



Supporting Online Material for

FGF19 as a Postprandial, Insulin-Independent Activator of Hepatic Protein and Glycogen Synthesis

Serkan Kir, Sara A. Beddow, Varman T. Samuel, Paul Miller, Stephen F. Previs, Kelly Suino-Powell, H. Eric Xu, Gerald I. Shulman, Steven A. Kliewer, David J. Mangelsdorf

*To whom correspondence should be addressed. E-mail:
davo.mango@utsouthwestern.edu (D.J.M.); steven.kliewer@utsouthwestern.edu (S.A.K.)

Published 25 March 2011, *Science* **331**, 1621 (2011)
DOI: 10.1126/science.1198363

This PDF file includes

Materials and Methods
Figs. S1 to S6
References

Supporting Online Materials for

**FGF19 as a postprandial, insulin-independent activator of
hepatic protein and glycogen synthesis**

Serkan Kir, Sara A. Beddow, Varman T. Samuel, Paul Miller, Stephen F. Previs, Kelly
Suino-Powell, H. Eric Xu, Gerald I. Shulman, Steven A. Kliewer*, David J.

Mangelsdorf*

*To whom correspondence should be addressed. E-mail:

steven.kliewer@utsouthwestern.edu or davo.mango@utsouthwestern.edu

Materials and Methods

Cell culture and reagents

HepG2 cells were maintained in MEM containing 10% FBS and penicillin/streptomycin. In experiments where cells were starved of amino acids, they were plated on collagen-coated plates to prevent detachment. All of the antibodies were purchased from Cell Signaling, except phospho-Ser³⁸⁰-p90RSK (Perseus proteomics), total GSK3 α/β (Invitrogen), and phospho-GS Ser⁶⁴¹/Ser⁶⁴⁵ (Invitrogen). Wortmannin, rapamycin and U0126 were also purchased from Cell Signaling. BI-D1870 was purchased from the University of Dundee, Division of Signal Transduction Therapy. Recombinant FGF19 was prepared as described (1).

Mouse Animal experiments

All animal experiments were approved by the Institutional Animal Care and Research Advisory Committee of the University of Texas Southwestern Medical Center and Yale University. Unless otherwise noted, mice were 6-8 weeks old males, wild type pure C57BL6 and were housed in a pathogen-free and a temperature-controlled environment with 12 hour light/dark cycles (6 am-6 pm) and fed standard irradiated rodent chow. *Fgf15*^{-/-} male mice and their matched wild-type controls were maintained in C57BL6/129 mixed strain background and used at 8-12 weeks of age (2). Since the use of recombinant FGF15 is limited by its decreased stability and variable bioactivity, the human ortholog, FGF19, was used for these studies. FGF19 protein was administered in a buffer (i.e., vehicle) containing PBS and up to 4% glycerol. Details of each experiment are described in figure legends. Vena cava and tail blood were collected and

transferred into EDTA-dipotassium tubes (Sarstedt) and centrifuged at 3000 rpm at 4 °C for 30 min, and total plasma albumin levels were measured using a Vitros 250 automated analyzer.

For oral glucose tolerance tests, overnight fasted animals were injected i.p. with vehicle or 1 mg/kg FGF19. 5 min. later animals were gavaged with 2 g glucose/kg body weight. Tail blood was taken at t = 0, 15, 30, 60, 90, 120 and 180 min and plasma glucose levels were measured and expressed as % basal (t=0) level.

Plasma glucose and glucagon, and insulin levels were measured with kits from Wako and Crystal Chem Inc. respectively. Hepatic cholesterol and triglyceride concentrations were measured using kits from Roche as previously described (3) except that Triton X-100 was used in place of Triton X-114.

Western blotting

Frozen and crushed liver samples were homogenized in liver lysis buffer containing 10 mM Tris HCl (pH 7.5), 150 mM NaCl, 0.5% NP40, 10% glycerol, 5 mM EDTA, 50 mM NaF, 10 mM β -glycerol phosphate, 1 mM Na_3VO_4 1 mM PMSF and complete protease inhibitor cocktail. The homogenates were centrifuges at 13,000 rpm for 10 min and the supernatants were used as whole cell lysates. Cultured cells were lysed in cell lysis buffer containing 20 mM Tris HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 50 mM NaF, 10 mM β -glycerol phosphate, 1 mM Na_3VO_4 1 mM PMSF and complete protease inhibitor cocktail. Protein concentration was determined by Bio-Rad Bradford assay and 30 μg of proteins were used in each SDS-PAGE run. Nitrocellulose membrane was used for blotting. Primary antibody incubation was performed in TBS containing 0.05% Tween and 5% BSA. For secondary antibody

incubation, TBS-T containing 5% milk was used. For visualization of the results, either SuperSignal West Pico or ECL western blotting substrates from Pierce were used. Quantification of the blots was performed by using ImageJ software. For each sample, integrated density of phospho-antibody band was divided by that of total antibody band and the values were normalized relative to the vehicle group.

Glycogen synthase activity assay

Frozen and crushed liver samples were homogenized in lysis buffer containing 50 mM Tris HCl (pH 7.5), 1% Triton X-100, 5 mM EDTA, 0.27M Sucrose, 50 mM NaF, 10 mM β -glycerol phosphate, 1 mM Na_3VO_4 . The homogenates were centrifuged at 13,000 rpm for 10 min. Protein concentration was determined by Bio-Rad Bradford assay. 300 μg of protein was used in each reaction and diluted with the lysis buffer to 50 μl volume. A reaction buffer which contains all the ingredients in the lysis buffer and 17.8 mM UDP-glucose (Sigma), 13.4 mg/ml rabbit liver glycogen (Sigma) and 0.07 μCi of ^{14}C -UDP-glucose (Perkin Elmer) per reaction was prepared and split into two tubes one of which is supplemented with 10mM glucose-6-phosphate (G6P) (Sigma). For each sample, two reactions (– and + G6P) in duplicates were set by adding 50 μl of reaction buffer into the diluted protein lysate making a final volume of 100 μl . Each sample was incubated at 30°C for 20 min. After the incubation, the samples were spotted onto Whatman Grade 3 circle filters and washed twice with 66% ethanol for 20 min at room temperature and once in acetone for 5 min. After drying, filters were put into scintillation vials with 10 ml of scintillation liquid and ^{14}C -glucose incorporation into glycogen was quantified. The glycogen synthase activity is defined as the activity measured in the absence of G6P divided by the activity measured in the presence of G6P.

Glycogen content determination

100 mg of liver samples were weighed and homogenized in 1 ml of 30% KOH and boiled for 15 min. After centrifugation at 3000 rpm, 75 μ l of homogenates were spotted on Whatman Grade 3 circle filters. Filters were washed once in 70% Ethanol at 4°C for 30 min and twice at room temperature for 15 min each. The filters were briefly rinsed with acetone. After drying, filters were placed in Fisher glass tubes and 1 ml of amyloglucosidase reaction mix which contains 2 mg of amyloglucosidase enzyme (Sigma) per 5 ml of 50 mM NaOAc was added. After incubation at 37°C for 90 min with periodic mixing, samples were used in Wako Autokit glucose assay to determine glucose concentration. The glycogen content results are calculated as mg glucose over 100 mg liver.

²H-Labeling of body water

The ²H-labeling of body water was determined by exchange with acetone (4, 5). Briefly, 10 μ l of sample or standard was reacted with 2 μ l of 10 N NaOH and 4 μ l of a 5% (v/v) solution of acetone in acetonitrile for 24 h. Acetone was extracted by addition of 600 μ l of chloroform followed by addition of ~ 0.5 g Na₂SO₄. Samples were vigorously mixed and a small aliquot of the chloroform was transferred to a GC-MS vial. Acetone was analyzed using an Agilent 5973N-MSD equipped with an Agilent 6890 GC system, a DB-17MS capillary column (30 m x 0.25 mm x 0.25 μ m) was used in all analyses. The temperature program was as follows: 60°C initial, increase by 20°C per min to 100°C, increase by 50°C per min to 220°C and hold for 1 min. The sample was injected at a split ratio of 40:1 with a helium flow of 1 ml per min. Acetone eluted at ~ 1.5 min. The mass

spectrometer was operated in the electron impact mode (70 eV). Selected ion monitoring of m/z 58 and 59 was performed using a dwell time of 10 ms per ion.

²H-Labeling of protein-bound alanine

The fractional rates of protein synthesis in liver and plasma albumin were determined from the incorporation of [²H]alanine using a precursor:product relationship (6, 7). Briefly, liver samples were homogenized in trichloroacetic acid (TCA, 0.1 g of tissue in 1000 µl of 10% TCA, w/v) and centrifuged for 10 min at 4000 rpm. The protein pellet was washed twice with 5% TCA and then hydrolyzed for 20 h in 1 ml of 6N HCl at 100°C. To determine rates of plasma albumin synthesis, ~ 200 µl of plasma was treated with 1 ml of 10% TCA (8). The protein pellet was washed twice with 5% TCA, albumin was then extracted from the pellet into 100% ethanol. Following the evaporation of ethanol, samples were hydrolyzed in 1 ml of 6N HCl at 100°C. An aliquot of a hydrolyzed protein sample was evaporated to dryness. The samples were then reacted to form the “methyl-8” derivative of alanine, made by mixing acetonitrile, methanol and “Methyl-8” reagent (Pierce, Rockford, IL; 1:2:3, v:v:v) and heating the sample at 75°C for 30 min (9). The sample was transferred to a GC-MS vial and analyzed using an Agilent 5973N-MSD equipped with an Agilent 6890 GC system. A DB17-MS capillary column (30 m x 0.25 mm x 0.25 µm) was used in all assays. The initial temperature program was set at 90°C and hold for 5 min, increased by 5°C per min to 130°C, increased by 40°C per min to 240°C and hold for 5 min, with a helium flow of 1 ml per min. Alanine elutes at ~ 12 min. The mass spectrometer was operated in the electron impact mode. Selective ion monitoring of m/z 99 and 100 (total ²H-labeling of alanine) was performed using a dwell time of 10 ms per ion.

Protein synthesis rate calculations

The rate of protein synthesis was calculated using the equation: ${}^2\text{H}$ -labeling protein-derived alanine (%) / [${}^2\text{H}$ -labeling body water (%) x 3.7 x time (h)], where the factor 3.7 represents an incomplete exchange of ${}^2\text{H}$ between body water and alanine, i.e. 3.7 of the 4 carbon-bound hydrogens of alanine exchange with water (6, 7). This equation assumes that the ${}^2\text{H}$ -labeling in body water equilibrates with free alanine more rapidly than alanine is incorporated into newly made protein and that protein synthesis is linear over the study (10). In cases where the mice were exposed to tracer for ~6h, we assumed a steady-state labeling of body water at a value equal to that measured at the end of the study. In cases where the mice were exposed to tracer for longer periods of time (e.g., overnight), we calculated the average water labeling determined using samples collected ~90 min post-injection and at the end of the study.

RT-qPCR

RNA was extracted from frozen liver samples using RNA-STAT60™ (Isotex diagnostics), DNase treated, and reverse transcribed using random hexamers. Resulting cDNA was analyzed by RT-qPCR. Briefly, 25 ng of cDNA and 150 nmol of each primer were mixed with SYBR® GreenER™ PCR Master Mix (Invitrogen). Reactions were performed in triplicates in 384-well format using an ABI PRISM® 7900HT instrument (Applied Biosystems). Relative mRNA levels were calculated using the comparative CT method normalized to cyclophilin. The following primers were designed using Primer Express® Software (Applied Biosystems): CYP7A1: 5'-agcaactaacaacctgccagtacta-3'; 5'-gtccggatattcaaggatgca-3'; LXR α : 5'-aagcaggtgccagggttct-3'; 5'-tgcattctgtctcgtggtt-3';

SREBP1c: 5'-ggagccatggattgcacatt-3'; 5'-ggccccgggaagtcactgt-3'; ACC: 5'-ggacagactgatcgcagagaaag-3'; 5'-tggagagccccacacaca-3'; FAS: 5'-gctgcggaaacttcaggaaat-3'; 5'-agagacgtgtcactcctggactt-3'; SCD-1: 5'-tgcccctgcggatctt-3'; 5'-gcccattcgtacacgtcatt-3'.

Hyperglycemic-Basal Insulin clamp study

Harlan male Sprague-Dawley rats (350g) underwent placement of chronic jugular vein and carotid artery catheters. After recovery from surgery (5-7 days), rats were fasted overnight prior to experiments. Continuous infusions of insulin [1 mU/(kg-min)] and somatostatin [4 mcg/(kg-min)] were started at T=0 min. Animals were continuously infused with insulin and somatostatin to maintain low levels of insulin and glucagon and variably infused with glucose to maintain hyperglycemia. Animals were given either vehicle or FGF19. A second group of animals were not clamped and were kept fasted throughout the experiment. The FGF19 group received 540 µg/kg total recombinant FGF19 administered i.v. in two divided doses (at T=0 and 90 min). A variable infusion of 20% glucose enriched with 20% [U-¹³C] glucose was started at 5 min and adjusted to maintain a plasma glucose concentration of ~250 mg/dL for a total of 180 min. Under these conditions, glycogen phosphorylase activity is inhibited. At 180 min, rats were euthanized with an i.v. bolus of pentobarbital and livers were freeze-clamped *in situ* using brass plated tongs pre-cooled in liquid nitrogen. Liver samples were stored at -80 until further analysis.

Net glycogen synthesis was determined by assessing the glycogen content in the clamped animals subtracted by the glycogen content of similar unclamped rats euthanized with the same duration of fasting, using methods previously described (11). Incorporation of glucose_{M+6} into glycogen to determine the percent of glycogen synthesized by the

direct pathway was assessed by GC/MS, as previously described (12). Insulin and glucagon were assayed by R.I.A. (Bio-Rad).

Statistical analysis

Values are expressed as mean \pm SEM. Significant differences between two groups were evaluated using two-tailed, unpaired *t* test.

Supplementary Figure Legends

Fig. S1. Insulin and FGF19 induce alternate signaling pathways to regulate protein synthesis in liver. Mice fasted overnight were injected i.p. with PBS or 1 U/kg insulin or i.v. with vehicle or 1 mg/kg FGF19. Animals in the insulin treatment group were sacrificed 15 min after the injection whereas those in the FGF19 treatment group were sacrificed 1 hour after the injection. Protein samples were prepared from livers. Western blotting was performed with the indicated antibodies.

Fig. S2. (A) FGF19 activates the Ras-ERK-RSK pathway at doses as low as 3 ng/ml. HepG2 cells and rat H4IIE hepatoma cells were overnight serum-starved and treated with 3, 10, 30, 100, and 300 ng/ml FGF19 for 10 min. Proteins were identified by western blotting with the indicated antibodies. **(B)** FGF19 does not activate Akt or p70S6K kinases in HepG2 cells. Overnight serum-starved HepG2 cells were incubated in the absence of amino acids in HBSS media for 1 hour. DMSO, wortmannin (200 nM), rapamycin (20nM), U0126 (10 μ M) or BI-D1870 (10 μ M) were added for a further 1 hour treatment. The cells were then treated with vehicle or 250 ng/ml FGF19 and harvested after 30 minutes. Proteins were identified by western blotting with the indicated antibodies. Asterisk represents non-specific band. **(C)** Effects of wortmannin and rapamycin on insulin signaling in HepG2 cells. Overnight serum-starved HepG2 cells were starved for aminoacids in HBSS media for 1 hour. DMSO, wortmannin (200 nM), or rapamycin (20 nM), were added for a further 1 hour treatment. The cells were treated with vehicle or 100 nM insulin and harvested after 30 minutes. Proteins were identified by western blotting with the indicated antibodies.

Fig. S3. Effects of FGF19 on hepatic lipids and plasma hormones. Mice fed ad libitum were injected subcutaneously with vehicle or 1mg/kg FGF19 at 6 pm and the next morning at 8 am. 6 hours after the last injection, the animals were sacrificed, liver cholesterol (**A**) and triglyceride (**B**) were determined (n=6). Separately, mice fed ad libitum were injected subcutaneously with vehicle or 1 mg/kg FGF19 and tail blood was taken at t = 0, 15, 30, 60, 120, 240 and 360 min. Plasma insulin (**C**), glucagon (**D**), and FGF19 (**E**) were determined (n=5). Values are means \pm SEM analyzed by two-tailed *t* test. n.s., not significant.

Fig. S4. (**A**) Plasma glucose, (**B**) urine glucose and (**C**) water intake of the animals in the STZ experiment described in Fig 4 were measured. Water intake is the amount of water consumed within the last 20 hours of the experiment and is expressed as ml/g body weight. Note that these mice were severely diabetic and their urine contained almost 10% glucose. Although plasma glucose was lowered only slightly in the FGF19-treated mice, these mice exhibited a significant reduction in water intake that is diagnostic of decreased urinary glucose excretion. Thus, while FGF19-induced glycogen synthesis was unable to completely correct plasma glucose concentrations, it did have a significant effect on overall glucose disposal as demonstrated by the decrease in glucose excretion. Values are means \pm SEM. Statistics by two-tailed *t* test. ***, $P < 0.0005$ is between control and STZ-vehicle or STZ-FGF19 groups; #, $P < 0.05$ is between STZ-vehicle and STZ-FGF19 groups.

Fig. S5. Three-hour-hyperglycemic clamp study was performed on overnight-fasted rats treated with vehicle or FGF19. Animals were continuously infused with insulin and somatostatin to maintain low levels of insulin and glucagon and variably infused with

glucose to maintain hyperglycemia. Animals were given either vehicle or 540 $\mu\text{g}/\text{kg}$ FGF19 i.v. injections at time 0 and 90 minutes. (A–C) Plasma glucose, insulin, and glucagon levels remained at similar levels in vehicle and FGF19 treated rats. (D) Plasma FGF19 levels were determined using a human FGF19 ELISA. Arrowheads indicate times of FGF19 injection. (E) Although the rate of glycogen synthesis was increased in FGF19-treated rats (see Fig. 4D), the percent of glycogen synthesized by the direct pathway (as assessed by GC/MS) remained the same in vehicle and FGF19 treated animals. Note, direct pathway refers to glycogen synthesized directly from glucose, rather than indirectly from gluconeogenesis. Values are means \pm SEM analyzed by two-tailed *t* test. n.s., not significant.

Fig. S6. Effects of FGF19 on lipogenic gene expression. Overnight-fasted mice were injected subcutaneously with vehicle or 1 mg/kg FGF19 protein. Animals were sacrificed 6 hours after injections. RNA was extracted from liver and reverse transcribed for qPCR analysis. Values are means \pm SEM analyzed by two-tailed *t* test. n.s., not significant.

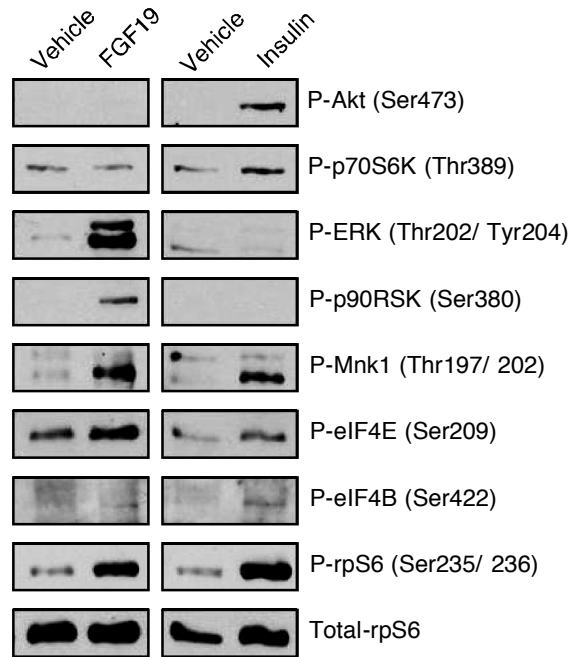


Fig. S1
Kir et al.

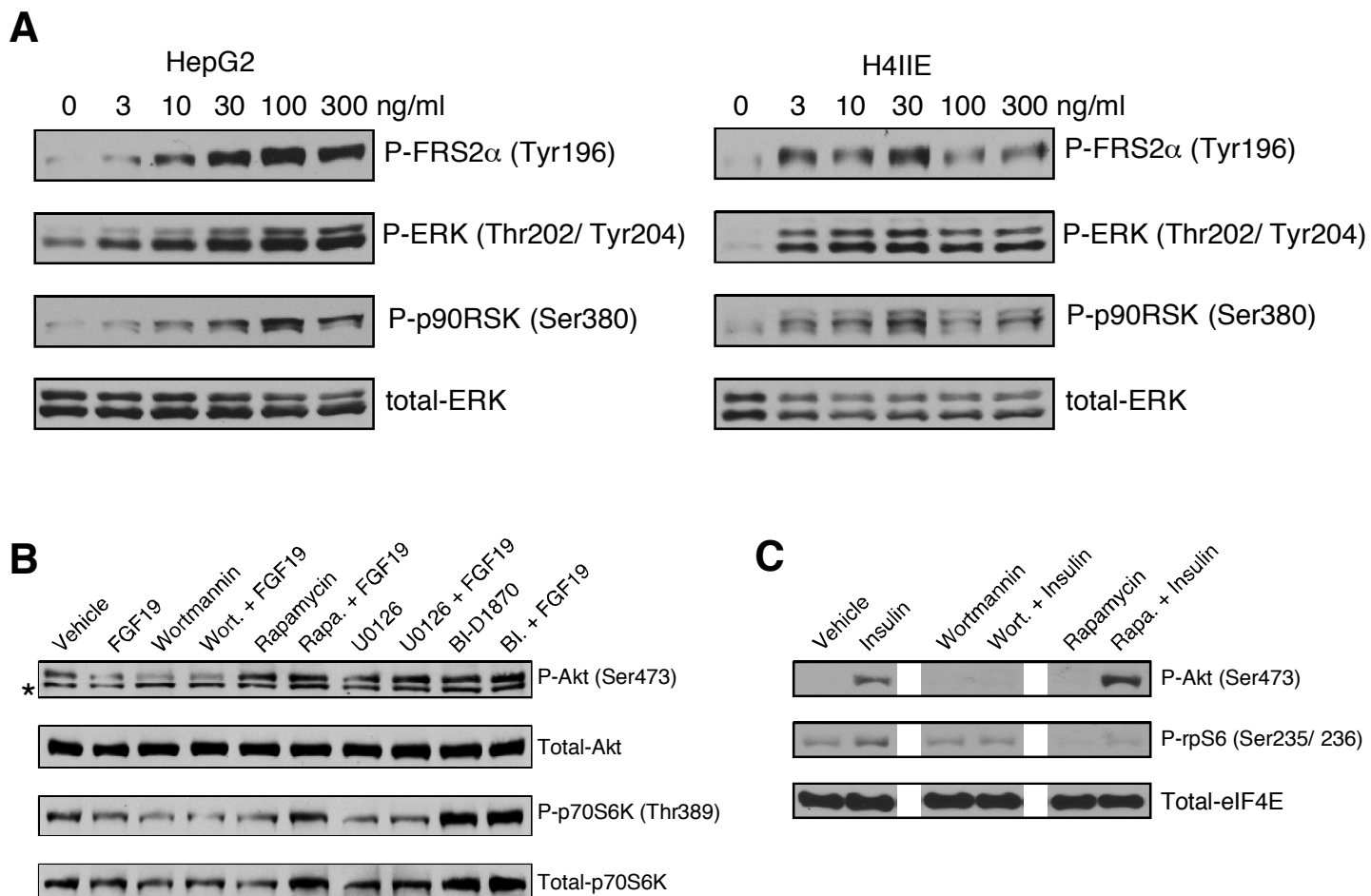


Fig. S2
Kir et al.

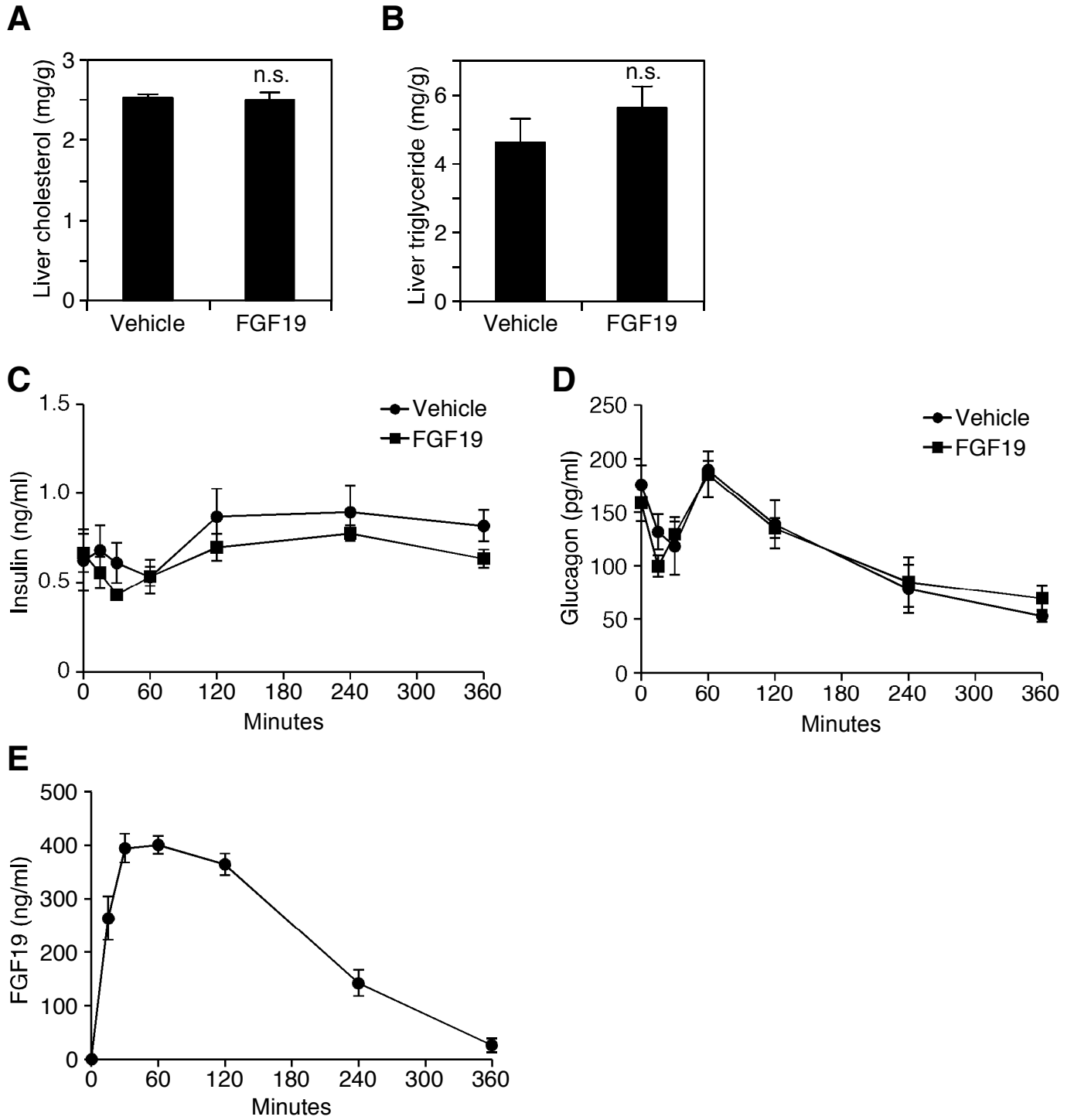


Fig. S3
Kir et al.

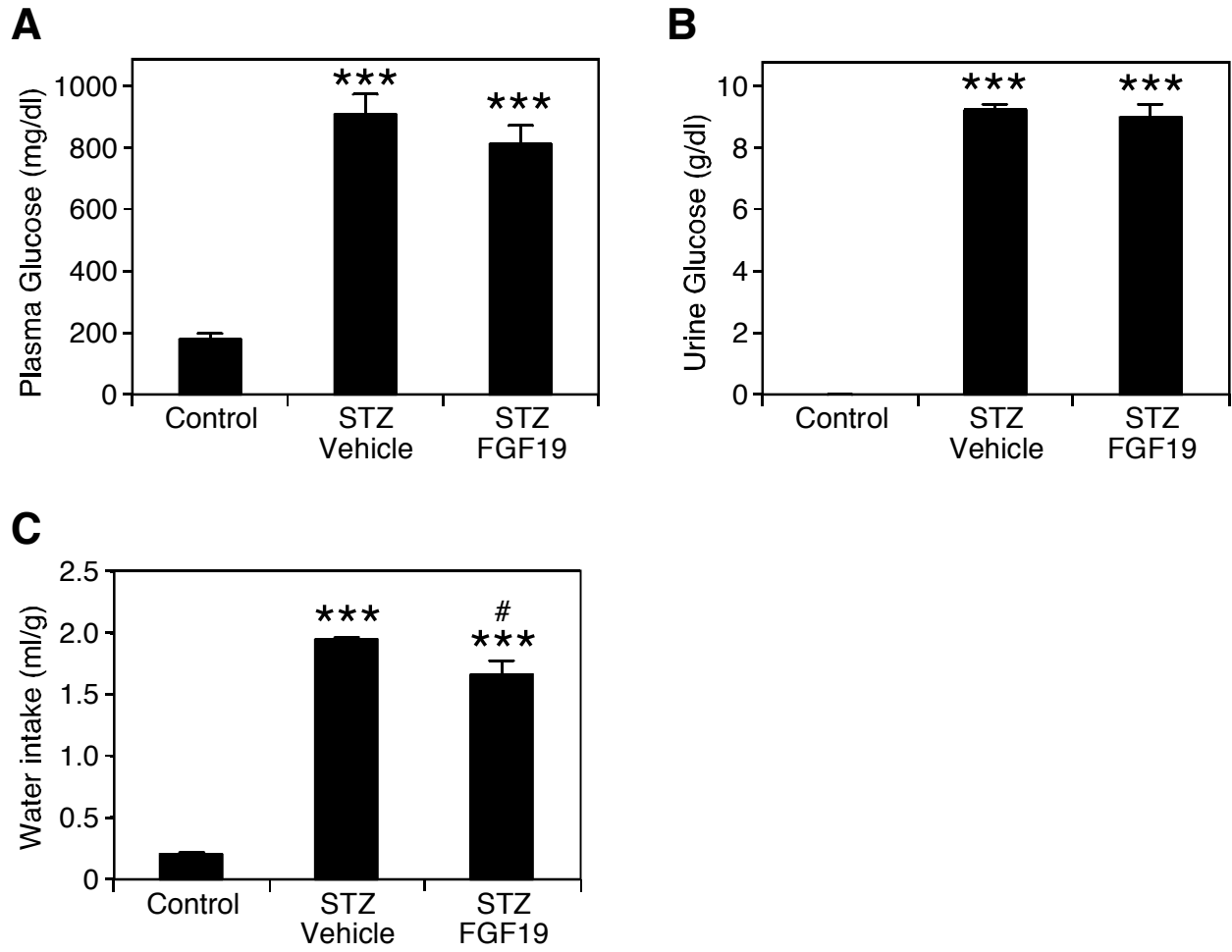


Fig. S4
Kir et al.

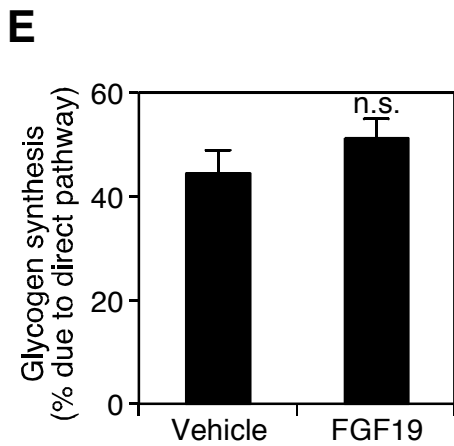
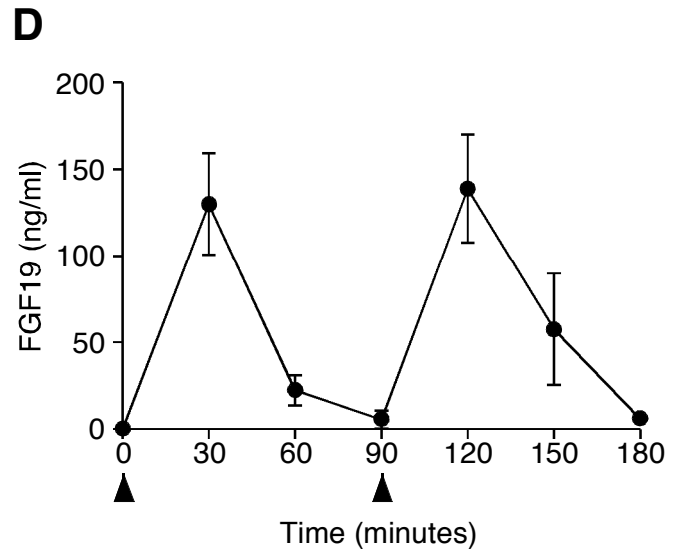
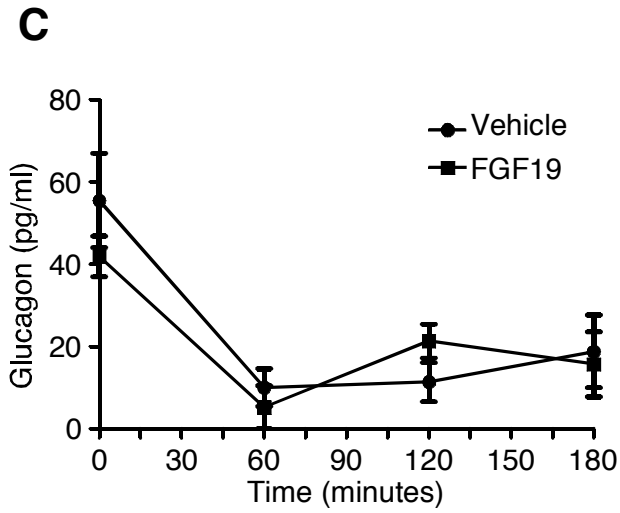
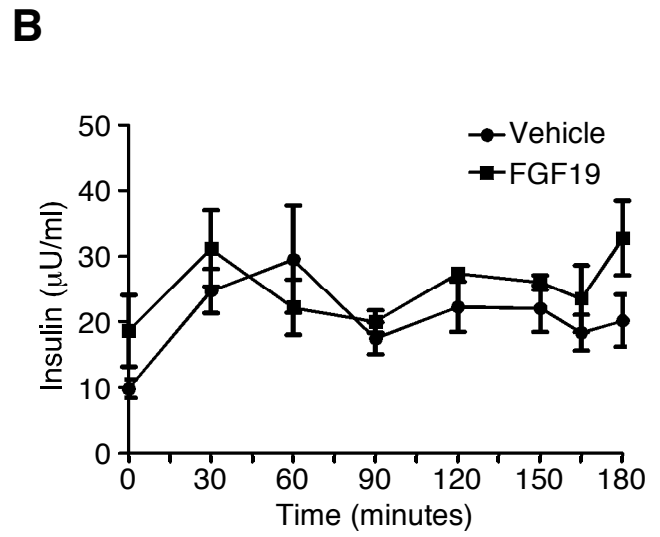
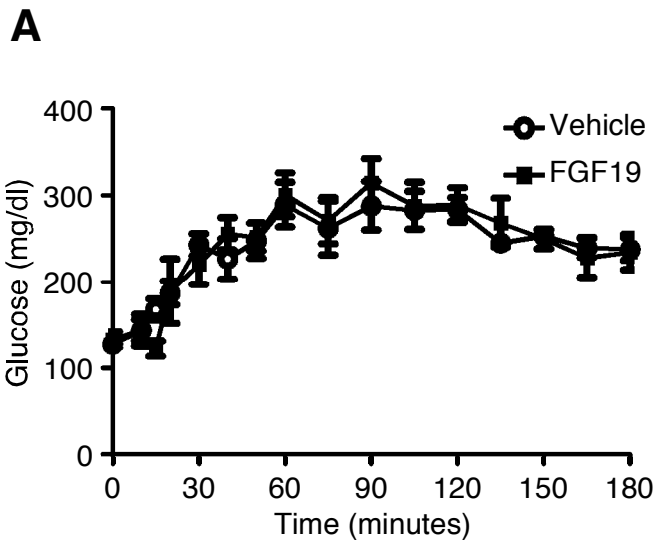


Fig. S5
Kir et al.

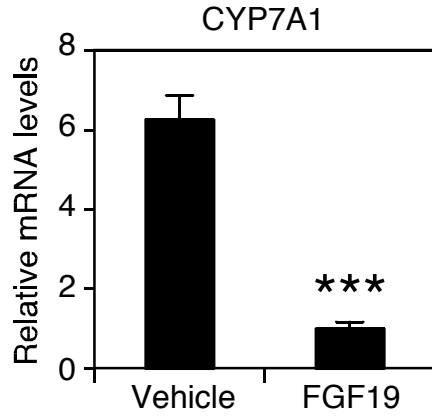
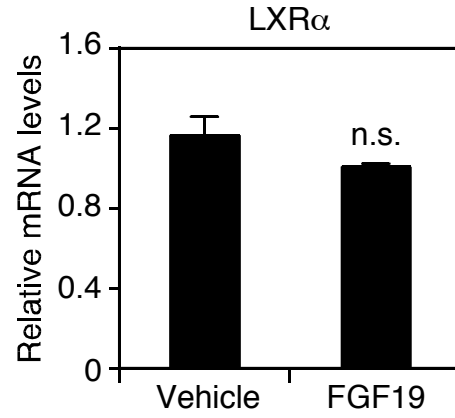
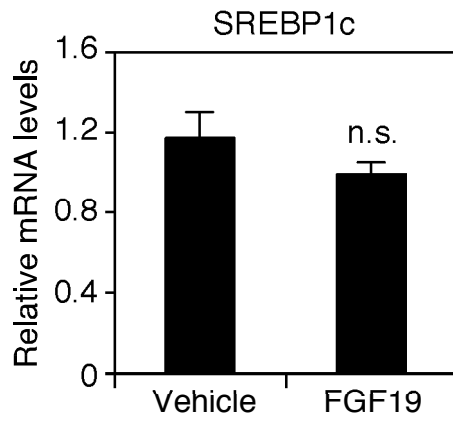
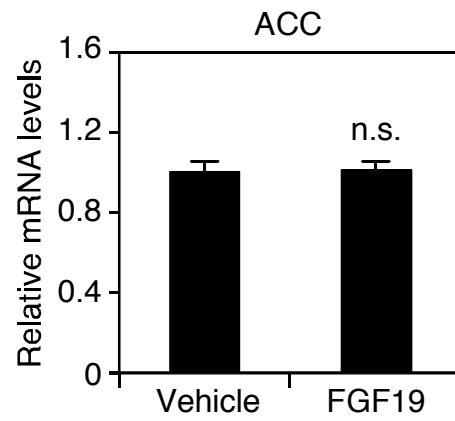
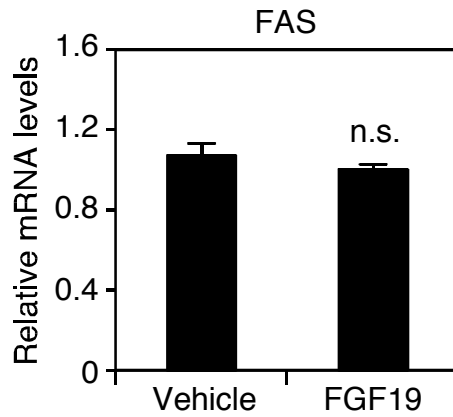
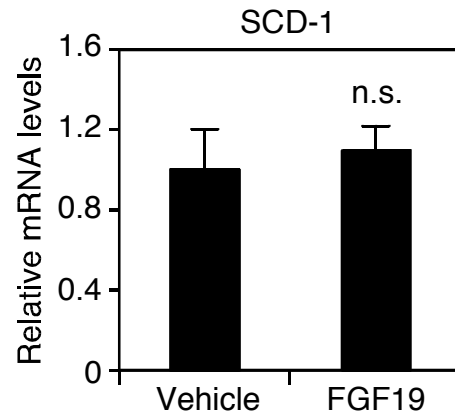
A**B****C****D****E****F**

Fig. S6
Kir et al.

References for Supporting Online Material

1. M. Choi *et al.*, Identification of a hormonal basis for gallbladder filling. *Nat Med* **12**, 1253-1255 (2006).
2. T. J. Wright *et al.*, Mouse FGF15 is the ortholog of human and chick FGF19, but is not uniquely required for otic induction. *Dev Biol* **269**, 264-275 (2004).
3. N. Y. Kalaany *et al.*, LXRs regulate the balance between fat storage and oxidation. *Cell Metab* **1**, 231-244 (2005).
4. B. J. McCabe *et al.*, Reproducibility of gas chromatography-mass spectrometry measurements of ²H labeling of water: application for measuring body composition in mice. *Anal Biochem* **350**, 171-176 (2006).
5. D. Yang *et al.*, Assay of low deuterium enrichment of water by isotopic exchange with [U-¹³C₃]acetone and gas chromatography-mass spectrometry. *Anal Biochem* **258**, 315-321 (1998).
6. S. R. Anderson, D. A. Gilge, A. L. Steiber, S. F. Previs, Diet-induced obesity alters protein synthesis: tissue-specific effects in fasted versus fed mice. *Metabolism* **57**, 347-354 (2008).
7. S. F. Previs *et al.*, Quantifying rates of protein synthesis in humans by use of ²H₂O: application to patients with end-stage renal disease. *Am J Physiol Endocrinol Metab* **286**, E665-672 (2004).
8. J. R. Debro, A. Korner, Solubility of albumin in alcohol after precipitation by trichloroacetic acid: a simplified procedure for separation of albumin. *Nature* **178**, 1067 (1956).
9. J. P. Thenot, E. C. Horning, Amino acid N-dimethylaminomethylene alkyl esters. New derivatives for gas chromatographic and gas chromatographic-mass spectrometric studies. *Analytical Letters* **5**, 519-529 (1972).
10. R. R. Wolfe, D. L. Chinkes, *Isotope Tracers in Metabolic Research: Principles and Practice of Kinetic Analyses* (Wiley-Liss, New York, 2004).
11. P. H. McNulty *et al.*, Glucose metabolism distal to a critical coronary stenosis in a canine model of low-flow myocardial ischemia. *J Clin Invest* **98**, 62-69 (1996).
12. V. T. Samuel *et al.*, Mechanism of hepatic insulin resistance in non-alcoholic fatty liver disease. *J Biol Chem* **279**, 32345-32353 (2004).