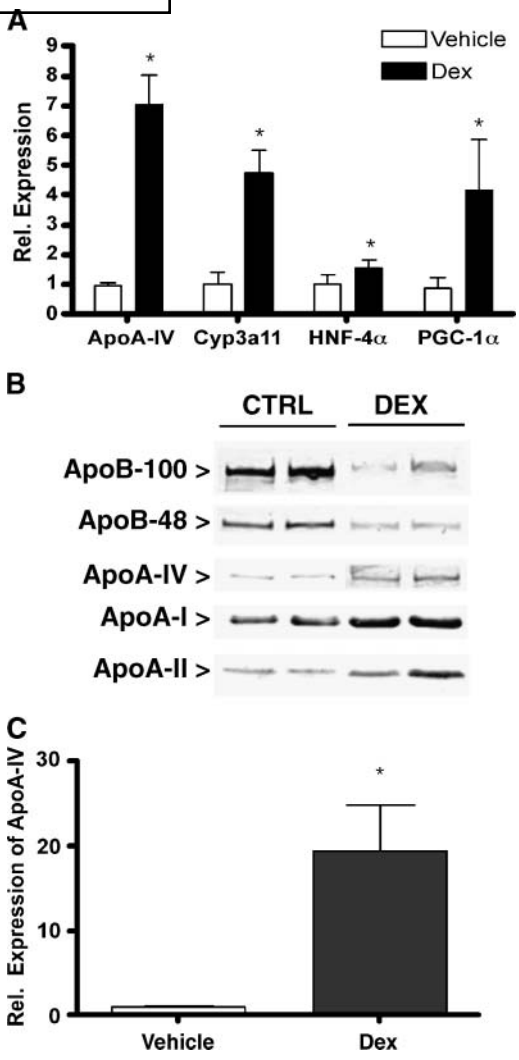

Fig. 4. Exogenous glucocorticoids increase apoA-IV expression. A: Real-time quantitative PCR analysis of hepatic apoA-IV, Cyp3a11, hepatic nuclear factor-4α (HNF-4α), and peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) mRNA expression in dexamethasone (Dex)-treated mice. All values were normalized to RNA polymerase II mRNA levels and are expressed as the fold difference relative to vehicle-treated mice. B: Western blot analysis of plasma lipoproteins from dexamethasone-treated mice. C: Real-time quantitative PCR analysis of hepatic apoA-IV mRNA expression in dexamethasone-treated, adrenalectomized mice. * $P < 0.05$ versus respective vehicle-treated controls (unpaired *t*-tests). All values are means ± SD (n = 4–5).

(Fig. 6B). To determine whether the regulatory mechanisms demonstrated with the murine apoA-IV promoter were conserved, the human apoA-IV construct was also examined. This construct contained the apoC-III enhancer fused to the apoA-IV promoter and was designated hEC3A4. Consistent with the pattern of regulation seen in the mouse promoter, transfection of the PGC-1α construct alone caused the activation of hEC3A4 by ~7-fold (Fig. 6C). Transfection of the HNF-4α construct alone did not activate hEC3A4, whereas the combination with the PGC-1α construct synergistically induced activity by ~11-fold (Fig. 6C).

The observation that PGC-1α could activate apoA-IV promoter activity in the absence of cotransfected receptor indicated an interaction with an endogenous nuclear receptor. Given that PGC-1α, when cotransfected with HNF-4α, led to a synergistic activation of the apoA-IV promoter, we hypothesized that this nuclear receptor was involved. To determine whether the basal activation of the apoA-IV promoter region by cotransfection of PGC-1α alone was attributable to endogenous HNF-4α in HepG2 cells, similar transfections were performed in Cos-7 cells, which do not express high levels of HNF-4α. Transfection of either the PGC-1α or HNF-4α construct alone did not cause the activation of the mEC3A4 construct in these cells (**Fig. 7A**). Consistent with data obtained in the HepG2 cells, cotransfection of both HNF-4α- and PGC-1α-express-

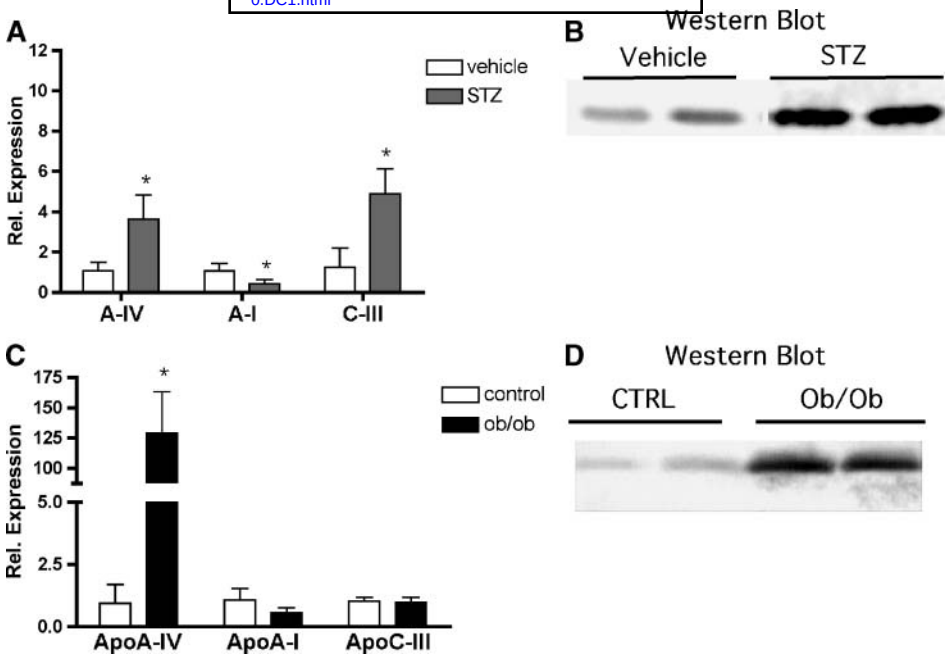

Fig. 5. ApoA-IV mRNA and serum protein levels are increased in type I and II diabetes. A: Real-time quantitative PCR analysis of hepatic mRNA expression of apoA-IV, apoA-I, and apoC-III in mice 4 weeks after streptozocin (STZ) injection. B: Western blot analysis of apoA-IV plasma protein levels in mice 4 weeks after streptozocin injection. C: Real-time quantitative PCR analysis of hepatic mRNA expression of apoA-IV, apoA-I, and apoC-III in 12 week old ob/ob mice. D: Western blot analysis of apoA-IV plasma protein levels in 12 week old ob/ob mice. CTRL, control. All values were normalized to RNA polymerase II expression and are expressed as the fold difference relative to vehicle-treated (for streptozocin-injected mice) and littermate (for ob/ob mice) controls. * $P < 0.05$ versus respective controls (vehicle-treated or littermate values). All values are means ± SD (n = 4–5).

ing constructs in Cos-7 cells caused a synergistic induction of mEC3A4 by ~170-fold (Fig. 7A). A similar pattern of regulation of the human apoA-IV promoter was seen in transfections of Cos-7 cells in which neither the PGC-1α nor the HNF-4α construct alone could induce the hEC3A4 construct, but cotransfection of the two led to a synergistic induction of ~40-fold (Fig. 7B). Therefore, the activating effect of PGC-1α alone on hEC3A4 in HepG2 cells was likely attributable to the presence of endogenous HNF-4α. To confirm the contribution of endogenous HNF-4α to mEC3A4 activation in HepG2 cells, two independent approaches were used to disrupt this effect. The first used a vector expressing a dominant-negative form of HNF-4α. Transfection of 75, 150, and 275 ng of this construct caused a dose-dependent decrease in the coactivation of mEC3A4 by PGC-1α (Fig. 7C). The second approach knocked down endogenous HNF-4α expression using a human HNF-4α shRNA expression vector. Transfection with the lowest amount of this construct (75 ng) abolished the coactivation of mEC3A4 by PGC-1α (Fig. 7C). Similar to results seen with the mEC3A4 construct, the dominant negative rat HNF-4α-expressing construct caused a dose-dependent decrease in coactivation of the hEC3A4 construct by PGC-1α (Fig. 7D). Similarly, cotransfection of the lowest amount of human HNF-4α shRNA-expressing construct (75 ng) abolished the coactivation of hEC3A4 by PGC-1α (Fig. 7D).

Further characterization of the role of HNF-4α and PGC-1α in the activation of apoA-IV was assessed in vivo by QPCR analysis of livers from 0, 6, 12, and 24 h fasted and 24 h fasted, refed mice. Hepatic expression of PGC-1α was induced in 6, 12, and 24 h fasted mice (~3-, 6-, and 3.5-fold, respectively) and decreased to control values after 24 h access to food (**Fig. 8A**). Similarly, hepatic mRNA expression of HNF-4α was induced by a 6 and 12 h fast, although it decreased to control values by a 24 h fast and remained at this level after 24 h access to food (Fig. 8A). Further insight into the in vivo roles of HNF-4α and PGC-1α was determined through a 24 h fast of HNF-4α liver-specific knockout mice. ApoA-IV mRNA expression in floxed mouse controls was induced by ~20-fold by a 24 h fast (Fig. 8B). Expression of apoA-IV mRNA was not detectable in HNF-4α liver-specific knockouts and was not induced by a 24 h fast (Fig. 8B). HNF-4α mRNA expression was increased by a 24 h fast in floxed controls (~2-fold) and was not detectable in knockout livers (Fig. 8B). Hepatic mRNA expression of PGC-1α in these mice was unaltered by the lack of HNF-4α expression in the knockout mice, compared with floxed controls, and was not induced by a 24 h fast in either genotype (Fig. 8B). To examine the role of corticosteroids in PGC-1α and HNF-4α expression in vivo, mRNA expression of these two genes was examined by QPCR analysis of 24 h fasted, adrenalectomized mice. Examination of liver mRNA expression of HNF-4α revealed no change in HNF-4α attributable

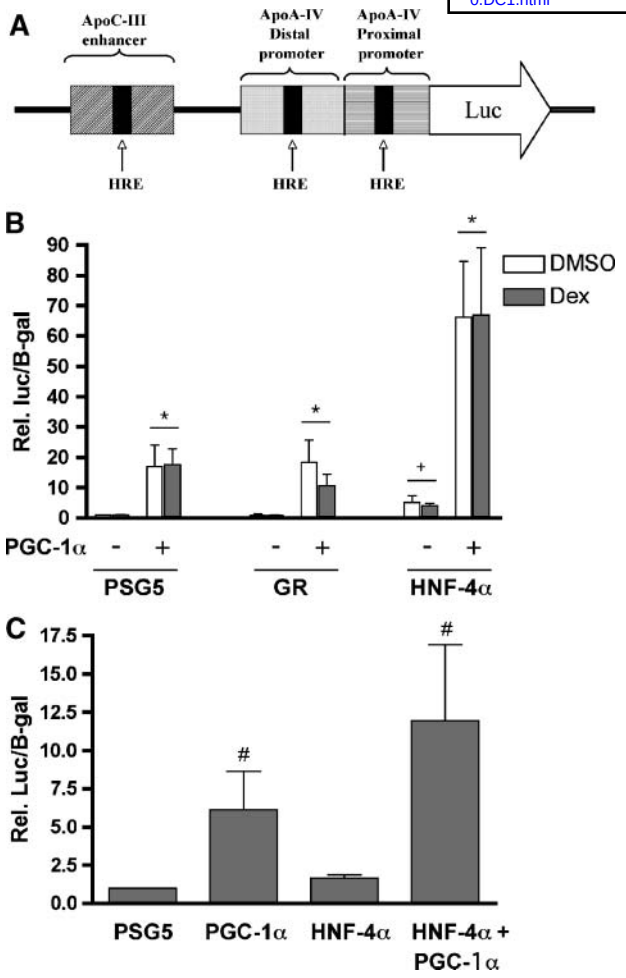

Fig. 6. HNF-4α and PGC-1α activate murine and human apoA-IV promoter activity. A: Scheme representing the mouse and human apoA-IV promoter constructs containing three hormone response elements (HREs) known to bind HNF-4α. B, C: Cotransfection of HepG2 cells with the mouse (B) or human (C) apoA-IV/C-III intergenic region containing the apoA-IV promoter with an intact apoC-III enhancer region in addition to vector (pSG5) or constructs expressing glucocorticoid receptor (GR) or HNF-4α. Cells were treated 24 h after transfection with either 50 μM dexamethasone (Dex) or DMSO and harvested 24 h later. Luciferase values were normalized to individual β-galactosidase activities for the same well. Promoter luciferase activities were reported as fold activation over activity with empty vector (pSG5). * $P < 0.05$ versus expression construct alone (pSG5, GR, or HNF-4α) with the same treatment. $^{+}$ $P < 0.05$ versus transfection with pSG5 or GR alone (unpaired *t*-tests). $^{\#}$ $P < 0.05$ versus all other groups. All values are means ± SD (n = 3).

to fasting in either the sham-operated or adrenalectomized mice (Fig. 8C). PGC-1α liver mRNA expression was increased as a result of fasting in sham-operated mice (~2.5-fold), and induction of this gene was even greater in fasted, adrenalectomized mice (~3.5-fold) (Fig. 8C).

DISCUSSION

This study demonstrates profound changes of hepatic and ileal apoA-IV expression during states of nutritional and metabolic stress. Challenging mice with a 24 h fast led to increases in liver and ileal apoA-IV mRNA and plasma apoA-IV protein levels. These increases did not occur in adrenalectomized mice, indicating a requirement for endogenous corticosteroids. Treatment with dexamethasone as well as induction of diabetes in mice also induced apoA-IV gene and protein expression. In vitro analyses revealed that the mouse and human apoA-IV promoters were induced by PGC-1α and HNF-4α but not by GR. Inhibition of endogenous HNF-4α function by expression of a dominant-negative HNF-4α or shRNA for HNF-4α abolished the coactivation of the promoter by PGC-1α. Consistent with a PGC-1α/HNF-4α mechanism of induction, the in vivo findings indicate that induction of these genes occurs in parallel with that of the apoA-IV gene during fasting.

Many physiological changes occur in the liver during fasting, including increased oxidation of fatty acids. This catabolic process involves many enzymatic steps, some of which are regulated at the transcriptional level by PPARα (40). PPARα is also known to regulate the expression of genes encoding apoA-I and apoC-III (37, 41), and PPARα$^{-/-}$ mice exhibit hepatic accumulation of lipids when challenged with clofibrate or Wy-14,643 (23). Examination of apoA-IV expression in PPARα$^{-/-}$ mice revealed that hepatic mRNA and plasma protein levels were increased by fasting to a level identical to that for wild-type mice. However, because of the high basal level of apoA-IV mRNA, the relative increase by fasting in PPARα$^{-/-}$ mice was lower than that seen for wild-type mice. Given that PPARα$^{-/-}$ mice did not exhibit superinduction of apoA-IV expression with fasting, it is likely that the mechanism(s) contributing to the increased basal expression of this apolipoprotein is also related to perturbations of energy homeostasis in these mice. Interestingly, a recent study found that female PPARα$^{-/-}$ mice had increased secretion of hepatic VLDL (42). Given that apoA-IV is a component of this lipoprotein, it is possible that the increased basal expression of mRNA and protein for apoA-IV detected in this study is functionally related to this increased VLDL synthesis and secretion. Further studies comparing apoA-IV expression in fasted and fed female as well as male PPARα$^{-/-}$ mice would help further characterize this phenotype.

Another physiological change that occurs during fasting is an increase in glucocorticoid levels. Glucocorticoids antagonize the physiological effects of insulin in large part by inhibiting the tissue uptake of glucose and by increasing hepatic gluconeogenesis (36). Glucocorticoids have also been known to cause increases in apoA-I expression levels through an unknown indirect mechanism (43, 44). In this study, fasting caused increases in plasma corticosteroid levels. Adrenalectomy abolished both the fasting-induced increases in plasma corticosteroids and apoA-IV expression. Furthermore, treatment of adrenalectomized mice with an exogenous glucocorticoid (dexamethasone) induced expression of the apoA-IV gene in adrenalectomized mice. This demonstrated that the transcriptional components required for glucocorticoid-induced expression of apoA-IV were present and functional in adrenalectomized mice. Together, these data strongly support the

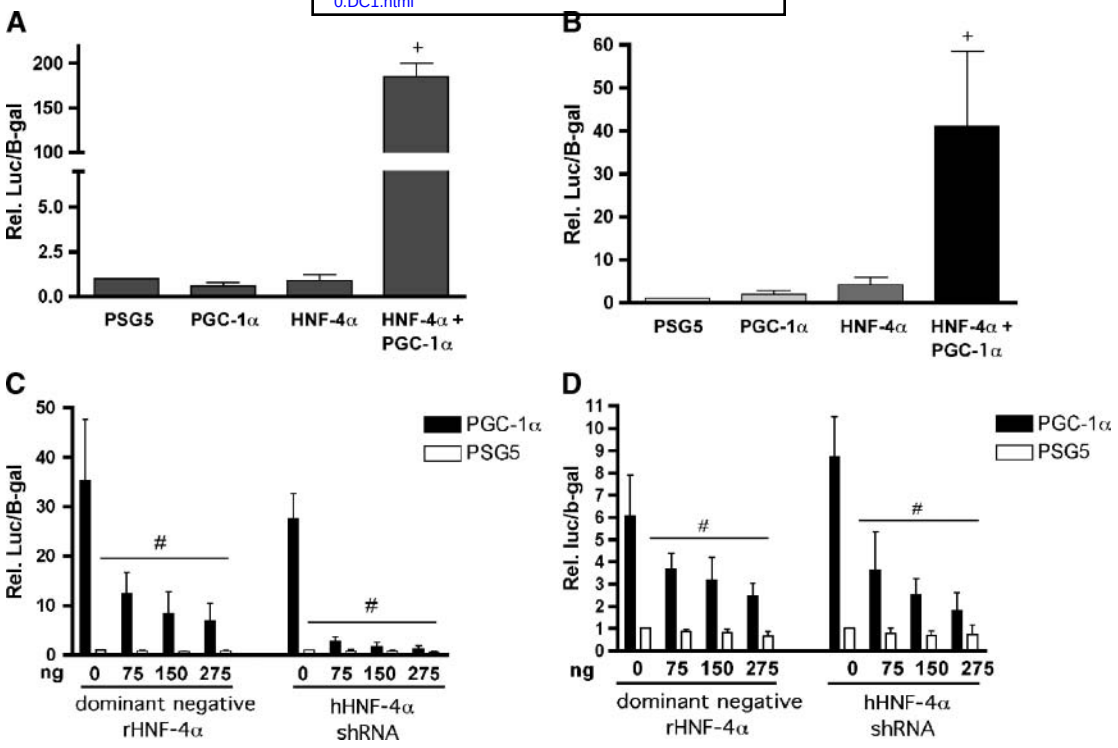

Fig. 7. HNF-4α is required for PGC-1α coactivation of the mouse and human apoA-IV promoters. A, B: Cos-7 cells were cotransfected with constructs containing the mouse (A) or human (B) apoA-IV/C-III regulatory regions in addition to either PGC-1α- or HNF-4α-expressing constructs, alone or in combination. Cells were harvested 24 h after transfection. C, D: HepG2 cells were cotransfected with a construct containing the mouse (C) or human (D) apoA-IV/C-III regulatory regions with or without the addition of a PGC-1α-expressing construct and 75, 150, and 275 ng of constructs expressing either a dominant-negative rat HNF-4α (rHNF-4α) or a human HNF-4α shRNA (hHNF-4α). Cells were harvested 36 h after transfection. Luciferase values were normalized to individual β-galactosidase activities (or renilla luciferase activity in the case of shHNF-4α) for the same well. Promoter luciferase activities were reported as fold activation over activity with control vector (pSG5, pShuttle, or the pENTR-lacZ construct that expresses shRNA for lacZ). $^{+}$ $P < 0.05$ versus all other groups. $^{\#}$ $P < 0.05$ versus PGC-1α plus 275 ng of control vector (either pENT-lacZ or TK-RL). Values are means ± SD (n = 3).

notion that the in vivo induction of apoA-IV is mediated by the release of an adrenal corticosteroid, most likely a glucocorticoid hormone. Although the fasting-induced expression of apoA-IV was relatively selective for this apolipoprotein, dexamethasone treatment was associated with increased expression of other apolipoproteins, such as apoA-I, apoA-II, and apoE. Thus, it is likely that the dose of dexamethasone used in this study was also associated with nonspecific effects independent of those involved in the fasting-associated induction of apoA-IV.

Glucocorticoids are increased in plasma by insulin deficiency (33) and are thought to contribute to many of the pathological changes that occur in diabetes, such as hyperlipidemia and hyperglycemia (20). Indeed, GR inactivation in liver or adipose leads to the amelioration of hyperglycemia as well as hyperlipidemia in animal models of diabetes (20, 45). Induction of type I diabetes in mice by streptozocin treatment led to increases in liver apoA-IV mRNA and plasma protein that persisted even at 4 weeks after injection. These streptozocin-treated mice also had increased plasma triglycerides and cholesterol. Consistent with current literature (5, 6), apoA-IV was detected solely in the HDL fraction of fast-performance liquid chromatography-separated plasma from mice. Therefore, increases in apoA-IV in type I diabetic mice are likely to be associated with HDL cholesterol and not triglycerides. ob/ob mice are leptin-deficient and become obese and insulin-resistant at an early age. Thus, these mice are often used as a model of type II diabetes (46). Interestingly, ob/ob mice have glucocorticoid levels 14-fold greater than controls, and adrenalectomy of these mice leads to a reversal of obesity (47, 48). In this study, liver apoA-IV mRNA and plasma protein levels were greatly increased in 12 week old ob/ob mice. Together, these results indicate that the induction of apoA-IV expression that occurs in fasting or diabetes entails a common glucocorticoid-dependent mechanism. Consistent with these in vivo findings in mice, apoA-IV protein levels are increased in diabetic patients (49).

Despite the glucocorticoid-dependent induction of apoA-IV observed in vivo, functional analyses of the murine and human apoA-IV promoters failed to provide evidence for a direct activation by the GR. The orphan nuclear receptor HNF-4α, in addition to PGC-1α, is known to be important in mediating gluconeogenic responses to fasting in the liver (35). The human apoA-IV promoter has two HNF-4α HREs in addition to one other HRE in the adjacent apoC-III enhancer (16). Studies with transgenic mice that express human apoA-IV under the control of the native promoter demonstrated that the distal HRE is required for basal expression in the intestine (28). Consis-

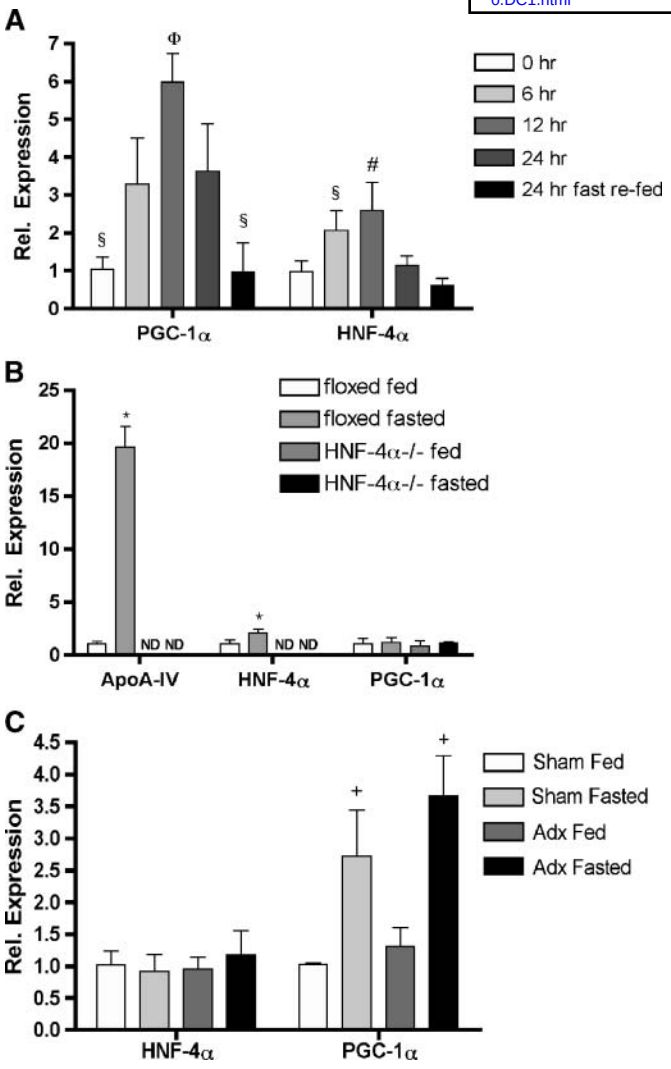

Fig. 8. Fasting induces HNF-4α and PGC-1α expression in vivo. Real-time quantitative PCR analysis of hepatic mRNA expression of PGC-1α and HNF-4α after 0, 6, 12, and 24 h fasts and 24 h fast followed by a 24 h refeed (A), apoA-IV, HNF-4α, and PGC-1α in fed or 24 h fasted HNF-4α$^{-/-}$ mice and floxed controls (B), and HNF-4α and PGC-1α in adrenalectomized (Adx) and sham-operated mice (fed or fasted for 24 h) (C). All values were normalized to RNA polymerase II mRNA levels and are expressed as the fold difference relative to controls (0 h fasted, floxed fed, or sham fed). § $P < 0.05$ versus 24 h. $^{\Phi}$ $P < 0.05$ versus 0, 6, and 24 h fasted refed. $^{\#}$ $P < 0.05$ versus 0, 24, and 24 h fasted refed. * $P < 0.05$ versus floxed fed. $^{+}$ $P < 0.05$ versus all other groups. Values are means ± SD (n = 3). ND, not detected.

tent with this, HNF-4α liver-specific knockout mice had no detectable basal expression of hepatic apoA-IV, and no induction of this gene was seen in this study when these mice were fasted. Interestingly, expression of PGC-1α alone was able to activate the expression of the murine and human apoA-IV/C-III promoter constructs in HepG2 cells that express endogenous HNF-4α but not in Cos-7 cells that lack expression of this nuclear receptor. Cotransfection of expression constructs for HNF-4α and PGC-1α synergistically increased the activation of these promoter constructs. Cotransfection of dominant-negative HNF-4α- or HNF-4α shRNA-expressing constructs abrogated the induction of the apoA-IV promoter by PGC-1α alone in HepG2 cells. This provided convincing evidence that HNF-4α and PGC-1α were mutually required for the activation of this promoter in vitro. This interaction of HNF-4α and PGC-1α at the mouse promoter is consistent with the recent findings of Rhee et al. (50). Our empirical demonstration that this interaction also activates the expression of human apoA-IV highlights the evolutionary conservation and likely physiological importance of this response across species.

Consistent with the promoter analyses, induction of hepatic HNF-4α and PGC-1α expression was observed in vivo as early as 6 h after the start of fasting. Maximal levels of HNF-4α and PGC-1α mRNA occurred at 12 h after the start of fasting and preceded the maximum levels of apoA-IV mRNA seen at 24 h. These results, in addition to the abrogation of response in liver-specific HNF-4α knockout mice, are consistent with an essential role for both HNF-4α and PGC-1α in fasting-induced hepatic apoA-IV mRNA expression in vivo. The coincident induction of PGC-1α, HNF-4α, and apoA-IV is also consistent with our in vitro findings that HNF-4α and PGC-1α interact to activate the expression of the apoA-IV promoter. In agreement with these findings, ecotopic overexpression of PGC-1α or RNA interference-mediated repression of endogenous PGC-1α expression was recently reported to increase or decrease, respectively, hepatic apoA-IV mRNA levels (50).

Intriguingly, examination of adrenalectomized mice revealed that although apoA-IV induction was absent, fasting-induced increases of PGC-1α mRNA levels were similar to those seen for sham-operated animals. Thus, although our data strongly support a role for PGC-1α in the induction of apoA-IV by fasting, increased expression of this transcription factor alone is clearly not sufficient for this response. It is possible that glucocorticoids contribute to apoA-IV induction through an independent signaling pathway or, alternatively, by modifying the transcriptional activating function of HNF-4α and PGC-1α. For example, a negative regulatory motif that represses transcriptional activity has been identified in the PGC-1α protein (51). Phosphorylation of this regulatory domain by p38 mitogen-activated protein kinase relieves this functional repression and permits transcriptional coactivation by PGC-1α. It is possible that adrenal glucocorticoid release acts upon an analogous regulatory mechanism that serves to activate the transcriptional activity of PGC-1α and/or HNF-4α in vivo. Alternatively, increases in glucocorticoid levels lead to altered insulin levels and activity, changes in lipid metabolism, and induction of gluconeogenesis in the liver. It is possible that apoA-IV gene activity is induced by an environmental stimulus (such as glucose or ketogenic products) that is required for the actions of HNF-4α and PGC-1α on the promoter. Studies of the complex regulation of the apoA-I gene have demonstrated that induction by prolonged fasting is correlated with increases in ketone bodies (52). In addition, the apoA-I gene has also been found to be responsive to glucocorticoid levels through an indirect mechanism thought to involve an in-

crease in the amount and/or activity of another nuclear receptor, HNF-3β (44). Therefore, the in vivo role for glucocorticoids in the induction of the apoA-IV gene in fasting and diabetes is likely mediated through an indirect mechanism that may include altered hormonal signaling, environmental stimuli, or the activation of another as yet unknown transcription factor.

In conclusion, this study demonstrates that hepatic and ileal apoA-IV gene expression are dramatically induced by fasting in a glucocorticoid-dependent manner. ApoA-IV is also induced in two established mouse models of diabetes. Despite the requirement for glucocorticoids, analysis of the mouse and human apoA-IV promoters indicates a direct regulatory role for HNF-4α and PGC-1α but not for GR. Although HNF-4α and PGC-1α are necessary for the induction of apoA-IV during fasting and diabetes, our data suggest a more complex and highly conserved in vivo mechanism for the regulation of apoA-IV during times of nutritional and/or metabolic stress.

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