Hepatic insig-1 or -2 overexpression reduces lipogenesis in obese Zucker diabetic fatty rats and in fasted/refed normal rats

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To determine whether the antilipogenic actions of insulin-induced gene 1 (insig-1) demonstrated in cultured preadipocytes also occur *in vivo*, we infected Zucker diabetic fatty (ZDF) (*fa*/*fa*) rats, with recombinant adenovirus containing insig-1 or -2 cDNA. An increase of both proteins appeared in their livers. In control ZDF (*fa*/*fa*) rats infected with adenovirus containing the β-galactosidase (β-gal) cDNA, triacylglycerols in the liver and plasma rose steeply whereas the insig-infected rats exhibited substantial attenuation of the increase in hepatic steatosis and hyperlipidemia. Insig overexpression was associated with a striking reduction in the elevated level of nuclear sterol regulatory element-binding protein (SREBP)-1c, the activated form of the transcription factor. The mRNA of SREBP-1c lipogenic target enzymes also fell. The mRNA of endogenous insig-1, but not -2a and -2b, was higher in the fatty livers of untreated obese ZDF (*fa*/*fa*) rats compared with controls, but the elevation was not sufficient to block the ≈3-fold increase in SREBP-1c expression and activity. In normal animals, adenovirus-induced overexpression of the insigs reduced the increase in SREBP-1c mRNA and its target enzymes caused by refeeding. The findings demonstrated that both insigs have antilipogenic action when transgenically overexpressed in livers with increased SREBP-1c-mediated lipogenesis. However, the increase in endogenous insig-1 expression associated with augmented lipogenesis may limit it, but is insufficient to prevent it.

Insulin-induced gene 1 (insig-1) was originally cloned by Peng *et al*. (1) in regenerating liver and was subsequently shown to be dramatically elevated in the fat tissue of rats at the onset of diet-induced obesity (DIO) (2). Its function was unknown until Yang *et al*. (3) demonstrated that it binds sterol regulatory element-binding protein (SREBP) cleavage-activating protein (SCAP), thereby preventing it from leaving the endoplasmic reticulum to escort SREBPs to the Golgi. SREBP proteins are inactive until converted by proteolytic processing in the Golgi into active transcription factors (4). In other words, insig-1 effectively blocks the activation of SREBPs.

Although this seminal discovery was directed at the liver's cholesterogenic transcription factor, SREBP-2, it obviously was relevant to other members of the SREBP family. It was subsequently shown that transfection of insig-1 into preadipocytes completely blocks glucose-derived lipogenesis in 3T3-L1 adipocytes (5). This finding led to the proposal that the rise in insig-1 in the expanding adipocytes of diet-induced obesity "brakes" the lipogenesis so as to avoid an overaccumulation of fat that would exceed their storage capacity and damage them.

This antilipogenic effect of insig-1 had been demonstrated *in vitro* without any corroborating evidence that the same antilipogenic effect also occurred *in vivo* in adult animals. To obtain such evidence, we chose to determine whether overexpression of insig-1 [and/or insig-2, a second SCAP-binding protein discovered by Yabe *et al*. (6)] could inhibit lipogenesis *in vivo*. We selected a rodent model in which SREBP-1c expression and lipogenesis were known to be abnormally high (7, 8), the Zucker diabetic fatty (*fa*/*fa*) rat. In addition to generalized obesity, these rats have a fatty liver (9), fatty heart (10), fatty skeletal muscles (9), and fatty pancreatic islets (11). We reasoned that a reduction in lipogenesis by overexpression of an insig gene would provide a stringent test of its putative antilipogenic function *in vivo*. To overexpress insig-1 or insig-2 *in vivo*, we injected recombinant adenovirus containing their cDNA and determined the effects on lipogenesis in the liver, the only organ to overexpress the transgenes.

Materials and Methods

Preparation of Recombinant Adenovirus Containing Mouse Insig-1 or Insig-2 cDNA. Mouse insig-1 and insig-2 cDNAs were obtained by RT-PCR by using mouse liver RNA, and the following primers: insig-1, forward 5′-CTG GAC GAC GAT GCC CAG GC-3′ and reverse 5′-GTC ACT GTG AGG CTT TTC CG-3′; and insig-2, forward 5′-CCT ACT GAA CTT ATG AAA CC-3′ and reverse 5′-TTC TTG ATG AGA TTT TTC AGC-3′. The PCR products were cloned into pcDNA3.1V5 vector (Invitrogen) and the sequences were verified by DNA sequencing. Insig-1 and insig-2 cDNA fragments obtained by *Bam*HI/*Pme*I excision from pcDNA3.1V5 vector were modified by addition of a V5 epitope tag and ligated to *Bam*HI-and *Eco*RV-restricted pBluescript II KS (Stratagene). Insig-1 and insig-2 cDNA fragments with a V5 epitope tag excised by *Bam*HI/*Hin*dIII digestion were ligated into pACCMVpLpA (12). The resulting plasmids were cotransfected with pJM17 (13) into human embryonic kidney (HEK) 293 cells by calcium phosphate/DNA coprecipitation to generate the new recombinant virus termed AdCMV-insig-1 and AdCMV-insig-2, by using described methods (14). It should be noted that the adenoviral vector expresses the mouse insig-2 protein common to both the insig-2a and insig-2b mRNA isoforms (15). Therefore, throughout this report, the exogenous protein will be referred to as insig-2, and the endogenous mRNAs as insig-2a and insig-2b. A virus containing the bacterial β-galactosidase gene (AdCMV-β-gal) was prepared and used as described (16).

Cell Culture. HEK 293 cells were propagated in 24-well plate (for immunoblot analysis), 60-mm (for cotransfection) or 150-mm (for amplification of viral stocks) culture dishes in DMEM supplemented with 10% FBS, 100 units of penicillin per ml, and

Abbreviations: insig, insulin-induced gene; ZDF, Zucker diabetic fatty; TG, triacylglycerol; SREBP, sterol regulatory element-binding protein; SCAP, SREBP cleavage-activating protein; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; SCD, stearoyl-CoA desaturase; GPAT, glycerol-3-phospate acyltransferase; HEK, human embryonic kidney; pfu, plaque-forming unit; β-gal, β-galactosidase.

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Table 1. Primers and probes used for quantitative real-time PCR

Gene	Accession no.	Forward/reverse primers	Probes
ACCα	J03808	5′-TCGAAGAGCTTATATCGCCTATGA	ACAGTGTACAGCATCGCCAGCTTAAGGACA
		5′-GGGCAGCATGAACTGAAATTC	
FAS	M76767	5′-GGAGGAGGCGGCTTCTGT	CCTGTTGTCTACACCACCGCCTAC
		5′-GCTGAATACGACCACGCACTAC	
SCD-1	J02585	5′-CGCTCCGCCACACTTGAT	CAACCGACAGCCACGATGCCG
		5′-GTGGTCGTGTAGGAACTGGAGAT	
GPAT	M77003	5′-CAACACCATCCCCGACATC	TCGTCATACCCGTGGGCATCTCGT
		5′-GTGACCTTCGATTATGCGATCA	
SREBP-1c	L16995	5′-GCAACACTGGCAGAGATCTACGT	CCCAGAGCCTTGCACTTCTTGACACG
		5′-TGGCGGGCACTACTTAGGAA	
Insig-1	NM_022392	5′-TGCAGATCCAGCGGAATGT	ACGCTCTTCCCGGACGAGGTGA
		5′-CCAGGCGGAGGAGAAGATG	
Insig-2a*	AY156086	5′-GACGGATGTGTTGAAGGATTTCT	
		5′-TGGACTGAAGCAGACCAATGTC	
Insig-2b*	AY156087	5′-CCGGCAGAGCTCAGGATTT	
		5′-AACTGTGGACTGAAGCAGACCAA	
36B4	NM_007475	5′-CACCTTCCCACTGGCTGAA	AAGGCCTTCCTGGCCGATCCATC
		5′-TCCTCCGACTCTTCCTTTGC	

*SYBR primers.

100 μg of streptomycin per ml at 37°C/5% CO_2. All medium components were from Sigma.

Animals. Zucker diabetic fatty (ZDF) (*fa/fa*) and lean wild-type (+/+) ZDF-drt male rats were bred in our laboratory from ZDF/drt-fa (F10) rats purchased from R. Peterson (University of Indiana School of Medicine, Indianapolis, IN). Animal experimentation was in accordance with institutional guidelines. All rats were fed standard chow (Teklad 6% fat mouse/rat diet, Harlan Teklad Premier Laboratory Diets, Madison, WI) ad libitum and had free access to water. Animals were killed under sodium pentobarbital anesthesia. Tissues were dissected immediately, frozen in liquid nitrogen, and stored at −80°C until analysis.

Plasma Measurements. Blood was collected from the tail vein of rats after a 4-h fast. Plasma was kept at −20°C until analysis. Plasma triacylglycerol (TG) levels were measured by the L-type TG H triglyceride kit (Wako Chemicals, Richmond, VA)

Adenovirus Transfer of Insig-1 or Insig-2 cDNA to Liver of ZDF (*fa/fa*) Rats. ZDF (*fa/fa*) rats were injected with 5×10^{11} or 1×10^{12} plaque-forming units (pfu) of AdCMV-insig-1, or AdCMV-insig-2, or AdCMV-β-gal as a control as described (17).

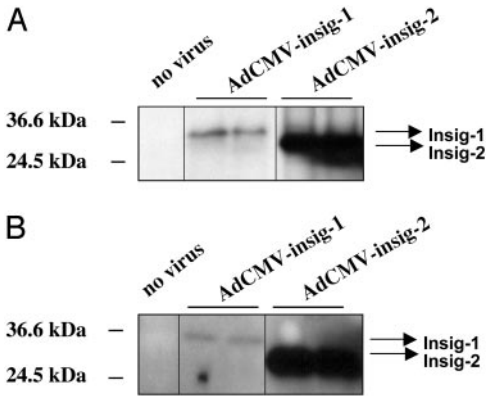

Fig. 1. Adenovirus-induced expression of insig-1 or insig-2 by recombinant adenovirus in HEK 293 cells and liver of ZDF (*fa/fa*) rats. (*A*) HEK 293 cells were exposed to 10 μl of unpurified recombinant adenoviruses containing the mouse insig-1 cDNA with a V5 tag (AdCMV-insig-1), or the mouse insig-2 cDNA with a V5 tag (AdCMV-insig-2). 293 cell extracts prepared 48 h after viral injection were subjected to immunoblot analysis with a mouse anti-V5 monoclonal antibody conjugated with HRP as described. (*B*) Seven-week-old ZDF (*fa/fa*) rats were injected with 5×10^{11} pfu of AdCMV-insig-1 or AdCMV-insig-2. Liver extracts prepared at 4 days after viral injection were subjected to immunoblot analysis as described above.

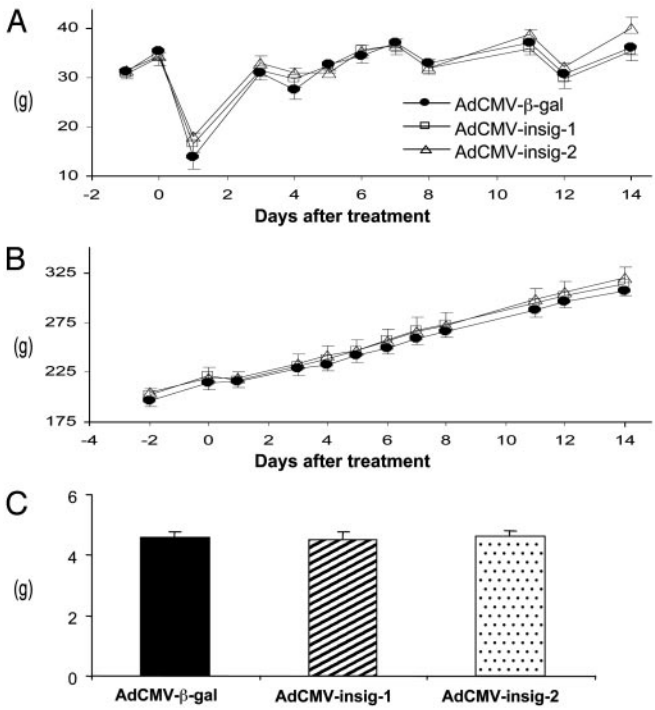

Fig. 2. Food intake, body weight, and wet weight of epididymal fat pads. Food intake (*A*), body weight (*B*), and fat pad weight (*C*) were measured in ZDF (*fa/fa*) rats injected with 5×10^{11} pfu of AdCMV-β-gal, AdCMV-insig-1, or AdCMV-insig-2 at 7 weeks of age. Wet weight of epididymal fat pads was measured at 14 days after viral injection. Values represent mean ± SE for six animals in each group.

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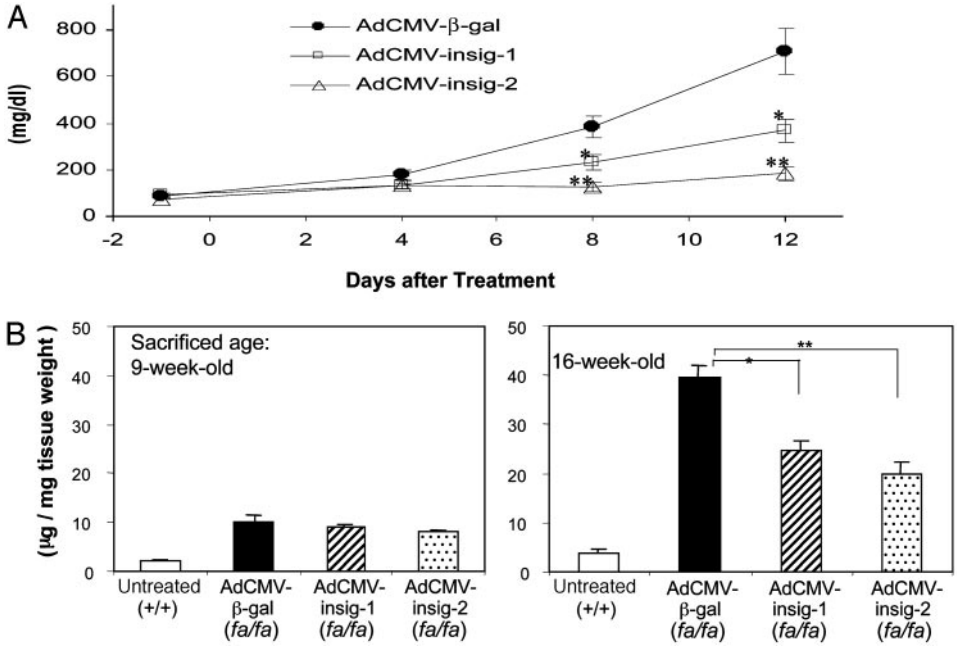

Fig. 3. Overexpression of insig-1 or insig-2 prevents increase of plasma TG and TG accumulation in liver of ZDF (*fa/fa*) rats. (*A*) Plasma TG levels in 7-week-old ZDF (*fa/fa*) rats injected with 5×10^{11} pfu of AdCMV-β-gal, AdCMV-insig-1, or AdCMV-insig-2. After a 4-h fast, blood was collected before and after viral injection Values represent mean ± SE for six animals in each group. *, $P \leq 0.05$; **, $P \leq 0.01$. (*B*) TG content in liver of 7- or 13-week-old ZDF (*fa/fa*) rats injected with 5×10^{11} or 1×10^{12} pfu, respectively, of AdCMV-β-gal, AdCMV-insig-1, or AdCMV-insig-2. TG content in liver was measured 2 weeks (in 7-week-old rats) or 3 weeks (in 13-week-old rats) after treatment as described. Values represent mean ± SE for five animals per group of ZDF (*fa/fa*) rats. *, $P \leq 0.005$; **, $P \leq 0.0005$. TG content of four lean, wild-type ZDF (+/+) rats was measured to indicate the normal value.

Preparation of Cell Total Extracts, Liver Total Extracts, and Nuclear Extracts. HEK 293 cells and livers from ZDF (*fa/fa*) rats were homogenized in Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA) containing 1 mM phenylmethylsulfonyl fluoride and then centrifuged at 10,000 × *g* for 20 min at 4°C to obtain supernatant for use as cell total extracts and liver total extracts. Nuclear extracts of ZDF (*fa/fa*) rat liver were prepared by a slight modification of the method of Sheng *et al*. (18). Liver was excised, rinsed in cold PBS, and suspended in 30 ml of buffer A (10 mM Hepes, pH 7.6/25 mM KCl/1 mM sodium EDTA/2 M sucrose/10% (vol/vol) glycerol/0.15 mM spermine/2 mM spermidine) supplemented with protease inhibitors (N-acetylleucylleucylnorleucinal at 50 μg/ml, 0.1 mM Pefabloc, pepstatin A at 5 μg/ml, leupeptin at 10 μg/ml, aprotinin at 2 μg/ml). The liver was homogenized briefly by using a Polytron. The homogenate (25 ml) was layered over 10 ml of buffer A, and the sample was centrifuged at 24,000 rpm for 1 h at 4°C by using a Beckman SW28 rotor. The resulting nuclear pellet was resuspended in 1 ml of buffer containing 10 mM Hepes at pH 7.6, 100 mM KCl, 2 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM DTT, and 10% (vol/vol) glycerol supplemented with protein inhibitors, after which 0.1 vol of 4 M ammonium sulfate (pH 7.9) was added. The mixture was agitated gently for 40 min at 4°C and then centrifuged at 55,000 rpm for 1 h at 4°C by using a Sorval RP100AT4 rotor and the supernatants used as nuclear extracts. Protein concentration of the extracts was determined by using the Bio-Rad Protein Assay kit.

Immunoblot Analysis. Aliquots of HEK 293 cell total extracts (30 μg), total liver extracts (30 μg), and nuclear extracts (50 μg) were subjected to SDS/PAGE on a 12%, 12%, or 7.5% gel, respectively. Immunoblot analysis was carried out with the ECL plus Western Blotting Detection System (Amersham Pharmacia Biosciences) according to the manufacturer's instructions. The following primary antibodies were used: mouse anti-V5 monoclonal antibody conjugated with horseradish peroxidase (Invitrogen) and rabbit anti-SREBP-1 (H-160, Santa Cruz Biotechnology). The anti-SREBP-1 antibody was visualized with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Pharmacia).

TG Content of Liver. Total lipids were extracted from ≈50 mg of liver by the method of Folch *et al*. (19). TG content of liver was measured by using the L-type TG H triglyceride kit (Wako).

Quantitative Real-Time PCR. Total RNA was extracted by the TRIzol isolation method according to the manufacturer's protocol (Life Technologies, Grand Island, NY). Total RNA was treated with DNase I (DNA-free; Ambion, Austin, TX), and first-strand cDNA was synthesized by using random hexamers. The real-time PCR contained in a final volume of 10 μl, 10 ng of reverse-transcribed cDNA, 900 nM forward and reverse primers, 250 nM of probe (Table 1), and 2× TaqMan PCR master mix (Applied Biosystems). We used 2× SYBR Green PCR Master Mix (Applied Biosystems) for insig-2a and -2b mRNA estimation. PCR reactions were carried out in 384-well plates by using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The relative amount of mRNA was calculated by comparative cycle time determination by using the standard curve method (20). 36B4 mRNA was used as the invariant control for all studies.

Fasting/Refeeding Experiments. Thirteen-week-old ZDF wild-type (+/+) rats were injected with 1×10^{12} pfu of AdCMV-β-gal, AdCMV-insig-1, or AdCMV-insig-2. Six days later, rats were divided into three groups: nonfasted, fasted, and refed. The nonfasted group was fed ad libitum, the fasted group was fasted for 15 h, and the refed group was fasted for 15 h and then refed a high carbohydrate/low fat diet (catalog no. 49918; Test Diet, Richmond, IN) for 7 h.

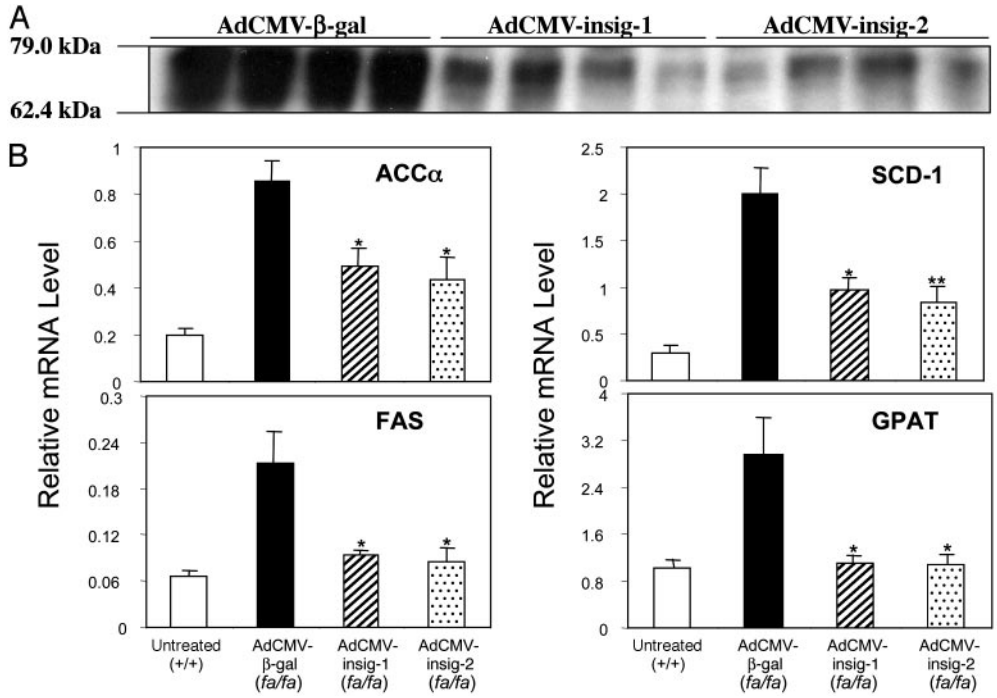

Fig. 4. Overexpression of insig-1 or insig-2 reduces nuclear SREBP-1c protein and lipogenic enzyme mRNA in liver of ZDF (*fa/fa*) rats. (*A*) Nuclear extracts from the livers of 7-week-old ZDF (*fa/fa*) rats that received injection with 5×10^{11} pfu of AdCMV-β-gal, AdCMV-insig-1, or AdCMV-insig-2 were prepared at 7 days after viral infection. Immunoblot analysis was carried out with rabbit anti-SREBP-1 polyclonal antibody. (*B*) Seven-week-old ZDF (*fa/fa*) rats were injected with 5×10^{11} pfu of AdCMV-β-gal, AdCMV-insig-1, or AdCMV-insig-2. Total RNA was isolated from the livers of age-matched untreated ZDF wild-type (+/+) rats and ZDF (*fa/fa*) rats 7 days after viral infection. mRNA levels of ACCα, FAS, SCD-1, and GPAT were measured by quantitative real-time PCR as described. The relative amount of mRNA was calculated by comparative cycle time determination by using the standard curve method. 36B4 was used as the invariant control. Values represent mean ± SE for five animals in a group of ZDF (*fa/fa*) rats and four animals in a group of ZDF wild-type (+/+) rats. *, $P \leq 0.05$; **, $P \leq 0.01$.

Statistical Analysis. All values shown are expressed as mean ± SE. Statistical analysis was performed by two-tailed unpaired Student's *t* test.

Results

Adenoviral Transfer of Insig-1 and -2 Genes. To establish that the insig-1 and -2 genes could be transferred by infection with recombinant adenovirus containing their cDNAs (AdCMV-insig-1 or -2), we first infected HEK 293 cells. Immunodetection of the V5 epitope revealed that both insig-1 and -2 proteins were present in the infected cells at their predicted sizes. Insig-1 protein levels were lower than those of insig-2 (Fig. 1*A*). It should be noted that the adenoviral vector expresses the mouse insig-2 protein common to both the insig-2a and insig-2b mRNA isoforms (15). Therefore, throughout this report, the exogenous protein will be referred to as insig-2, and the endogenous mRNAs as insig-2a and insig-2b. To determine whether *in vivo* infection would also result in expression of insig protein, we injected intravenously 5×10^{11} pfu of AdCMV-insig-1 or -2 into 7-week-old ZDF (*fa/fa*) rats and measured their protein in liver by immunoblot analysis. Both were expressed in liver, although, again, insig-1 protein was far less abundant than insig-2 (Fig. 1*B*).

Effect of Hepatic Insig-1 and -2 Overexpression on Food Intake, Body Weight, and Wet Weight of Epididymal Fat Pads. To assess the consequences of treatment of the ZDF (*fa/fa*) rats with AdCMV-insig-1 and -2 on energy balance we measured food intake, body weight, and wet weight of epididymal fat pads 14 days later. There were no differences in any of these parameters between AdCMV-insig-treated and control ZDF (*fa/fa*) rats treated with AdCMV-β-gal (Fig. 2). A transient decrease in food intake immediately after viral injection was exhibited by all treated rats.

Effect of Hepatic Insig-1 and -2 Overexpression on Plasma TG and Liver. Before treatment, plasma TG levels were virtually identical in the three groups, but 12 days later TG levels in the controls had risen to 707 ± 100 mg/dl, compared with 369 ± 48 mg/dl and 183 ± 32 mg/dl in the insig-1 and -2 overexpressing rats, respectively (Fig. 3*A*). These differences were significant ($P < 0.05$; $P < 0.005$). All obese ZDF (*fa/fa*) rats had steatotic livers at 9 weeks of age. At 16 weeks of age, TG content averaged 40 ± 2 μg/mg of wet weight in the AdCMV-β-gal-treated ZDF (*fa/fa*) rats, compared with 4 ± 1 μg/mg of wet weight in the lean, wild-type ZDF (+/+) rats. In insig-1 and -2 overexpressing rats, it averaged only 25 ± 2 and 20 ± 3 μg/mg of wet weight, respectively (Fig. 3*B*). The 4-fold increase in hepatic TG that occurred in the AdCMV-β-gal-treated control rats between 9 and 16 weeks of age (Fig. 3*B*) had been markedly reduced by overexpression of an insig gene. Thus, in ZDF (*fa/fa*) rats that overexpress an insig protein in their liver, the hyperlipidemia and hepatic steatosis increase at a much slower rate than in untreated ZDF (*fa/fa*) rats.

Effect of Hepatic Insig-1 and -2 Overexpression on Nuclear SREBP-1c. If the mechanism of the apparent antilipogenic action of insig-1 and -2 overexpression is, as determined by Yang *et al*. (3), the result of reduced proteolytic processing of SREBP, nuclear SREBP-1c levels in livers of AdCMV-insig-1 and -2-treated rats should be reduced. We therefore compared nuclear SREBP-1c levels in livers of AdCMV-β-gal-treated rats with those of the two insig-treated groups (Fig. 4*A*). The antibody detects both SREBP-1a and -1c, but SREBP-1c is the major form present in liver (21). A substantial decrease was observed in the livers of both groups of the insig-overexpressing rats, confirming the work of Yang *et al*. (3).

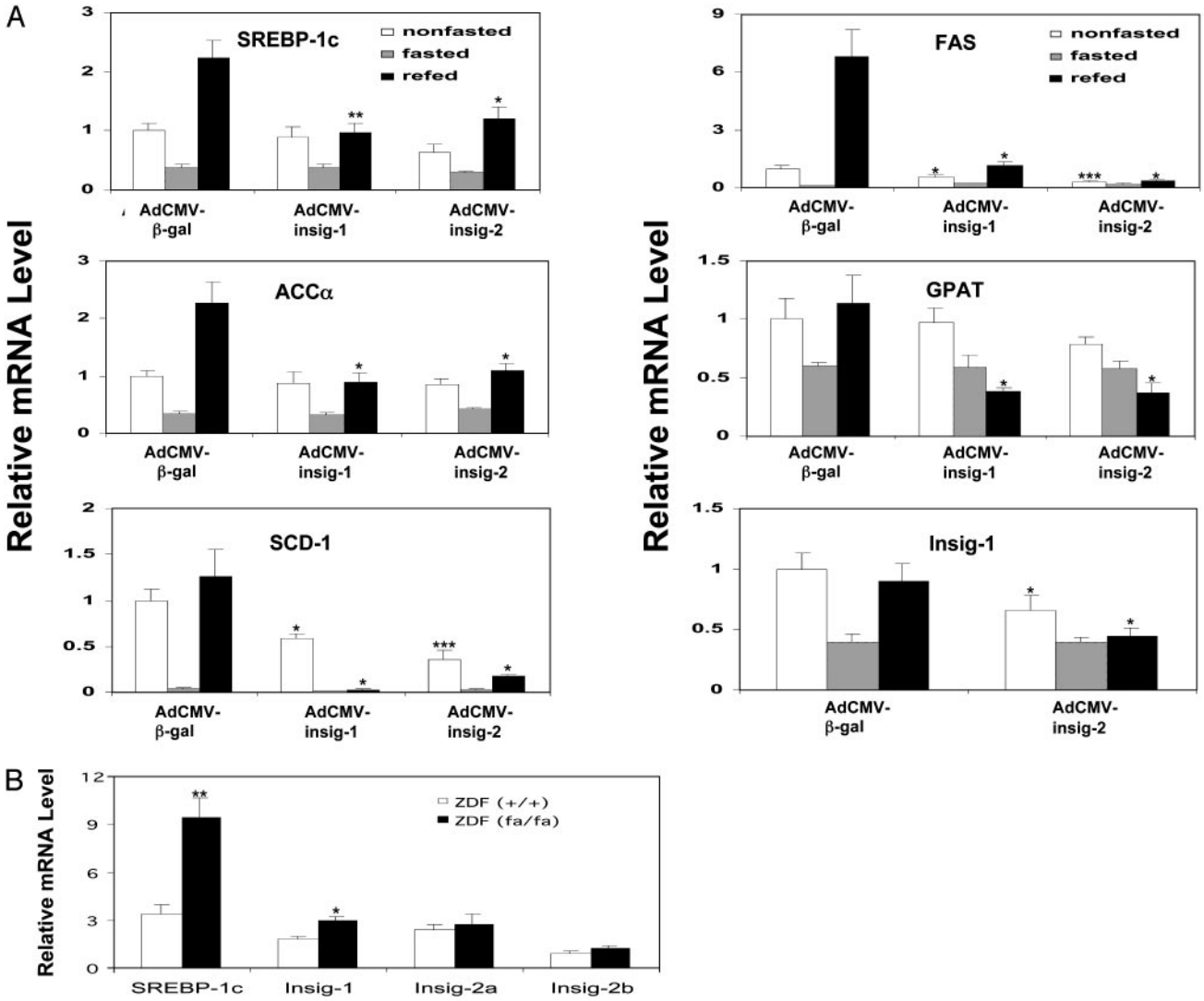

Fig. 5. (*A*) Hepatic response to fasting/refeeding by overexpression of insig-1 or insig-2. Thirteen-week-old ZDF wild-type (+/+) rats were injected with 1×10^{12} pfu of AdCMV-β-gal, AdCMV-insig-1, or AdCMV-insig-2. They were subjected to fasting and refeeding 6 days after viral infection as described in *Materials and Methods*. Total RNA from livers was isolated and subjected to quantitative real-time PCR as described. Each value represents the amount of mRNA relative to that in the nonfasted rats injected with AdCMV-β-gal, which is arbitrarily defined as 1. Data are mean ± SE for four animals in each group. Asterisks denote the level of statistical significance between the AdCMV-β-gal-treated and AdCMV-insig-1-treated or -2-treated groups. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$. (*B*) Comparison of endogenous SREBP-1c and insig-1, -2a, and -2b mRNA in lean, wild-type ZDF (+/+) rats and obese ZDF (*fa*/*fa*) rats. Total RNA from livers of 7-week-old ZDF wild-type (+/+) rats and ZDF (*fa*/*fa*) rats was isolated and subjected to quantitative real-time PCR as described. The relative amount of mRNA was calculated by comparative cycle time determination by using the standard curve method. 36B4 was used as the invariant control. Values represent mean ± SE for four animals in a group of ZDF wild-type (+/+) rats and six animals in a group of ZDF (*fa*/*fa*) rats. *, $P \leq 0.05$; **, $P \leq 0.001$.

Effect of Hepatic Insig-1 and -2 Overexpression on the Expression of Lipogenic Enzymes. To determine the mechanism of the antilipogenic effect of insig overexpression, we compared the expression of lipogenic target enzymes of SREBP-1c, acetyl-CoA carboxylase (ACC) α, fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD)-1, and glycerophosphate acyl transferase (GPAT) in β-gal and insig-overexpressing livers. In the β-gal group, the mRNA of all four enzymes averaged at least 2.9 times those of the wild-type ZDF (+/+) controls (Fig. 4*B*). The expression of all four enzymes was lower in insig-1 and -2 overexpressing rats ($P < 0.05$; $P < 0.01$), and FAS and GPAT mRNAs were as low as in normals. Thus, the reduction in nuclear SREBP-1c was associated with a dramatic reduction in the expression of the target enzymes responsible for the steatosis.

Effect of Hepatic Insig-1 or -2 Overexpression in Fasted and Refed Normal Rats. The foregoing results indicate that insig-1 and -2 overexpression in the liver can reduce the steatosis and hypertriglyceridemia in congenitally obese ZDF (*fa*/*fa*) rats by down-regulating the lipogenic enzymes that are up-regulated when the processed nuclear SREBP-1c enters the nucleus. Horton *et al.* (22) have reported that these enzymes are increased in livers of fasted/refed mice by means of changes in SREBP-1c, whereas in SREBP-1c$^{-/-}$ mice the lipogenic target enzymes of SREBP-1c are reduced (23). In SCAP-deficient mice, the response of target enzymes to refeeding is profoundly reduced or completely blocked (24) because none of the SREBP isoforms can reach their target genes within the nucleus. Based on the findings of Liang *et al.* (23), one would predict that overexpression of the insigs in liver would, by binding SCAP and preventing the processing of the SREBPs, also reduce the response of lipogenic enzymes to refeeding.

In normal rats treated with AdCMV-β-gal, fasting and refeeding had the expected effects on SREBP-1c mRNA and its target enzymes; the suppression by fasting and the brisk rebound with

feeding confirmed the findings of Horton *et al*. (22) (Fig. 5*A*). However, when rats were treated with AdCMV-insig-1 or -2, the rebound in SREBP-1c, ACCα, GPAT, FAS, and SCD-1 was reduced or completely suppressed (Fig. 5*A*). Perhaps the insigs reduced SREBP-1c expression by blocking a feed-forward effect on its own expression (25). Interestingly, AdCMV-insig-2 treatment prevented a rebound in endogenous insig-1 mRNA that otherwise occurs with refeeding; because SREBP-1c can stimulate insig-1 expression (6), we speculate that, by lowering SREBP-1c activity, the overexpression of insig-2 blocked the rise in endogenous insig-1.

Comparison of Endogenous Insig-1, -2a, and -2b mRNA in Lean, Wild-Type ZDF (+/+) and Obese ZDF (*fa*/*fa*) rats. In lean, wild-type ZDF (+/+) rats injected with AdCMV-β-gal, insig-1 declined with fasting and returned with refeeding (Fig. 5*A*). In addition, insig-1, but not -2a and -2b, was significantly higher in the obese steatotic rats than in lean controls (Fig. 5*B*). However, the increase in SREBP-1c mRNA in the livers of the obese ZDF (*fa*/*fa*) rats was approximately three times that of the ZDF (+/+) controls, whereas the increase in insig-1 was only $\approx$50% above the controls. This finding suggests that the higher expression of insig-1 was not enough to block the effect of the proportionally much higher expression of SREBP-1c on its target enzymes, although it might have limited it.

Discussion

This study indicates that the antilipogenic effects of the insigs demonstrated previously *in vitro* in preadipocytes also occur *in vivo* in the steatotic livers of obese ZDF (*fa*/*fa*) rats. Overexpression of insig-1 and -2 reduced significantly hepatic steatosis and hypertriglyceridemia. The mechanism of this effect on lipogenesis seems to be precisely that postulated by Yang *et al*. (3) for cholesterogenesis, i.e., a block in the processing of SREBP in the Golgi. This action results from the binding of SCAP in the endoplasmic reticulum, making it unavailable to escort inactive SREBP to the Golgi, where proteolytic cleavage converts it to an active form that enters the nucleus to induce its target genes (3). Indeed, the phenotype of the SCAP-deficient mice of Matsuda *et al*. (24) is qualitatively similar to that of the insig-overexpressing rats reported here.

The results of this study demonstrate that increased expression of insig-1 and -2 can reduce the abnormally high TG levels in the liver and plasma of ZDF (*fa*/*fa*) rats (Fig. 3). These rats exhibit generalized steatosis as the result of increased expression of SREBP-1c and its target enzymes (7, 8). The hepatic overexpression of insig proteins reduced the expression of the four lipogenic enzymes to a level comparable to that seen in wild-type controls (Fig. 4*B*). It is of interest that the endogenous level of insig-1 mRNA in ZDF (*fa*/*fa*) rats is significantly above that of wild-type controls (Fig. 5*B*) but clearly is not sufficient to reduce the increased activity of the up-regulated SREBP-1c on its elevated target enzymes. Insig-2a and -2b were not increased above the controls (Fig. 5*B*).

Thus, there was no evidence for a protective role of the insigs in this form of pathological hyperlipogenesis. However, it can be imagined that, in the absence of the insigs, the steatosis would be far more severe. As postulated for adipocytes, the insigs may limit the increased hepatic lipogenesis when it becomes excessive.

In contrast to their antilipogenic effects in obese ZDF (*fa*/*fa*) rats, overexpression of insig-1 and -2 in normal rats had very little effect on lipogenic enzyme expression in the nonfasted or fasted states when SREBP-1c mRNA was relatively low. Only after refeeding, which dramatically increases SREBP-1c mRNA, did the insig transgenes influence lipogenic enzyme expression (Fig. 5*A*). This result indicates that these proteins are most effective when SREBP activity is increased, whether in response to physiological stimuli, such as refeeding, or because of pathological steatosis.

Note Added in Proof. Overexpression of insig-1 in livers of transgenic mice also reduces insulin-stimulated lipogenesis (26).

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