A gut—brain axis regulating glucose metabolism mediated by bile acids and competitive fibroblast growth factor actions at the hypothalamus

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ABSTRACT

Objective: Bile acids have been implicated as important regulators of glucose metabolism via activation of FXR and GPBAR1. We have previously shown that FGF19 can modulate glucose handling by suppressing the activity of hypothalamic AGRP/NPY neurons. As bile acids stimulate the release of FGF19/FGF15 into the circulation, we pursued the potential of bile acids to improve glucose tolerance via a gut—brain axis involving FXR and FGF15/FGF19 within enterocytes and FGF receptors on hypothalamic AGRP/NPY neurons.

Methods: A 5-day gavage of taurocholic acid, mirroring our previous protocol of a 5-day FGF19 treatment, was performed. Oral glucose tolerance tests in mice with genetic manipulations of FGF signaling and melanocortin signaling were used to define a gut—brain axis responsive to bile acids.

Results: The taurocholic acid gavage led to increased serum concentrations of taurocholic acid as well as increases of FGF15 mRNA in the ileum and improved oral glucose tolerance in obese (ob/ob) mice. In contrast, lithocholic acid, an FXR antagonist but a potent agonist for GPBAR1, did not improve glucose tolerance. The positive response to taurocholic acid is dependent upon an intact melanocortinergic system as obese MC4R-null mice or ob/ob mice without AGRP did not show improvements in glucose tolerance after taurocholate gavage. We also tested the FGF receptor isoform necessary for the bile acid response, using AGRP:Fgfr1–/– and AGRP:Fgfr2–/– mice. While the absence of FGFRI in AGRP/NPY neurons did not alter glucose tolerance after taurocholate gavage, manipulations of Fgfr2 caused bidirectional changes depending upon the experimental model. We hypothesized the existence of an endogenous hypothalamic FGF, most likely FGF17, that acted as a chronic activator of AGRP/NPY neurons. We developed two short peptides based on FGF8 and FGF17 that should antagonize FGF17 action. Both of these peptides improved glucose homeostasis after a 4-day course of central and peripheral injections. Significantly, daily average blood glucose from continuous glucose monitoring was reduced in all tested animals but glucose concentrations remained in the euglycemia range.

Conclusions: We have defined a gut—brain axis that regulates glucose metabolism mediated by antagonistic fibroblast growth factors. From the intestine, bile acids stimulate FGF15 secretion, leading to activation of the FGF receptors in hypothalamic AGRP/NPY neurons. FGF receptor intracellular signaling subsequently silences AGRP/NPY neurons, leading to improvements of glucose tolerance that are likely mediated by the autonomic nervous system. Finally, short peptides that antagonize homodimeric FGF receptor signaling within the hypothalamus have beneficial effects on glucose homeostasis without inducing hypoglycemia. These peptides could provide a new mode of regulating glucose metabolism.

Keywords Bile acids; FGF15; Hypothalamus; FGF receptors; AGRP; Melanocortins

1. INTRODUCTION

Bile acids modulate glucose homeostasis by direct actions on two receptors, GPBAR1 (G-protein coupled bile acid receptor 1; TGR5) [13] and FXR (farnesoid X receptor) [18,24,36]. GPBAR1’s ability to stimulate incretin secretion, such as GLP1 and GLP2, from enteroendocrine cells is readily understood from its role as a G-protein coupled receptor [12]. While FXR has been thoroughly studied as a nuclear hormone receptor, FXR’s sites and modes of action relevant to glucose homeostasis remain poorly understood [27]. One well known role for FXR is as a bile acid sensor [15] within the enterocyte that increases the production of FGF15 (FGF19 is the human ortholog). FXR acts as a direct transcriptional regulator of FGF15 and FGF19 due to binding sites within the promoter of the human FGF19 gene [20]. FGFRA,

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among the four known FGF receptors, is the major hepatic isoform and is activated by circulating FGF15/19 to inhibit hepatic synthesis of bile acids from cholesterol [40]. However, FGF19 can significantly improve glucose handling even in mice that do not express FGF4, indicating that other FGF receptors found in extrahepatic tissues are critical. Antibodies to FGFRI [32], which have mixed agonist/antagonist activity, improve glucose tolerance while accumulating within the median eminence, pointing to a central site of action. We have shown that FGF19 suppresses the activity of hypothalamic neurons which express Agouti gene related peptide (AGRP) and Neuropeptide Y (NPY) neurons [19], suggesting that modulation of central melanocortinergic tone is the critical mechanism for the ability of FGF19 to improve glucose homeostasis.

Previous genetic studies have revealed pleiotropic actions for FGF receptors, principally during development [9]. Activating mutations of FGFR1, FGFR2, and FGFR3 cause syndromes with craniosynostosis as a major feature [34]. Complete absence of FGFR1 or FGFR2 causes developmental abnormalities of the central nervous system and is typically incompatible with life. As an interesting side note, the FGF8/FGFR1 ligand/receptor pair [26] is critical for the migration and maturation of gonadotrophin releasing hormone cells from the oligodendrocyte placode with resultant hypothalamic hypogonadism associated with mutations in FGF8 or FGFR1.

In this report, we conduct studies with oral bile acid supplementation to provide evidence that bile acid signaling via FXR affects ileal FGF15/19 production, causing activation of FGFRI on melanocortinergic neurons to significantly improve glucose tolerance. We propose that there is a gut–brain axis that can modulate glucose metabolism, an axis that incorporates 1) small molecules (bile acids) that are synthesized by the liver and undergo biotransformation by gut bacteria and the enterohpatic circulation and 2) a receptor, FGFR1, on hypothalamic neurons which can activate intracellular signaling pathways overlapping those triggered by leptin and insulin. Further testing of our hypothesis suggested that some FGF ligands might worsen glucose handling. Postulating that antagonism between FGF ligands (FGF15/19/21) that bind to beta Klotho-FGF receptor heterodimers and FGF ligands (FGF8/17/18) that bind to FGF receptor homodimers, we tested the ability of small peptides (predicted to block FGF receptor signaling) based on FGF8 and FGF17 to improve glucose handling. These short peptides improved glucose handling as measured with glucose tolerance tests as well as lowering daily average blood glucose after central and peripheral administration. In conclusion, we have identified a gut–brain axis wherein bile acid and FGF15/19 can improve glucose handling as well as identifying short FGF-derived peptides that improve glucose homeostasis.

2. MATERIALS AND METHODS

2.1. Animals and animal handling — genotyping, gavage, GTT and ITT

Animals were housed and bred in the Einstein barrier facility with 12 h light/12 h dark (6 am EST lights on) and provided with water and chow (Purina 5058) ad lib in polycarbonate cages with corn cob bedding. Heterozygous ob+/ mating pairs generated ob/ob animals with Agrp-IRES-CRE [35] segregating in the colony. A separate colony of ob/+ Agrp–/– mice were used to generate ob/ob Agrp–/– mice [11]. Heterozygous Mc4r+/– mice were used to generate Mc4r–/– animals. The Mc4r null allele was generated from CRE-mediated deletion of the Mc4r allele [1]. Fgfr1-1/flox/flox Agrp-CRE mice were generated from female Fgfr1-1/flox/flox [25] and Fgfr1-1/flox/flox Agrp-CRE males. While it has been suggested that the Agrp-IRES-CRE knock-in allele causes fewer instances of extrahypothalamic deletion, the presence of both Agrp (Chr. 8, 53 cM) and Fgfr1 (Chr. 8, 14 cM) on mouse Chromosome 8 made the production of the double mutant mice extremely difficult, necessitating the use of the Agrp-CRE BAC-based transgene. Fgfr1-1/flox/flox Agrp-IRES-CRE mice were generated from female Fgfr1-1/flox/flox [6] and male Fgfr1-1/flox/flox Agrp-IRES-CRE mice. Genotypes were determined with DNA isolated from ear clips. For bile acid gavage, sodium taurocholate (Sigma–Aldrich, St. Louis, MO) dissolved in water was dosed at 3 mg/g of body weight [10] while lithocholic acid [29] (sigma-Aldrich, St. Louis, MO) suspended in peanut oil was dosed at 0.1 mg/g of body weight. These doses were based on published reports of known efficacy for activation of FXR and GPBAR1, respectively. Bile acid gavages were given daily in the morning for 5 days. On the 5th day, overnight fasted mice were challenged with glucose. Oral glucose tolerance tests were done as previously described [19] with glucose loads of 0.5 mg/g for genetically obese mice (ob/ob, ob/ob Agrp–/– and Mc4r–/–) and 2 mg/g for mice without genetic obesity mutations. Glucose was determined from tail vein blood samples using the glucose oxidase method (Control AST, US Diagnostics, New York, NY). Insulin was determined from sera obtained from fasting mice using a quantitative ELISA kit with mouse insulin standards (Crystal Chem, Downers Grove, IL).

Peptide injections (FGF8 and FGF17; Novoprotein, Summit, NJ) were performed ivc via an indwelling third ventricular cannula. FGF8 was resuspended in aCSF at 1 mg/ml, and mice were infused with 1 μg of the peptide via syringe pump. FGF-derived peptides (F8b13, F8b13ProPro; sequences in Figure 6) were custom synthesized by GenScript (Piscataway, NJ) at 90% purity. These peptides, initially prepared at 1 mg/ml, were diluted to 100 μg/ml in aCSF, and mice were infused with 100 ng of peptide via syringe pump. For ip injections, these peptides were injected ip at 10 μg/g (body weight). Continuous glucose monitor devices from Data Scientific International (DSI, St. Paul, Minnesota) were implanted into the ascending aorta (glucose detector) and the temperature monitor, activity monitor, and battery were implanted into the abdomen. Signals were collected continuously for >4 weeks or until the batteries were discharged. Calibration was performed with tail blood using a portable glucometer weekly. Data were analyzed in 24 h windows. Daily average blood glucose values were the mean of glucose values collected per minute over 24 h (1440 data points per mouse per day) although the device can be programmed for data collection at shorter time intervals. All procedures had been approved by the Einstein Institutional Animal Care and Use Committee.

2.2. Quantitative RT-PCR and hormone assays

RNA isolated from the terminal ileum was used for quantification of FGF15 mRNA by quantitative RT-PCR. All RNA samples underwent quality checks by agarose gel electrophoresis and quantitation by absorbance readings at 260 nm. Briefly, RNA was reverse transcribed to generate first strand cDNA with Mo-MuLV reverse transcriptase (Superscript III, Life Technologies, Carlsbad, CA) which was used as a template for quantitation by SYBR Green fluorescence during Taq polymerase based amplification on a Roche LightCycler 480II. All primer pairs (synthesized from Integrated DNA technologies, Coralville, IA) spanned at least on intron to prevent amplification of genomic DNA. Relative quantitation was based on the delta–delta Ct method using beta 2 microglobulin mRNA as a loading control. Untreated control means were set to 1.0 for comparison purposes and treated samples were expressed relative to those means.
2.3. Bile acid assay
Serum samples (100 µL) were used for bile acid composition assay by ultraperformance liquid chromatography mass spectrometry [38]. Free and taurine conjugated bile acids were quantified. Taurocholic acid concentrations were compared between control and gavaged states in a pairwise comparison (paired t-test). As there were no effects due to the Agpr-CRE transgene, we pooled samples from the two genotypes.

2.4. Immunofluorescence staining
Mice were anesthetized with isoflurane and transcardially perfused with pre-perfusion solution (9 g NaCl, 5 g sodium nitrate, 1000 u heparin in 1 L distilled water). Brains were fixed overnight at 4 °C with 4% paraformaldehyde and 50 µm sections were cut with a vibratome on the following day. For pS6 immunostaining, sections were blocked in 0.1 M PBS buffer containing 0.2% Triton X-100, 5% normal donkey serum, and 5% bovine serum albumin for 2 h at room temperature and then incubated with an anti-pS6 Ribosomal Protein (S240/244) (1:1000, Cell Signaling, cat# 2215) antibody for 72 h in a cold room. Sections were washed 3 times in PBS and incubated with Alexa 568-conjugated anti-rabbit IgG (1:500; Life Technologies, cat# A10042) for 2 h at room temperature. Tissues were washed, dried, and mounted with VECTASHIELD media containing DAPI. Images were acquired using the AxioObserver CLEM fluorescence microscope (Carl Zeiss) at the Einstein Analytical Imaging Center.

2.5. Analysis of RNA from hypothalamic NPY neurons by flow sorting and microarray analysis
The mediobasal hypothalamus from Npy-hr animals was dissected and dissociated for 30 min with Papain enzyme. After washes and filtration, cells were treated with DAPI (20 µg/mL) to label dead cells and sorted on a Beckman-Coulter (Dako) MoFlo XDP FACS cell sorter for FITC signals (detecting GFP) and Indo1 signals (detecting DAPI). Live cells that are GFP positive (GFP+) and DAPI negative (D−) were sorted for RNA extraction. RNA quality was assessed with Agilent Bioanalyzer 2100. The amplification and hybridization on the Affymetrix Mouse Gene 1.0 ST were performed by the core facility of Albert Einstein College of Medicine. Raw data were normalized using the robust multiarray average algorithm using the Affymetrix Expression Console Software 1.1 and data from three independent replicates (each replicate represents a pool of at least three individuals) were used to assess expression. A cut-off of 32 (mean ± 2 SD) was calculated from negative controls and signals below this detection limit indicate no or low expression.

2.6. Statistical analyses
The data are presented as means with standard deviations. Differences were significant with p values less than 0.05. Paired t-tests were conducted where appropriate and indicated in the legends. For small groups (n = 3 or 4), we performed non-parametric tests, such as the rank sum test.

3. RESULTS

3.1. Gavage feeding with taurocholic acid induces FGF15 in the ileum and improves glucose tolerance in ob/ob mice
We used a 5-day protocol of oral gavages with sodium taurocholate in obese leptin deficient mice followed by an oral glucose tolerance test on the fifth day. This protocol increases circulating concentrations of taurocholic acid in the blood by slightly greater than two-fold (Table 1). Deoxycholic acid was also increased in the blood although unconjugated cholic acid was not significantly elevated (Table 1).

Table 1 – Bile acid composition analysis after short term taurocholate gavage. Mice were gavaged with either saline (control; n = 10) for 4 days or taurocholic acid (taurocholate gavage; n = 10) for 5 days. Serum bile acid concentrations are presented in mM with standard deviations for the groups. A * symbol denotes a significant difference (p < .01; paired t-test) and a ** symbol denotes a significant difference (p < .005, paired t-test).

|                   | Control | Taurocholate gavage |
|-------------------|---------|---------------------|
| Primary conjugates|         |                     |
| Taurocholic acid  | 25.2 ± 9.4 | 59.6 ± 28.2*** |
| Tauro-x-muricholic | 17.3 ± 7.2 | 11 ± 6.6  |
| Tauro-B-muricholic | 11.5 ± 3.7 | 10 ± 6   |
| Taurochenodeoxycholic acid | 1.9 ± 0.8 | 2.2 ± 1.7  |
| Cholic acid       | 52.1 ± 63.4 | 74.4 ± 59.4 |
| Cholic acid       | 52.1 ± 63.4 | 74.4 ± 59.4 |
| Primary unconjugates |       |                     |
| Chloroacetic acid | 4.0 ± 1.6  | 1.2 ± 1.2  |
| Deoxycholic acid  | 6.4 ± 2.6  | 13.6 ± 8.3* |
| Lithocholic acid  | 1.5 ± 0.2  | 1.5 ± 0.4  |
| Deoxycholic acid  | 6.4 ± 2.6  | 13.6 ± 8.3* |
| Lithocholic acid  | 1.5 ± 0.2  | 1.5 ± 0.4  |

Serum concentrations of other bile acids were not altered by the gavage. The taurocholate gavages did not alter body weights or food intake (data not shown). Oral glucose tolerance was significantly improved by taurocholate gavage of leptin deficient obese (ob/ob) mice (Figure 1). We also found that taurocholate gavage increased FGF15 and preproglucagon mRNA (the precursor for GLP1 and GLP2) around 2-fold in the ileum (Figure 2), suggesting that stimulation of two different bile acid receptors (FXR and GPBAR1) during the gavage may be involved.

We performed oral glucose tolerance tests in ob/ob female mice to determine whether taurocholic acid could improve glucose handling (Figure 1). The taurocholate treatment produced a dramatic improvement in glucose tolerance, with blood glucose concentrations returning to baseline values within 30 min of glucose gavage whereas, in the control condition, glucose concentrations did not return to normal until 90 min after the glucose gavage. While the initial studies were done in mixed sex ob/ob animals, it is unlikely that gender would have a strong effect since ob/ob mice are infertile due to a lack of progression through puberty, suggesting that sex differences would be minimal relative to lean adult mice. Nevertheless, we studied the effects of taurocholate in a second and separate cohort of 9 ob/ob females. We observed similar decrements in the areas under the curve during an oral glucose tolerance test following 5 days of taurocholate treatment (Figure 1B).

To determine whether stimulation of incretin secretion by activation of GPBAR1 by any bile acid ligand was involved in the improved glucose tolerance, we gave lithocholic acid (Figure 1A), a potent agonist of GPBAR1 but a known antagonist of FXR, to ob/ob mice with the same 5-day protocol of oral gavage. In this case, lithocholic acid did not have a significant effect on glucose tolerance. These results are highly suggestive of a causal association between taurocholate supplementation, FXR activation of FGF15 expression, and improved glucose tolerance, whereas GPBAR1 activation may be of lesser significance or remained uncaptured with our protocol due to timing of the GTT, use of ob/ob mice or some other factors. Alternatively, it is possible that both arms of bile acid signaling, via both FXR and TGR5/GPBAR1 concurrently, may be necessary for effective improvement in glucose handling.
3.2. Melanocortin signaling is necessary for the improved glucose tolerance induced by taurocholate

We tested our prior observations that the ability of FGF19 to ameliorate glucose intolerance is dependent on melanocortins. Using the same 5-day gavage of taurocholate, we tested the responses of ob/ob Agrp⁻/⁻ and Mc4r⁻/⁻ mice (Figure 1B, C). We observed no improvement in glucose tolerance for either of these obese mutant models, indicating that suppression of inhibitory melanocortinergic signaling, likely within the hypothalamus, is necessary for the beneficial effects of taurocholic acid on glucose tolerance. In support of this provisional conclusion, taurocholate treatment of wild type mice caused a reduction in hypothalamic AGRP and NPY mRNA whereas POMC mRNA was not affected (Figure 2).

3.3. FGF receptors in hypothalamic AGRP/NPY neurons modulate glucose handling and the ability of taurocholate to improve glucose tolerance

We examined the expression of FGF receptors in hypothalamic AGRP/NPY neurons. Using hypothalami from Npy-hrGFP mice, we isolated a dual positive (GFP-positive and DAPI-positive) fraction by fluorescence
activated cell sorting. RNA isolated from this material was used to probe microarrays by hybridization (Figure 3). The data indicate the expression of three FGF receptors: FGFR1, FGFR2 and FGFR3 in hypothalamic AGRP/NPY neurons. The signal for FGFR4 was below the detection limit of the assay.

In order to define the receptor isoform required for the response to taurocholate, we generated mice that lacked either FGFR1 or FGFR2 in hypothalamic AGRP/NPY neurons (AGRP: \( Fgfr1^{-/-} \) are mice with the following genotype: \( Fgfr1^{-/-} Agrp-CRE \) and AGRP: \( Fgfr2^{-/-} \) are mice with the following genotype: \( Fgfr2^{-/-} Agrp-CRE \)). We have not been able to examine \( Fgfr1^{-/-} \) or \( Fgfr2^{-/-} \) mice due to embryonic lethality. We tested the ability of taurocholate to improve glucose tolerance in AGRP: \( Fgfr1^{-/-} \) mice. While \( Fgfr1^{-/-} \) mice showed an improvement in glucose tolerance after taurocholate gavage, the AGRP: \( Fgfr1^{-/-} \) mice did not show a reduction in glucose tolerance (Figure 4). Paradoxically, we did not see a worsened glucose tolerance in these mutant FGFR1 mice relative to their control littermates, despite the lack of response to taurocholate. Fasting insulin concentrations did not differ between the control (0.48 ± 0.17 ng/ml) and taurocholate (0.41 ± 0.17 ng/ml) treated states and the presence of the CRE transgene did not affect insulin concentrations. High fat feeding did not affect glucose tolerance in these mice as the areas under the curve of the GTTs were similar (\( 24,843 \pm 4187 \text{ mg-min/dL}; \) AGRP: \( Fgfr1^{-/-} \) − 21,993 ± 3064 mg-min/dL).

AGRP: \( Fgfr2^{-/-} \) mice improved their glucose tolerance after taurocholate gavage in a manner similar to their FGFR2-intact littermates (Figure 4). However, the loss of FGFR2 worsened glucose handling in the saline gavage state compared to their control littermates, suggesting that an FGF ligand which is not regulated by bile acids is an important signaling component for glucose control. We did not see any effect of taurocholate gavage on body weight in these mice.

Figure 2: Taurocholate affects gene expression in the intestine and the hypothalamus. Male and female obese mice (ob/ob; group sizes were 5–6 mice with at least 2 mice of each sex per group) were gavaged with sodium taurocholate or water for 4 days. At day 4, the mice were sacrificed. RNA was isolated from the ileum [panel A] and hypothalamus [panel B] for quantification of FGF15, Glucagon (precursor for GLP1), AGRP, NPY, and POMC mRNAs by quantitative RT-PCR. An asterisk (*) denotes a statistically significant difference from the control state (water gavage) (p < .05, one sided t-test).
on standard chow (changes in body weights after the gavage were \( Fgfr2^{-/-} / Fgfr2^{-/-} 0.98 \pm 0.91 \) and AGRP: \( Fgfr2^{-/-} / Fgfr2^{-/-} 1.00 \pm 1.72 \)). We also investigated the responses of these mice with absence of FGFR2 in AGRP/NPY neurons in terms of their glycemic responses to a high fat diet. The mice with absence of FGFR2 in AGRP/NPY neurons showed a remarkably better glucose tolerance than their control littermates in both the oral glucose tolerance test and insulin tolerance test (Figure 5). There did not appear to be any differences in insulin secretion among the two genotypes either at baseline or 15 min after a glucose load. These data indicate better insulin sensitivity due to the absence of FGFR2 in AGRP/NPY neurons. In addition, the AGRP: \( Fgfr2^{-/-} / Fgfr2^{-/-} \) mice weighed slightly less due to a lower amount of fat mass. Taken together, the bidirectional glucose homeostatic responses of mice lacking FGFR2 in specific

**Figure 3:** Expression of FGF receptors in FACS sorted hypothalamic NPY neurons. (A) FACS plot obtained from brain dissociation of control and Npy-hrGFP mice. (B) qRT-PCR analysis of Npy and Pomc mRNA in neurons isolated from MBH of Npy-hrGFP animals, that are DAPI negative (D\(^{-}\)) and GFP\(^{-}\) or GFP\(^{+}\). (C) Expression profiles of Fgfr1, Fgfr2, Fgfr3, Fgfr4, Npy, Agrp, and Pomc genes determined by microarray in sorted NPY/AgRP neurons (GFP\(^{+}\)) from Npy-hrGFP mice. Signals below the detection limit (red line) indicate no or low expression.
hypothalamic neurons point to an important regulatory function of FGF receptor ligands, potentially with a multiplicity of actions by endogenously produced FGFs within the hypothalamus as well as endocrine FGFs, such as FGF15/19 and FGF21. Locally produced FGFs would be retained within the extracellular matrix due to binding to heparin conjugates whereas FGF15/19 are produced in association with meals, pointing to potential differences in chronicity of the signaling modalities for different FGFs.

Figure 4: FGFR2 on AGRP/NPY neurons is necessary for normal glucose homeostasis. Mice (8–9 months old; group sizes were 4–5 male mice per group) without FGFR1 [panels A and B] or FGFR2 [panels C and D] in AGRP/NPY cells were treated with taurocholate or saline for 5 days. An oral glucose tolerance test (2 mg/g of glucose) was administered on day 5. A # symbol signifies a statistically significant difference due to taurocholate (p < .05, paired t-test). An asterisk (*) denotes a statistically significant difference due to genotype (p < .05, t-test). A summary of the areas under the curves is presented in panel E.
3.4. A model of antagonism between FGF receptor homodimers and FGF receptor-beta Klotho heterodimers

While FGF15/19 and FGF21 require binding to beta Klotho FGF receptor heterodimers, the FGF8 family members (FGF8, FGF17, FGF18) bind to FGF receptor homodimers (Figure 6). Indeed, structural studies indicate that the docking site for beta Klotho to FGF receptors occupies the same space as the binding site for FGF8 family members. While FGF8 is not expressed in the adult murine hypothalamus, FGF17b and FGF18b mRNAs are expressed in the adult mouse hypothalamus (based on RT-PCR of hypothalamic mouse RNA, data not shown). Based on the requirements of the N terminal helix of FGF8b and FGF17b for binding to the IIIc splice isoforms of FGF receptors, the ones that are predominantly expressed within the CNS, we tested two short peptides (F8b13 and F17b13) with predicted helical structure as well as a mutated peptide F8b13-ProPro for their capacities to affect glucose homeostasis (Figure 6). The mutated peptide substitutes two helix breaking prolines for the two critical residues known to be required for ligand-receptor interaction. When injected for 4 days into the third ventricles of lean mice on normal chow, F8b13 produced consistent improvements in glucose tolerance (Figure 7). The mutant F8b13-ProPro did not cause any change in glucose tolerance. We saw no differences in body weight over the treatment periods due to treatments with the peptides (aCSF 1.3 g/C6 2.1 g; F8b13/C0 0.5 g/C6 2.1 g; F8b13ProPro 2.7 g/C6 2.8; data are means for the change in

Figure 5: FGFR2 renders mice susceptible to insulin resistance and fat accumulation from a high fat diet. Mice (3–4 months old, group sizes were 5–8 male mice per group) without FGFR2 in AGRP/NPY neurons were fed a high fat (60% fat by calories) diet from Research Diets for 2–3 months started around weaning. Body weights and compositions [panels A and B] were ascertained by NMR spectroscopy of hydrogen atoms. Glucose tolerance tests [panel C] were performed after an overnight fast and blood was withdrawn from a tail vein during the GTT for insulin determinations [panel E]. An insulin tolerance test was also performed on the same animals [panel D]. An asterisk (*) represents a significant difference (p < .05) due to genotype for panels A–D. For panel E, an asterisk (*) signifies a difference due to the glucose load during the GTT.
body weights over the treatment periods and standard deviations for 4 mice in each treatment group). We also tested the potential of F17b13 as a therapeutic agent with intraperitoneal injections into mice implanted with continuous glucose monitoring (CGM) devices (Figure 8). Peripheral administration of F17b13 in mice implanted with CGMs showed that the mice experienced a decrease in daily average blood glucose as well as the anticipated improvement of glucose clearance during an oral glucose tolerance test. Observation of the continuous glucose monitor data did not show any episodes of hypoglycemia. As the devices incorporate a temperature sensor, we were also able to measure core body temperature during treatment with F18b13. We did not observe any change in average daily body temperature as telemetrically recorded by the CGM device (control: 36.277°C vs F17b13: 36.225°C; NS).

We also tested the ability of FGF8 and FGF17 injected icv to affect glucose metabolism during an oral GTT. Neither FGF17 (AUC FGF17: 21,691 ± 2961 min-mg/dL, n = 6 male mice vs AUC aCSF: 22,663 ± 1094 min-mg/dL, n = 4 male mice) nor FGF8 (AUC FGF8: 16,850 ± 1038 min-mg/dL, n = 6 male mice vs aCSF: 15,785 ± 1710 min-mg/dL, n = 6 male mice) produced any change in glucose tolerance. This is not a surprising result as secreted FGFs of the FGF8 family are bound to heparan sulfates within the extracellular matrix and are likely to act in a persistent fashion due to this localization. Endogenously produced FGF17 could be exerting a ceiling effect such that exogenous FGF17 may not have contributed any additional effect.

We also investigated the specificity of F8b13 when delivered peripherally. After a 4-day treatment with F8b13 injected ip, a group of Npy-hrGFP mice were fasted overnight along with a control group of Npy-hrGFP mice that had been injected ip with saline (Figure 9). Using phosphorylated S6 ribosomal protein as a marker for neuronal activation, similar to Fos-ir as such a marker, we determined that F8b13
Figure 7: F8b13 injected icv improves oral glucose tolerance in lean mice. Lean mice (n = 6 lean males) were implanted with third ventricular cannulae and injected daily for 4 days with F8b13, F8b13-ProPro, FGF8, or aCSF. An oral glucose tolerance test [panel A] was performed on day 4. Glucose tolerance was improved for mice injected with F8b13, compared to the aCSF injected state. In a separate trial with 6 mice, F8b13 also reduced the AUC during an oGTT compared to the aCSF injected state (panel B). The summary AUC comparison is provided in panel C. A symbol (#) represents a significant difference due to F8b13 treatment (p < .05, paired t-test). The mutated peptide with two proline to disrupt the helical structure did not alter glucose tolerance. Mice injected with FGF8 [panel D] also did not show any alteration in glucose tolerance.
Figure 8: F17b13 lowers daily average blood glucose and improves glucose tolerance. Lean mice \((n = 4)\) were implanted with a continuous glucose monitors from DSL. After recovery, the mice were used in a cross-over design to test the effect of peripherally injected F17b13. Compared to the saline injected state [panel A], F17b13 improved oral glucose tolerance in the same mice [data presented as means and standard deviations] when injected daily for 4 days \((p < .01, \text{paired t-test})\). Concurrent data collected from the continuous glucose monitor showed a lowering of daily average blood glucose for day 5 [panels B showing plots for one mouse and C showing the means and standard deviations for the two treatments]. We discounted days 3 and 4 due to the manipulations of diet involved in performance of the glucose tolerance test. A representative daily record is shown in panel B but all 4 mice in the test showed a decrease in daily average blood glucose associated with F17b13 treatment \((p < .05, \text{paired t-test})\).
and GPBAR1. Within the gut, GPBAR1 is expressed on enteroendocrine cells and stimulate the release of multiple gut hormones [4] such as GLP1, GLP2, and secretin, while FXR is expressed principally in the absorptive enterocytes of the ileum and, as a heterodimerizing partner of RXR, stimulates the expression and secretion of FGF15 in mice and FGF19 in humans [14]. In the circulation, FGF19 activates FGF receptors in the liver to down-regulate hepatic bile acid synthesis [17]. FGF19 can also access hypothalamic neurons that send projections outside of the blood brain barrier, principally AGRP/NPY neurons [5,23]. We [19] and others [22] have shown that FGF19 acts centrally to improve glucose handling, primarily via an insulin-independent mechanism.

In this report, we have extended our prior studies on FGF19 actions to involve bile acids and implicate bile acid signaling as a major factor in glucose homeostasis. Prior studies have shown that dramatic alterations in bile acid composition are achieved by exogenous provision of single bile acids. Our current study indicates that short 5-day treatment is sufficient to only alter taurocholic acid concentration. Taurocholate, a taurine conjugate of cholic acid, is a primary bile acid and a major component of bile in both rodents and humans. Cholic acid feeding in mice shifts bile acid composition to mainly cholate and deoxycholate and their conjugates [31]. Endogenous cholate and muricholate is supplanted by exogenous cholate which undergoes bacterial biotransformation to deoxycholate. During enterohepatic circulation, the deoxycholate can be further conjugated to taurine. It is possible that some of the beneficial effects are due to the absence of muricholates [30] as muricholic acid has been shown to be an FXR antagonist although our data indicated that muricholic acids were not reduced by our 5-day gavage (Table 1). Due to the taurocholate supplementation, the composition of the bile acid pool has been shifted to contain more of an FXR agonist. Our initial model, leptin signaling deficient mice, is predisposed to identification of insulin-independent mechanisms of glucose control due to the severe insulin resistance of ob/ob mice and their high circulating insulin concentrations [3]. For this model, small changes in incretin release are unlikely to affect glucose tolerance. Moreover, we attempted to mimic our prior 5 daily intracerebroventricular injections of FGF19 [19] with 5 daily gavages of taurocholate. We have been unable to quantify FGF15 peptide due to a paucity of reagents and the low degree of homology (50%) between FGF15 and FGF19 at the amino acid level, precluding the use of FGF19 antibodies.

We implicated melanocortins as a critical component of a gut—brain signaling axis for bile acids by demonstrating the inability of mice without AGRP or MC4R to improve glucose tolerance upon taurocholate treatment. While it might appear to be counterintuitive that the absence of MC4R would mimic the absence of AGRP, AGRP has been identified as a biased agonist that causes MC4R to activate downstream signals independent of the classical stimulatory Gs complex [7,21]. We have previously shown that the presence of AGRP and MC4R is required for FGF19 to improve glucose handling [19]. Due to the co-expression of Neuropeptide Y and GABA with AGRP in hypothalamic neurons, it is possible that other neurotransmitter systems (NPYergic and GABAergic) are similarly involved.

FGF signaling involves activation of FRS1/2, ERK1/2, and STAT3 [8], signaling pathways that overlap with those relevant to insulin receptor and leptin receptor. Moreover, FGF19 suppresses AGRP/NPY neurons [19] in a manner analogous to leptin [39], suggesting that FGF19/FGF15 could partially mimic the effects of leptin. However, unlike leptin, FGF19 had no effect on POMC transcription. FGF receptors can function as homodimers or as heterodimers with beta Klotho, indicating unexpected complexity of the FGF ligand and FGF receptor

4. DISCUSSION

Bile acids were recognized as signaling molecules that have effects in multiple tissues due to the discovery of two bile acid receptors, FXR

Figure 9: F8b13 decreases S6 phosphorylation in hypothalamic AGRP/NPY neurons. Lean Npy-hrGFP mice (n = 3 per group) were injected ip with saline [panel A] or F8b13 [panel B] for 4 days. On the night of the 4th day, food was removed and mice were subjected to an overnight fast. The brains were harvested on the morning of day 5 or F8b13 [panel B] for 4 days. On the night of the 4th day, food was removed and mice were subjected to an overnight fast. The brains were harvested on the morning of day 5

Reduced the number of NPY neurons by nearly 50% that were positive for pS6. Indeed, much of the pS6 labeled neurons were not GFP+, suggesting that some other neuronal types were activated by the F8b13 treatment.

Figure 9: F8b13 decreases S6 phosphorylation in hypothalamic AGRP/NPY neurons. Lean Npy-hrGFP mice (n = 3 per group) were injected ip with saline [panel A] or F8b13 [panel B] for 4 days. On the night of the 4th day, food was removed and mice were subjected to an overnight fast. The brains were harvested on the morning of day 5 and processed for S6 phosphorylation. While native GFP were subjected to an overnight fast. The brains were harvested on the morning of day 5 or F8b13 [panel B] for 4 days. On the night of the 4th day, food was removed and mice were subjected to an overnight fast. The brains were harvested on the morning of day 5 and processed for S6 phosphorylation. While native GFP

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system. Another revelation was the diminished glucose tolerance of mice without FGFR2 in AGRP/NPY neurons. This result suggests that other FGF ligands, perhaps FGF17 or some other FGF expressed in the hypothalamus, can modulate glucose handling.

Our findings may have relevance to several clinical presentations. In cases of ileal resection due to inflammatory bowel disease, necrotizing enterocolitis or intestinal tumors, the loss of FGF19 secretion would make such individuals prone to develop glucose intolerance, an additional burden that could complicate their fragile nutritional state. After gastric bypass, principally Roux-en-Y and biliary diversion, the rapid presentation of bile acids to the ileum after a meal is likely to shift the time of release of FGF19/15 and incretins after a meal. This phenomenon is likely to be enhanced after intestinal adaptation after gastric bypass increases the mass of absorptive enteroctyes and enteroendocrine cells [33]. The long-term effects of enhanced actions of FGF19/15 and incretins is likely to account for the beneficial effects of gastric bypass on glucose handling.

Despite the beneficial effects of bile acids on glucose control, long-term treatment with taurocholate has detrimental effects due to the suppression of bile acid synthesis. As bile acid synthesis constitutes a significant route of cholesterol disposal due to fecal excretion of bile acids, suppression of bile acid synthesis, either by bile acid supplementation or FGF15/19 treatment, will cause cholesterol to accumulate in the liver and the circulatory system, potentially increasing cardiovascular risk. In addition, chronic overexpression of FGF19 in mice leads to the development of hepatic tumors, principally by the phosphorylation of STF3 from activation of hepatic FGFR4 [41]. Our results suggest that suppression of hypothalamic AGRP/NPY neurons or enhancing positive melanocortinergic signaling could be of potential benefit in improving glucose homeostasis in both lean and obese states.

Finally, we have tested the potential beneficial effects of blocking FGF signaling in the hypothalamus with short peptides that prevent formation of homodimers and heterodimers of FGF receptors. The end result is an improvement in glucose metabolism as shown by improved glucose tolerance, improved insulin sensitivity and lower daily average blood glucose. While these results might be counter-intuitive, there are reports that antibodies to FGF receptors with activating activity or with blocking activity have intuitively been predicted to reduce FGF receptor activity or with blocking activity [16,28,32,37] have been reported to improve glucose metabolism. However, antibodies are dimeric and could force the dimerization of FGF receptors with initiation of signal transduction via the ligand independent enforced dimerization. In a slightly different perspective, our peptides specifically bind to FGF receptor monomers and reducing the number of free FGF receptor monomers would enhance the likelihood of FGF receptors forming heterodimers with beta Klotho. This can be heuristically understood in the reductionist situation where only one FGF receptor monomer molecule on a given cell was not blocked by the peptide. This free monomer can only partner with beta Klotho as there is no available FGF receptor for homodimer formation. We would emphasize that this is a complex system with multiple ligands and receptor complexes operating simultaneously for which simple models are likely to be inadequate. Further long-term testing with these peptides might reveal the real therapeutic potential of manipulating hypothalamic FGF signaling.

5. CONCLUSION

We have provided evidence for a gut–brain axis that is likely engaged during and after meals to improve glucose homeostasis, insulin sensitivity, and metabolism. The axis involves bile acid receptors in the gut, secretion of the endocrine FGF19/15 from enterocytes with secretion of incretins from enteroendocrine cells, and silencing of hypothalamic AGRP/NPY neurons. In addition, the FGF signaling within the hypothalamus is likely to involve FGFs produced and secreted endogenously within the hypothalamus that antagonize the action of FGF19/15. Short peptides derived from FGF8 and FGF17 are able to block homodimeric FGF receptors and alleviate the antagonism between classical FGFs and endocrine FGFs, thus providing a means of improving glucose handling and minimizing episodes of hyperglycemia.

ACKNOWLEDGMENTS

This work was supported by the Einstein-Sinai Diabetes Center (P60DK020541), a pilot grant from Novo Nordisk and the New York Obesity Nutrition Research Center (P01DK026687). We would like to acknowledge the assistance of the Einstein-Sinai Diabetes Center’s Animal Phenotyping Core and the Metabolomic Core of the Einstein-Sinai Diabetes Center in the conduct of this project.

CONFLICT OF INTEREST

This work was partially funded by a pilot grant from Novo Nordisk to SC.

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