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RESEARCH ARTICLE | *Role of Gut Microbiota and Gut-Brain and Gut Liver Axes in Physiological Regulation of Inflammation, Energy Balance, and Metabolism*

Differential effects of a 40-hour fast and bile acid supplementation on human GLP-1 and FGF19 responses

F. Samuel van Nierop,[1]* Emma C. E. Meessen,[1]* Kyra G. M. Nelissen,[1] Roos Achterbergh,[2] Laureen A. Lammers,[3] Frédéric M. Vaz,[4] Ron A. A. Mathôt,[3] Heinz-Josef Klümpen,[5] Steven W. Olde Damink,[6,7] Frank G. Schaap,[6,7] Johannes A. Romijn,[2] E. Marleen Kemper,[3] and Maarten R. Soeters[1]

[1]*Department of Endocrinology and Metabolism, Amsterdam University Medical Centers, The Netherlands;* [2]*Department of Internal Medicine, Amsterdam University Medical Centers, The Netherlands;* [3]*Department of Hospital Pharmacy, Amsterdam University Medical Centers, The Netherlands;* [4]*Department of Clinical Chemistry, Amsterdam University Medical Centers, The Netherlands;* [5]*Department of Medical Oncology, Amsterdam University Medical Centers, The Netherlands;* [6]*Department of Surgery, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University, Maastricht, The Netherlands; and* [7]*Department of General, Visceral and Transplantation Surgery, RWTH University Hospital Aachen, Aachen, Germany*

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van Nierop FS, Meessen ECE, Nelissen KGM, Achterbergh R, Lammers LA, Vaz FM, Mathôt RAA, Klümpen HJ, Olde Damink SW, Schaap FG, Romijn JA, Kemper EM, Soeters MR. Differential effects of a 40-hour fast and bile acid supplementation on human GLP-1 and FGF19 responses. *Am J Physiol Endocrinol Metab* 317: E494–E502, 2019. First published June 25, 2019; doi:10.1152/ajpendo.00534.2018.—Bile acids, glucagon-like peptide-1 (GLP-1), and fibroblast growth factor 19 (FGF19) play an important role in postprandial metabolism. In this study, we investigated the postprandial bile acid response in plasma and its relation to insulin, GLP-1, and FGF19. First, we investigated the postprandial response to 40-h fast. Then we administered glycine-conjugated deoxycholic acid (gDCA) with the meal. We performed two separate observational randomized crossover studies on healthy, lean men. In *experiment 1*: we tested 4-h mixed meal after an overnight fast and a 40-h fast. In *experiment 2*, we tested a 4-h mixed meal test with and without gDCA supplementation. Both studies measured postprandial glucose, insulin, bile acids, GLP-1, and FGF19. In *experiment 1*, 40 h of fasting induced insulin resistance and increased postprandial GLP-1 and FGF19 concentrations. After an overnight fast, we observed strong correlations between postprandial insulin and gDCA levels at specific time points. In *experiment 2*, administration of gDCA increased GLP-1 levels and lowered late postprandial glucose without effect on FGF19. Energy expenditure was not affected by gDCA administration. Unexpectedly, 40 h of fasting increased both GLP-1 and FGF19, where the former appeared bile acid independent and the latter bile acid dependent. Second, a single dose of gDCA increased postprandial GLP-1. Therefore, our data add complexity to the physiological regulation of the enterokines GLP-1 and FGF19 by bile acids.

bile acids; FGF-19; GLP-1; type 2 diabetes; postprandial metabolism

INTRODUCTION

Bile acids (BA) are known for their role in hepatobiliary cholesterol secretion (37) and detergent properties that enable enteral lipid uptake (28). Recently, BAs have gained interest for their role as hormone-like mediators involved in energy metabolism (33). Their effects are mainly mediated by the nuclear Farnesoid X Receptor (FXR) and the transmembrane Takeda G-protein-coupled receptor (TGR5) (24, 33). BA dynamics in the enterohepatic and systemic circulation are responsive to nutrient intake, and BA receptors are ideally positioned in the digestive system to function as postprandial metabolic integrators by sensing and conveying information about nutrient status (2, 43).

The two main primary human BAs, cholic acid (CA) and chenodeoxycholic acid (CDCA), are synthesized from cholesterol in the hepatocyte, conjugated with either glycine or taurine, and subsequently stored in the gallbladder. After a meal, these BAs facilitate the absorption of lipids and lipid-soluble vitamins. The microbiota in the gut are involved in BA deconjugation and dehydroxylation, yielding the secondary BAs deoxycholic acid (DCA) and lithocholic acid from CA and CDCA, respectively. BA reabsorption occurs primarily through active transport in the ileum, and BAs reach the liver via the portal vein (26, 28, 44). A fraction of BAs escapes hepatic clearance and enters the systemic circulation in a pattern of postprandial peaks that may be high enough to activate BA receptors in the systemic circulation (9).

TGR5 is expressed by entero-endocrine L-cells in the intestine, and its activation by BAs results in the release of the incretin glucagon-like peptide 1 (GLP-1) (45). GLP-1, in turn, increases glucose-dependent insulin release from the pancreatic beta cells. BAs may also directly stimulate insulin release, as both FXR and TGR5 are expressed by the beta cell (25, 38). During enterohepatic BA cycling, FXR activation induces a negative feedback loop comprising ileal fibroblast growth factor 19 (FGF19) that represses hepatic BA production.

* F. S. van Nierop and E. C. E. Meessen contributed equally to this work.
Address for reprint requests and other correspondence: M. Soeters, K2-283, Dept. of Endocrinology & Metabolism, Internal Medicine, Amsterdam Univ. Medical Center, Meibergdreef 9, 1105AZ, Amsterdam, The Netherlands (e-mail: m.r.soeters@amsterdamumc.nl).

FGF19 has been suggested to inhibit gluconeogenesis and hepatic lipogenesis, whereas it stimulates glycogen synthesis (22, 36). In contrast, it was recently shown in rodents that FXR is an activator of fasting hepatic gluconeogenesis (35). In humans, administration of a FGF19 analog diminished liver fat without effects on plasma glucose levels (7, 18).

In this study, we investigated the postprandial BA response in plasma in relation to insulin, GLP-1, and FGF19 levels. First, we analyzed the effects of mixed meal tests after an overnight and a 40-h fast as model of insulin resistance, because BA metabolism is altered in various models of insulin resistance (5, 14, 47). We subsequently assessed the effects of oral administration of glycine-conjugated DCA (gDCA) on postprandial glucose, insulin, and GLP-1 levels in a second separate experiment.

MATERIALS AND METHODS

Subjects

We performed two experiments with different subjects. We recruited 9 lean young men in *experiment 1* and 10 lean young men in *experiment 2*. Exclusion criteria were body mass index (BMI) > 25 kg/m^2, history of gallstones or biliary surgery, use of medication, substance abuse (nicotine or drugs, alcohol >2 units/day), liver test abnormalities (aspartate aminotransferase, alanine aminotransferase, bilirubin, gamma-glutamyl transferase, alkaline phosphatase), or abnormal fasted levels of plasma glucose or insulin. Written informed consent was obtained from all subjects before the start of the study procedures, and the studies were approved by the Academic Medical Center (AMC) Medical Ethics Committee (Amsterdam, The Netherlands). The experiments were conducted in accordance with the principles of the Declaration of Helsinki (sixth revision, 2008). *Experiment 1* was filed at the ethical committee under ABR number NL4083401812. *Experiment 2* was prospectively registered at www.trialregister.nl (NTR5849).

Study Design

Both separate, but related, experiments were mono center and conducted on the Experimental and Clinical Research Unit of the AMC, Amsterdam, The Netherlands.

Experiment 1. Each subject ($n = 9$) had two mixed meal tests on separate study days in a randomly assigned crossover design. Prior to each study day, subjects were instructed to either consume their regular diet followed by an overnight fast leading up to the study day (14 h FAST) or remain fasted for a total of 40 h (40 h FAST).

Experiment 2. Each subject ($n = 10$) had two mixed meal tests after an overnight fast on separate study days in a randomly assigned crossover design. In balanced assignment, subjects underwent the control meal test (−gDCA) or received 750 mg of gDCA in capsules concomitantly with the standard meal (+gDCA). We administered a similar dose as used for ursodeoxycholic acid in earlier studies (11, 15). gDCA capsules were prepared for each subject individually under responsibility of the AMC hospital pharmacy.

Mixed Meal Test

For both experiments, subjects were admitted to the unit after an overnight or 40-h fast. The liquid test meal (Nutridrink Compact, Nutricia, Zoetermeer, The Netherlands) contained 16% protein, 35% fat, and 49% carbohydrates. Subjects ingested the caloric equivalent of 25% of their estimated daily energy expenditure calculated using the Harris-Benedict equation. The test meal was ingested at time point 0 ($t = 0$). In both experiments, blood was sampled from a catheter inserted in a forearm vein. In *experiment 1*, blood samples were obtained at 0, 30, 60, 90, 120, 180, and 240 min after meal ingestion and in *experiment 2* at −20, −10, 0, 15, 30, 45, 60, 75, 90, 120, 180, and 240 min. Samples were collected into chilled EDTA or heparin-coated tubes on ice and immediately centrifuged and subsequently stored at −20°C until analysis. For GLP-1 samples, a dipeptidyl peptidase inhibitor (Ile-Pro-Ile, Sigma-Aldrich, St. Louis, MO) was added to the collection tube at 0.01 mg/mL, and plasma was stored at −80°C.

Laboratory Analysis

Plasma glucose concentrations were analyzed bedside using the glucose oxidation method (EKF Diagnostics, Barleben/Magdeburg, Germany). Insulin was determined on an Immulite 2000 system (Siemens Healthcare Diagnostics, Breda, The Netherlands). Active GLP-1 concentrations were measured by ELISA (EMD Millipore, Billerica, MA). FGF19 was measured at the University of Maastricht using an in-house developed ELISA as published previously (40).

Bile Acid Analysis

Taurine- and glycine-conjugated internal standards ([2,2,4,4-$^2H_4$]taurocholic acid (tauro-CA), [2,2,4,4-$^2H_4$] taurochenodeoxycholic acid (tauro-CDCA), [2,2,4,4-[$^2H_4$]glycocholic acid (glyco-CA), and [2,2,4,4-$^2H_4$] glycochenodeoxycholic acid (glyco-CDCA) were synthesized as described by Mills et al. (30). Plasma (50 μL) was diluted with 50 μL internal standard solution (30) and 500 μL acetonitrile was added while mixing. After centrifugation, the sample was dried under N_2 and reconstituted in 100 μL methanol:H_2O (1:3). Subsequently, 10 μL of this solution was injected onto a UPLC column (Waters Acquity BEH C18; length 10 cm, internal diameter 2.1 mm, particle size 1.7 μm). The BAs were separated using a gradient from 98% 5 mM ammoniumformate (pH 8.1):methanol (3:1 v:v) to 98% acetonitrile:H_2O (9:1 v:v) in 5 min at a flow rate of 350 μL/min.

BAs were detected with a Waters Quattro Premier XE tandem mass spectrometer in the negative electrospray ionization mode. For the detection of glycine- and taurine-conjugated BAs, cone voltage was set at 60 V and 90 V, respectively, using 40 eV and 60 eV collision energy, respectively. Overall collision gas pressure was 3×10^{-3} mbar argon, and the source temperature was kept at 120 °C. Glycine-conjugated BAs were detected by multiple reaction monitoring using specific transitions with a mass difference of *m/z* 74, since all glycine conjugates specifically lose the *m/z* 74 fragment from the quasimolecular ion after fragmentation (30). Transitions: glyco-CDCA, glyco-DCA, and glyco-ursodeoxycholic acid (UDCA) (448→74); [$^2H_4$]glyco-CDCA (452→74); glyco-CA (464→74); [$^2H_4$]glyco-CA (468→74). Taurine-conjugated BAs were detected in a similar manner using specific transitions with a mass difference of *m/z* 80 due to the loss of a part of the taurine moiety. Transi\tions: tauro-CDCA, tauro-DCA, and tauro-UDCA (498→80); [$^2H_4$]tauro-CDCA (502→80); tauro-CA (514→80); [$^2H_4$]tauro-CA (518→80). Unconjugated BAs were detected using selected ion recording (cone voltage 70 V, collision energy 10 eV): CDCA and DCA *m/z* 391, CA *m/z* 407.

Absolute concentrations of either taurine- or glycine-conjugated CA and CDCA were calculated using calibration curves by relating the peak area to the peak area of the respective $^2H_4$ internal standard. For DCA and UDCA conjugates, the [$^2H_4$]CDCA internal standards were used. Calibration curves were labeled linear (r 0.98) from 0.1 to 100 μmol/L.

Energy Expenditure

Energy expenditure (REE) was determined by indirect calorimetry before (at −30 min), 90, and 240 min after the meal using a ventilated hood system (Vmax Encore 29; SensorMedics, Anaheim, CA). Substrate oxidation was calculated as described by Frayn (12). The abbreviated Weir equation was used to calculate 24-h energy expenditure.

Statistical Analysis

Concentrations and area under the curves (AUCs) are presented as median and interquartile range (IQR) unless otherwise stated. For visual purposes, data in graphs are displayed as means ± SE. AUCs and incremental AUCs (iAUCs), by using baseline subtracted values, were calculated with the trapezoidal rule. The total bile acid (TBA) concentration is the sum of the unconjugated and conjugated forms of CA, CDCA, DCA, and UDCA. The lithocholic acid (LCA) concentrations were all below the detection limit and therefore not included in the data analysis. A P value below 0.05 is considered as statistically significant. Comparisons between two conditions were evaluated with either paired t-testing for normally distributed data or the Wilcoxon signed rank test for nonparametric distribution. We used a two-way repeated measurement ANOVA (2-way RM-ANOVA) for the analyses of differences in postprandial curves between the mixed meal tests, including fasted concentrations ($t = 0$). When the two-way RM-ANOVA was statistically significant, we performed post hoc Bonferroni testing to detect the specific differences in the individual time points. Correlations between individual BA species, insulin, GLP-1, and FGF19 were tested on all time points using Pearson's correlation for normally distributed data or Spearman's Rho for nonparametric data. The Bonferroni test was used to correct for multiple testing and only highly significant results are reported. Statistical analysis was performed using IBM SPSS Statistics 24 (IBM, Armonk, NY) and GraphPad Prism 7.02 (GraphPad Software Inc., La Jolla, CA). Graphs were made using GraphPad Prism 7.02.

RESULTS

Subjects Characteristics

We included 9 healthy, lean men (median [IQR] age 23.1 [2.8] years, 79.0 [14.0] kg, height 187.0 [9.5] cm, BMI 23.1 [2.8] kg/m^2) for the *experiment 1* and 10 healthy lean men (age 24.5 [5.0] years, 74.4 [10.2] kg, height 183.0 [7.8] cm, BMI 22.1 [2.6] kg/m^2) for *experiment 2*. In both experiments, all subjects completed the study procedures.

Experiment 1

Plasma glucose and insulin concentrations after 40 h of fasting. Forty hours of fasting lowered premeal plasma glucose concentrations (baseline 14 h FAST: 5.3 [0.35] mmol/L vs. 40 h FAST: 4.2 [1.0] mmol/L; $P < 0.05$) but had no effect on premeal insulin concentrations (baseline 14 h FAST: 7.5 [15.0] pmol/L vs. 40 h FAST: 7.5 [0] pmol/L; $P > 0.05$). Extended fasting increased postprandial glucose and insulin concentrations (AUC_{0-240} glucose 14 h FAST: 1,401.0 [240.5] mmol/L × min vs. 40 h FAST: 1,579.5 [498.2] mmol/L × min; $P < 0.05$, 2-way RM-ANOVA. $P < 0.01$; post hoc analysis time (T) 60 $P < 0.05$ and T90 $P < 0.01$ AUC_{0-240} insulin 14 h FAST: 40,447.0 [8,775.0] pmol/L × min vs. 40 h FAST: 64,807.5 [67320.0] pmol/L × min; $P < 0.01$, 2-way RM-ANOVA $P < 0.05$, post hoc analysis T90, T120, and T180 $P < 0.01$) (Fig. 1, *A* and *B*), denoting that 40 h fasting induced glucose intolerance. iAUCs yielded the same results (data not shown).

Plasma BA concentrations after 40 h of fasting. Postprandial TBA concentrations increased in both conditions (Fig. 1*C*). Fasting and postprandial BA levels showed large interindividual variations in BA composition (Table 1). The 40-h fast did not affect baseline (14 h FAST: 0.70 [0.63] μmol/L vs. 40 h FAST: 0.54 [0.60] μmol/L; $P > 0.05$) or postprandial total BA levels in plasma (AUC_{0-240} 14 h FAST: 989.3 [848.4] μmol/L × min vs. 40 h FAST: 987.5 [516.0] μmol/L × min; $P > 0.05$, 2-way RM-ANOVA $P > 0.05$) (Table 1, Fig. 1*C*). There was no significant effect of the 40-h fast on baseline levels or postprandial excursion of other individual BA species (Table 1). We did not find any differences in the fasted CA:CDCA ratio (14 h FAST 0.54 ± 0.3 vs. 40 h FAST 0.44 ± 0.1; $P > 0.05$) and the primary (CA and CDCA): secondary bile acid (DCA and UDCA) ratio (14 h FAST 1.52 ± 0.7 vs. 40 h FAST 1.31 ± 0.1; $P > 0.05$). Furthermore, the postprandial CA:CDCA ratio (AUC 14 h FAST 0.39 ± 0.2 vs. 40 h FAST 0.36 ± 0.1; $P > 0.05$) and the primary: secondary bile acid ratio (AUC 14 h FAST 2.6 ± 1.6 vs. 40 h FAST 2.6 ± 1.9; $P > 0.05$) did not differ.

Since we were interested in the correlations between plasma levels of BAs and insulin, we performed correlation analyses at different time points after ingestion of the meal. We observed a strong positive correlation between post absorptive insulin and gDCA levels at $t = 60$ min after an overnight fast (Spearman's Rho 14 h FAST: $r = +0.88$, $P < 0.01$, Fig. 2*A*, 40 h FAST: $r = +0.42$, $P = 0.27$) and $t = 90$ min (14 h FAST: $r = +0.75$, $P < 0.05$; 40 h FAST: $r = +0.73$, $P < 0.05$). The correlation at 60 min was strongest after an overnight fast, where the correlation remained significant after correction for multiple testing. iAUCs showed no differences (data not shown).

Plasma GLP-1 and FGF19 concentrations after 40 h of fasting. Forty hours of fasting did not change GLP-1 baseline concentrations (baseline 14 h FAST: 4.4 [8.7] pmol/L vs. 40 h FAST: 7.1 [15.0] pmol/L; $P > 0.05$) but increased postprandial AUC of GLP-1 (AUC_{0-180} 14 h FAST: 1,896.0 [2,463.8] pmol/L × min vs. 40 h FAST: 2,232.0 [2,470.5] pmol/L × min; $P < 0.05$, 2-way RM-ANOVA $P = 0.059$) (Fig. 1*G*). The iAUC analysis for GLP-1 showed no differences (data not shown).

Forty hours of fasting increased FGF19 baseline concentrations (baseline 14 h FAST: 0.10 [0.07] ng/mL vs. 40 h FAST: 0.22 [0.40] ng/mL; $P < 0.05$), thereby tremendously increasing postprandial AUC of FGF19 (AUC_{0-240} 14 h FAST: 58.6 [44.3] ng/mL × min vs. 40 h FAST: 123.8 [124.1] ng/mL × min; $P < 0.05$, 2-way RM-ANOVA $P < 0.01$, post hoc analysis T60 and T180 ($P < 0.05$), T90 and T120 $P < 0.001$) (Fig. 1*E*). However, postprandial iAUC of FGF19 was not increased after 40 h of fasting (AUC_{0-240} 14 h FAST: 33.4 [28.2] ng/mL × min vs. 40 h FAST 65.8 [49.8] ng/mL × min; $P > 0.05$). Additionally, we measured 7α-hydroxy-4-cholesten-3-one (C4) as a marker of bile acid synthesis. In contrast to FGF19, 40 h of fasting did not affect C4 baseline levels (baseline 14 h FAST: 10.0 [12.6] ng/mL vs. 40 h FAST: 7.1 [10] ng/mL; $P > 0.05$). However, postprandial C4 was lower after a 40-h fast (AUC_{0-240} 14 h FAST 3,419 [2,099] vs. 40 h FAST 2,058 [952] ng/mL × min, $P < 0.05$, 2-way RM-ANOVA $P < 0.01$, post hoc analyses T120 $P < 0.05$ and T180 $P < 0.01$) (Fig. 1*F*)**.** TBA did not correlate with FGF19, but we observed a strong positive correlation between baseline glycochenodeoxycholic acid and FGF19 levels after 40 h of fasting (Spearman's Rho +0.98, $P < 0.01$) and baseline glycocholic acid and FGF19 (Spearman's Rho +0.81, $P < 0.05$) (Fig. 2, *B* and *C*).

Experiment 2

The changes in GLP-1 and FGF19 and correlation between gDCA and insulin levels in *experiment 1* prompted us to

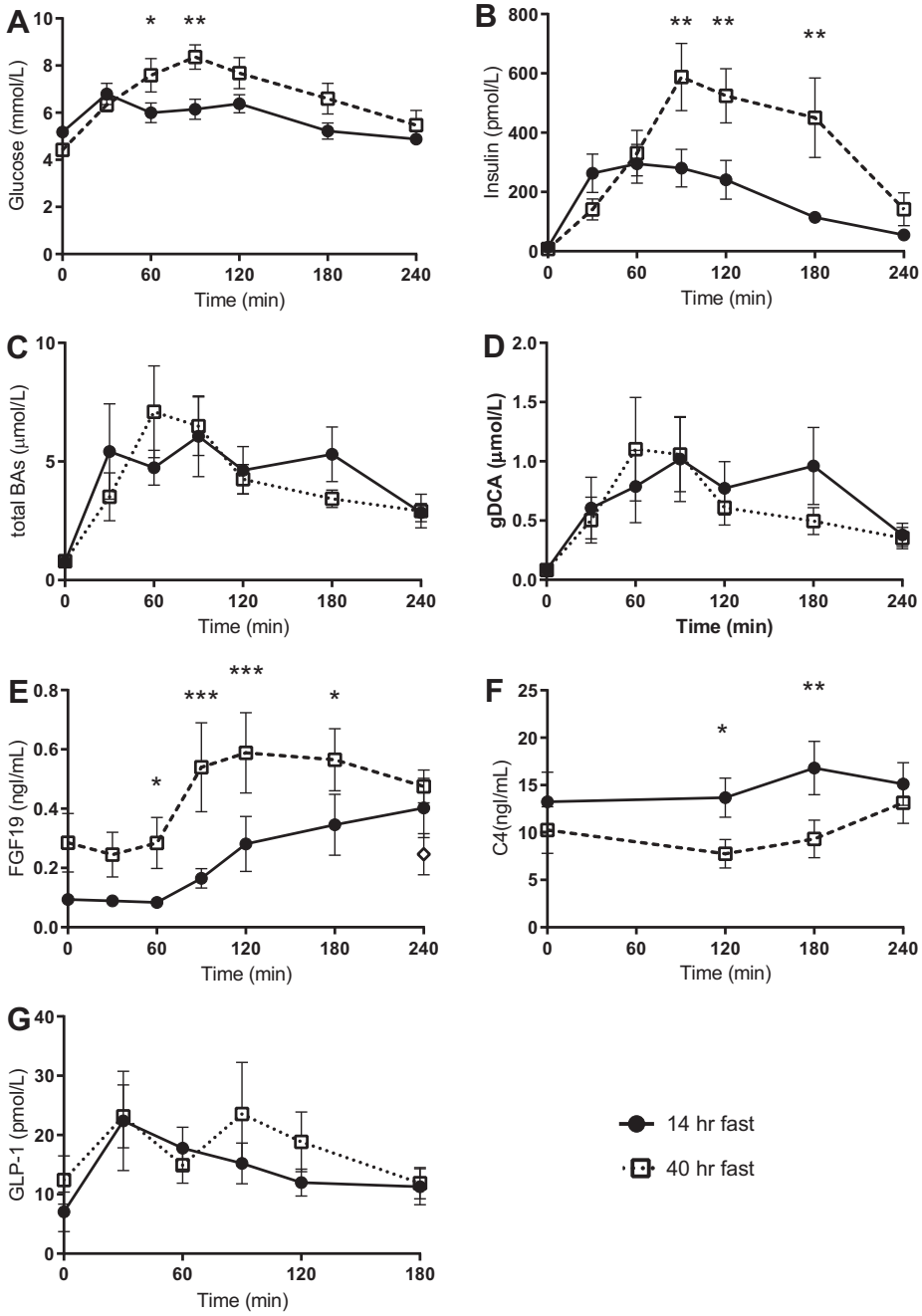

Fig. 1. Postprandial homeostasis in fasting-induced insulin resistance in healthy men. In a crossover design, healthy men ($n = 9$) consumed a standardized liquid meal test at $t = 0$ after an overnight (14-h FAST) or 40 h fast (40-h FAST). Postprandial excursion of glucose (*A*), insulin (*B*), total bile acids (BA; *C*), glycine-conjugated deoxycholic acid (gDCA; *D*), fibroblast growth factor 19 (FGF19; *E*), C4 (7α-hydroxy-4-cholesten-3-one; *F*). ●, 14 h FAST; □, 40 h FAST. *$P <$ 0.05, **$P < 0.01$, and ***$P < 0.001$ after post hoc analysis. Data are means ± SE.

explore the effect of gDCA supplementation on these postprandial parameters.

Plasma glucose and insulin concentrations after gDCA administration. Administration of gDCA had no effect on the total postprandial excursion of glucose (AUC_{0-240} −gDCA: 1,176.9 [173.0] mmol/L × min vs. +gDCA: 1,145.1 [63.3]; $P > 0.05$, 2-way RM-ANOVA $P > 0.05$) (Fig. 3*A*). Because the postprandial curve showed a glucose lowering effect after gDCA administration between T75 and T180, we calculated the AUC of glucose for these specific time points and we found that gDCA administration decreased the AUC_{75-180} before glucose levels returned to baseline (−gDCA: 616.5 [76.3] mmol/L × min vs. +gDCA: 544.4 [109.7]) mmol/L × min; $P < 0.05$).

However, gDCA administration did not affect postprandial insulin concentrations (AUC_{0-240} −gDCA: 46,188.8 [30,275.9] vs. +gDCA 45,978.8 [4,4045.6] $P > 0.05$; 2-way RM-ANOVA $P > 0.05$) (Fig. 3*B*). Analyses of the iAUCs of glucose and insulin yielded the same results (data not shown).

Plasma BA concentrations after gDCA administration. TBA excursions after the test meal were not affected by gDCA administration (AUC_{0-240} −gDCA: 1,126.6 [621.0] μmol/L × min vs. +gDCA: 1,104.3 [686.7] μmol/L × min; $P > 0.05$, 2-way RM-ANOVA $P > 0.05$), although some differences in BA composition were uncovered (Table 1)**.** In line with our expectations, postprandial gDCA concentrations (AUC) were increased after gDCA administration (AUC_{0-240} −gDCA: 152.0 [141.4] μmol/L × min vs. +gDCA: 231.1 [133.0]

Table 1. *Effects of short-term fasting and single-dose gDCA administration on plasma bile acid composition in healthy men*

		Experiment 1, n = 9 men			*Experiment 2, n* = 10 men		
Bilogram, μmol/L		14 h FAST	40 h FAST	*P*	−gDCA	+gDCA	*P*
Total bile acids	baseline	0.70 [0.63]	0.54 [0.60]	0.86	1.10 [2.14]	0.73 [1.13]	0.11
	AUC	987.1 [859.1]	987.5 [515.2]	0.31	1126.7 [621.0]	1104.3 [686.7]	0.88
	2-way RM-ANOVA			0.20			0.63
Unconj. CA	baseline	0.04 [0.04]	0.02 [0.02]	0.14	0.10 [0.20]	0.11 [0.75]	0.25
	AUC	10.13 [3.78]	7.4 [2.69]	0.14	22.1 [106.6]	27.8 [27.5]	0.28
	2-way RM-ANOVA			0.11			0.16
G-conj. CA	baseline	0.05 [0.04]	0.04 [0.07]	0.91	0.13 [0.25]	0.05 [0.09]	0.37
	AUC	170.3 [130.2]	159.3 [158.6]	0.31	155.4 [143.2]	92.7 [112.1]	0.72
	2-way RM-ANOVA			0.55			0.69
T-conj. CA	baseline	0.02 [0.01]	0.01 [0.02]	0.77	0.0 [0.07]	0.0 [0.01]	0.16
	AUC	14.7 [17.6]	16.4 [27.4]	0.77	21.8 [22.5]	15.3 [20.8]	0.33
	2-way RM-ANOVA			0.87			0.63
Unconj. CDCA	baseline	0.04 [0.04]	0.01 [0.03]	0.18	0.10 [0.34]	0.09 [0.29]	0.21
	AUC	21.2 [46.0]	16.7 [10.8]	0.31	26.8 [58.6]	23.6 [30.0]	0.06
	2-way RM-ANOVA			0.46			0.62
G-conj. CDCA	baseline	0.14 [0.26]	0.06 [0.11]	0.86	0.35 [0.28]	0.22 [0.15]	0.37
	AUC	353.5 [399.6]	452.9 [279.6]	0.44	414.1 [285.3]	454.3 [169.1]	0.72
	2-way RM-ANOVA			0.28			0.34
T-conj. CDCA	baseline	0.01 [0.02]	0.02 [0.02]	0.44	0.02 [0.05]	0.0 [0.02]	0.08
	AUC	40.9 [40.0]	35.4 [58.8]	0.86	48.7 [31.8]	54.6 [35.3]	0.39
	2-way RM-ANOVA			0.28			0.50
Unconj. DCA	baseline	0.11 [0.21]	0.09 [0.42]	0.83	0.22 [0.39]	0.23 [0.34]	0.31
	AUC	66.8 [160.5]	108.7 [144.2]	0.26	69.0 [61.9]	48.8 [101.3]	0.58
	2-way RM-ANOVA			0.30			0.84
G-conj. DCA	baseline	0.03 [0.12]	0.28 [0.74]	0.51	0.10 [0.14]	0.07 [0.04]	0.24
	AUC	121.1 [227.1]	108.1 [171.1]	0.44	152.0 [141.4]	231.1 [133.0]	0.02*
	2-way RM-ANOVA			0.28			0.17
T-conj. DCA	baseline	0.0 [0.01]	0.01 [0.03]	0.91	0.01 [0.08]	0.0 [0.0]	0.04*
	AUC	17.1 [45.3]	17.4 [34.4]	0.77	33.4 [47.4]	21.0 [18.2]	0.06
	2-way RM-ANOVA			0.21			0.78

Data are median and [interquartile range]. AUC, area under the curve; TBA, total bile acids; G-conj., glycine conjugated; T-conj., taurine conjugated; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA: deoxycholic acid; RM, repeated measures. *$P < 0.05$.

μmol/L × min, $P < 0.05$, 2-way RM-ANOVA $P > 0.05$) (Fig. 3*C*). Correlation analyses showed no significant correlations after gDCA administration. Analysis of the iAUC of TBAs yielded the same results (data not shown).

Plasma GLP-1 and FGF19 concentrations after gDCA administration. The AUC of GLP-1 was similar between the study days (AUC_{0-240} −gDCA: 3,110.6 [3,471.7] pmol/L × min vs. +gDCA: 3,232.5 [2,865.8] pmol/L × min; $P > 0.05$, 2-way RM-ANOVA $P > 0.05$) (Fig. 2*E*), whereas the postprandial incremental AUC of the first phase secretion of GLP-1 was higher after gDCA ($iAUC_{0-60}$: −gDCA: 56.8 [483.8] pmol/L × min vs. +gDCA: 365.7 [388.8] pmol/L × min; $P < 0.01$) (Fig. 2*E*).

Finally, postprandial excursion of the BA/FXR-induced enterokine FGF19 was not affected by gDCA administration (AUC_{0-240} −gDCA: 48.8 [16.9] ng/mL × min vs. +gDCA: 46.7 [12.9] ng/mL × min, $P > 0.05$, 2-way RM-ANOVA $P > 0.05$) (Fig. 2*D*). We found no correlations between BAs, GLP-1, and FGF19 (data not shown). The iAUCs of both GLP-1 and FGF19 yielded the same results (data not shown).

Pre- and postprandial energy expenditure. Baseline REE per kilogram body weight was slightly less in the control setting

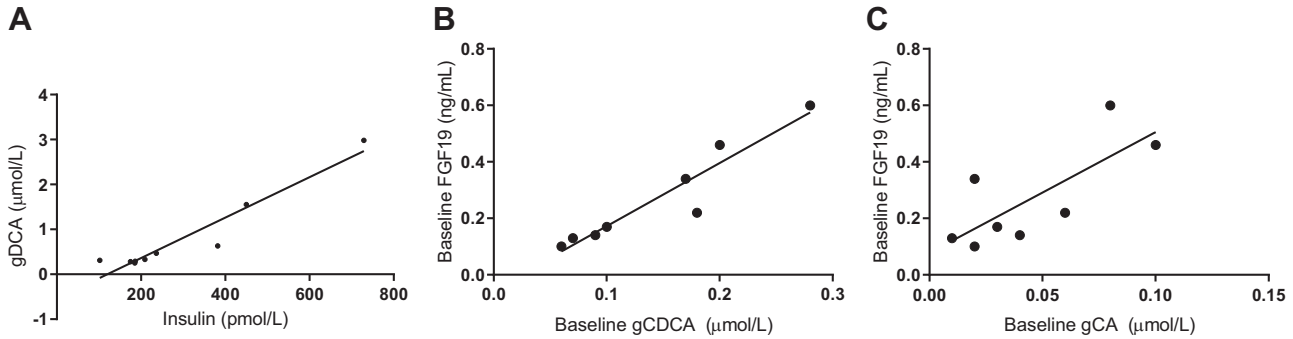

Fig. 2. Correlations of enteroendocrine factors in healthy men. In a crossover design, healthy male subjects ($n = 9$) consumed a standardized liquid meal test at $t = 0$ after an overnight (14-h fast) or 40-h fast (40-h fast). Depicted are the postprandial Spearman's Rho correlation between insulin and glycine-conjugated deoxycholic acid (gDCA) 60-min postprandial after 14 h of fasting ($P < 0.01$) (*A*), the Spearman's Rho correlation between baseline fibroblast growth factor 19 (FGF19) and glycochenodeoxycholic acid (GCDCA) ($P < 0.01$) (*B*), and between baseline FGF19 and glycocholic acid (gCA) ($P < 0.05$) (*C*).

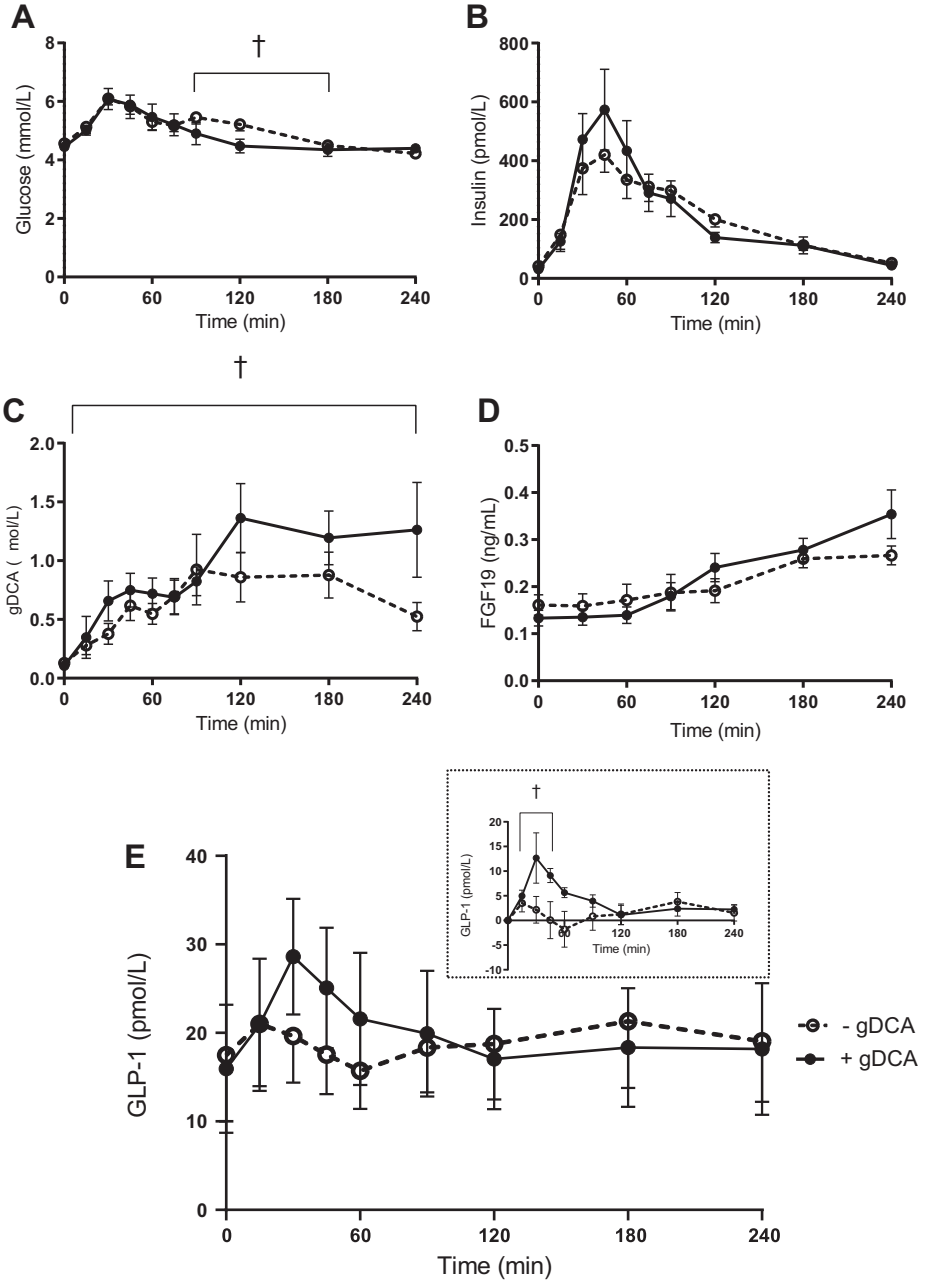

Fig. 3. Postprandial glucose homeostasis after oral glycine-conjugated deoxycholic acid (gDCA) administration in healthy men. In a crossover design, healthy men ($n = 10$) consumed a standardized liquid meal test at $t = 0$ with or without 750 mg gDCA. Postprandial excursion of glucose (*A*), insulin (*B*), gDCA (*C*), fibroblast growth factor 19 (FGF19; *D*), and (incremental) glucagon-like peptide 1 (GLP-1; *F*). ○, −gDCA; ●, +gDCA. † at the curve represents significant effect on area under the curve. Data are means ± SE.

(−gDCA: 21.0 ± [2.51] vs. +gDCA: 21.7 ± [2.7] kcal·kg^{-1}·day^{-1}; $P < 0.05$), whereas total REE at baseline was not different between the groups (−gDCA: 1655.0 [244.5] vs. +gDCA: 1720.0 [164.0] kcal·kg^{-1}·day^{-1}; $P > 0.05$). Total energy expenditure (REE) and REE per kilogram body weight increased 90 min after meal ingestion (REE −gDCA: 1,899.0 [130.0] vs. +gDCA: 2,057.5 [483.3] kcal·kg^{-1}·day^{-1}; $P > 0.05$; REE per kilogram body weight −gDCA: 24.1 [4.2] vs. +gDCA: 26.1 [4.7] kcal·kg^{-1}·day^{-1}; $P > 0.05$) and leveled off again after 240 min (REE −gDCA: 1605.0 [301.8] kcal·kg^{-1}·day^{-1} vs. +gDCA: 1,750.5 [228.8] kcal·kg^{-1}·day^{-1}; $P > 0.05$; REE per kilogram body weight −gDCA: 20.6 [3.1] kcal·kg^{-1}·day^{-1} vs. +gDCA: 22.0 [3.6] kcal·kg^{-1}·day^{-1}, $P > 0.05$). However, gDCA did not alter the postprandial response of REE. Furthermore, we did not find effects of gDCA supplementation on oxidation of individual macronutrients (data not shown).

DISCUSSION

BAs and their targets GLP-1 and FGF19 have received interest as hormone-like mediators that modulate energy metabolism. Here we explored these axes in response to 40 h fasting-induced insulin resistance and by administering oral gDCA. In the first experiment, we assessed the effects of a 40-h fast on BA metabolism because this induces insulin resistance (42) and BA metabolism is altered in various models of insulin resistance (5, 14, 47). More specifically, CA synthesis is increased and the DCA pool is relatively enlarged in patients with type 2 diabetes (5), most likely leading to the observed increased postprandial BA peaks (43, 47). Postprandial BA concentrations in plasma are suppressed in subjects with obesity (14).

We reproduced the effects of 40 h fasting on insulin sensitivity from Horton and Hill al. (19), but we found no effects of

40 h fasting on postprandial BA concentrations. Obviously, there are important differences between fasting-induced insulin resistance and the metabolic changes seen in obesity and type 2 diabetes (42). It was shown previously that insulin resistance affects the BA pool composition via upregulation of CYP8B1 (12a-hydroxylase, engaged in synthesis of CA) by diminished FoxO1 inhibition (16, 17, 20). Here we found no differences in the ratio of 12a-hydroxylated and non-12a-hydroxylated bile acids, which may either negate an effect on CYP8B1 or may be due to considerable intraindividual variation. Moreover, only small changes in BA levels may be seen after a diet intervention of 2 wk (32), whereas after bariatric surgery beneficial changes in glucose metabolism appeared after changes in BA pool composition (8).

We found positive correlations between gDCA and insulin levels at multiple postprandial time points in both overnight fasted and 40-h fasted states. TGR5 mediates release of GLP-1 by enteroendocrine L cells in response to stimulation with BAs (45, 46, 49). GLP-1 can in turn lead to increased insulin release. Alternatively, activation of TGR5 on human pancreatic beta cells leads to rapid basal and glucose-dependent insulin secretion in vitro (25). DCA is a secondary BA that makes up around 30% of the total BA pool in humans, where it is mainly found in its glycine-conjugated form (5). In patients with type 2 diabetes, the relative contribution of DCA to the total BA pool is increased (5). This could be interpreted as an adaptive response to counter declining beta cell insulin secretion. Sato and colleagues (39) showed that gDCA is a particular strong TGR5 agonist, activating 50% of this receptor at a concentration of 1.18 μM.

Forty hours of fasting actually did increase postprandial GLP-1 concentrations. BAs continue to cycle in the enterohepatic cycle during fasting, but it is unclear if increased luminal stimulation by BAs such as gDCA increased the GLP-1 levels after the 40-h fast, because we did not witness increased BA levels in our peripheral samples. Preclinical work has linked an increase of GLP-1 production to an increase of energy status, i.e., AMPK-dependent regulation of GLP1 expression in L-like cells (21).

Likewise, it may be difficult to explain both the increased basal and postprandial FGF19 concentrations after 40 h of fasting. Here, FGF19 may be high to suppress hepatic BA biosynthesis while starving despite absence of stimuli that would normally induce FGF19 release. 7α-Hydroxy-4-cholesten-3-one (C4) reflects hepatic CYP7A1 activity and, indeed, we found suppressed postprandial C4 levels after 40 h of fasting as seen in other studies where high FGF19 levels lower C4 (13, 41). However, increased basal FGF19 after 40 h of fasting did not result in statistically lower basal C4 concentrations. This may be due to lack of power, insufficient basal FGF19 concentrations to inhibit C4, or different circumstances (i.e., glucose, insulin, and bile acid composition) in the fasted state compared with the postprandial state (9). Alternatively, FGF19 is not obligatory for reduced BA biosynthesis (1). Forty hours of fasting induces profound reciprocal changes in energy metabolism that may have influenced FGF19 biology (42). It is also tempting to speculate that ongoing cycling of BAs such as CDCA, which correlated with FGF19 levels, may have led to increased FGF19 production as shown earlier in other models (29, 50).

In the second experiment, we further explored this relationship and administered a single dose of 750 mg gDCA with the meal. The dose is similar to that recommended for ursodeoxycholic acid, prescribed for treatment of primary biliary cholangitis and used in translational studies (11, 15), but lower than the dose of CDCA (15 mg/kg) used in the study by Broeders et al. (4). Unconjugated DCA has been suggested to exert carcinogenic effects in in vitro studies employing supraphysiological concentrations (31). However, in our study, we administered glycine-conjugated DCA, which is abundantly present in the human BA pool and enters the gut lumen in high concentrations after a meal (5).

We found slightly lower plasma glucose levels in our healthy volunteers after gDCA administration between 75 and 180 min after the meal. Although this could be perceived as a chance finding, lower glucose levels were very reproducible between subjects, showed marginal spread, and reached a mean difference of ~0.5 mM at time points 90 and 120 min. We could not attribute this to a relevant increase in insulin levels. Moreover, gDCA supplementation acutely increased GLP-1 levels. This is likely explained by increased secretion and not diminished clearance, because GLP-1 normally is broken down quickly by dipeptidyl peptidase-4 (3). The key question remains then: how does gDCA increase GLP-1 secretion? Stimulation of TGR5 receptors in the gut by gDCA is most likely responsible for this phenomenon, because the GLP-1 effects were rather acute (after 30 min, Fig. 3*E*) and preceded the increased gDCA plasma levels, which only became evident after ~120 min (Fig. 3*C*). Our data support previous work that shows bile acids as important regulators of appetite- and metabolism-regulating hormones by activation of basolateral intestinal TGR5 (23). However, the combination of a short GLP-1 surge and healthy lean volunteers may have prevented clear effects on glucose levels. The fact that we found no effects on FGF19 levels after gDCA administration may be explained by lower, but not absent, affinity of gDCA for FXR compared with CDCA (34).

Contrary to our expectations, we did not detect an effect of oral gDCA on energy expenditure in this study, despite observing significantly increased gDCA plasma concentrations at the end of the meal. TGR5/DIO2 coexpression has been confirmed to be present in both human skeletal muscle myoblasts and brown adipose tissue in humans, suggesting that this pathway is functionally active (4). Broeders et al. (4) described a 5% increase in early morning basal metabolic rate in response to two consecutive doses of unconjugated CDCA given over the course of 24 h. Despite the fact that unconjugated CDCA is a BA with weaker TGR5 affinity compared with gDCA, the increased brown adipose tissue activity was attributed to TGR5 activation (39).

Our study had a few limitations. First, the experiments were designed to assess acute effects of BAs mediated by TGR5 and not by changes in FXR stimulation or changes to the composition of the circulating BA pool. Plasma BA concentrations are probably not under short-term regulation but instead are a function of their portal concentration and a relatively constant hepatic uptake (27). However, longer duration of BA supplementation may lead to more profound changes in the BA pool and downstream targets, including prolonged TGR5 and FXR activation (10, 48). Another limitation of this study was the high interindividual variability of BA curves, which is a

recurring finding in postprandial BA studies (14, 47) and may warrant the inclusion of a larger number of subjects. Also, the results of *experiment 1* may be affected by the rate of stomach emptying, which is slower after a 40-h fast (6). Finally, we were not able to measure portal vein concentrations of the substrates and hormones of interest, and peripheral sampling may not provide optimal information about changes within the enterohepatic cycle during our studies (9).

In this study, we show the different effects of 40 h fasting and gDCA administration on BAs and their downstream targets GLP-1 and FGF19. Unexpectedly, 40 h fasting increased both GLP-1 and FGF19, where the former appeared BA independent and the latter BA dependent. Therefore, our data add complexity to the physiological regulation of the enterokines GLP-1 and FGF19 by BAs.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

F.S.V.N. and M.R.S. conceived and designed research; F.S.V.N., E.C.E.M., K.G.M.N., R.A., and L.A.L. performed experiments; F.S.V.N., E.C.E.M., K.G.M.N., S.W.O.D., F.G.S., and M.R.S. analyzed data; F.S.V.N., E.C.E.M., K.G.M.N., S.W.O.D., F.G.S., and M.R.S. interpreted results of experiments; F.S.V.N., E.C.E.M., R.A., L.A.L., F.M.V., R.A.M., H.-J.K., S.W.O.D., F.G.S., J.A.R., E.M.K., and M.R.S. edited and revised manuscript; F.S.V.N., E.C.E.M., K.G.M.N., R.A., L.A.L., F.M.V., R.A.M., H.-J.K., S.W.O.D., F.G.S., J.A.R., E.M.K., and M.R.S. approved final version of manuscript; E.C.E.M. and M.R.S. prepared figures; E.C.E.M. and M.R.S. drafted manuscript.

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