endocrines

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Bitter Phytochemicals Acutely Lower Blood Glucose Levels by Inhibition of Glucose Absorption in the Gut

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Abstract: For early hominids, frequent encounters with plant foods necessitated the ability to discern bitter poisons and adjust the activity of the gastrointestinal system in anticipation of carbohydrate-rich meals. Plants bitters were also used historically to manage a variety of metabolic and digestive disorders despite an immense structural diversity of bitter phytochemicals without a common molecular target. Our study confirms these observations in a standardized C57BL/6J prediabetic mouse model using 24 model compounds by demonstrating acute lower peak blood glucose values and improved glucose tolerance following intragastric, but not intraperitoneal, treatment. The administration of the synthetic bitter compound denatonium benzoate yielded similar results that were attenuated by co-application of the allosteric inhibitor of the bitter TAS2R receptors. We also show that these effects occur dose-dependently; associate with reduced glucose uptake, increased intracellular [Ca^{2+}] fluxes, and enhanced GLP-1 expression; and are attenuated by the TAS2R inhibitor in the neuroendocrine STC-1 intestinal cells. These findings support the view that inhibition of glucose transport from the intestinal lumen to the blood by TAS2R bitter receptor signaling in the gut may represent a common mechanism in the acute response to oral ingestion of bitter phytochemicals.

Keywords: diabetes; pre-diabetes; glucose management; glycemic control; plant metabolites; antidiabetic plants; glucose homeostasis

Citation: Palatini Jackson, K.M.; Mhawish, R.; Komarnytsky, S. Bitter Phytochemicals Acutely Lower Blood Glucose Levels by Inhibition of Glucose Absorption in the Gut. *Endocrines* **2024**, *5*, 304–322. https://doi.org/10.3390/endocrines5030022

Academic Editor: Daniela Foti

Received: 11 June 2024
Revised: 11 July 2024
Accepted: 15 July 2024
Published: 25 July 2024

1. Introduction

Health issues related to type 2 diabetes (T2D) frequently arise prior to the formal diagnosis of the condition. Elevated fasting blood sugar levels below the threshold of T2D (100–125 mg/dL or 5.6–6.9 mmol/L) is defined as an important metabolic risk factor in the association between obesity, insulin resistance, dyslipidemia, and hypertension [1]. Although a useful diagnostic tool, impaired fasting plasma glucose alone is often not sufficient to identify up to 31% of diabetic subjects [2], and the capillary whole blood measures on home glucose monitoring devices are about 15% lower [3]. Likewise, more than half of diabetic subjects are missed when using 6.5% HbA1c as the primary diagnostic test [4]. For these reasons, the oral glucose tolerance test (OGTT) standardized to the oral glucose load of 75 g and the 2h post-load glycemia (2hPG more than 200 mg/dL or 11.1 mmol/L) was widely adopted for detecting prediabetes and T2D [5]. The addition of the 1h post-load glycemia (1hPG more than 155 mg/dL or 8.6 mmol/L) as a surrogate measurement of insulin sensitivity and pancreatic β-cell function was also substantiated [6].

T2D typically trails the rise of obesity with an average delay of 10–15 years [7], and several oral and injectable glucose-lowering medications have been developed to achieve and maintain T2D glycemic control, including subcutaneous insulin (Lantus, NovoRapid, Humalog), insulin sensitizers (metformin, rosiglitazone), insulin secretagogues (glipizide), alpha-glucosidase inhibitors (acarbose), sodium-glucose cotransporter 2 (SGLT2) inhibitors

(empagliflozin, dapagliflozin), and incretin mimetics such as glucagon-like peptide 1 (GLP-1) receptor agonists (semaglutide, dulaglutide) and dipeptidyl peptidase-4 (DPP-4) inhibitors (sitagliptin) [8]. While effective, these medications are often associated with gastrointestinal side-effects, risk a developed tolerance, and can be costly [9]. Prediabetes hyperglycemia, however, can often be managed with lifestyle interventions to change diet and physical activity [10]. Early diagnosis of impaired glucose tolerance or fasting glucose in clinical and home settings is therefore intended to give a person the option to reverse the onset of T2D via lifestyle modifications before requiring a prescription drug.

The tradition of managing elevated blood glucose with botanical preparations as a part of dietary modification dates back to antiquity and probably earlier [11]. In fact, some glucose-lowering drugs have been developed based on natural product pharmacophores, including metformin derived from the alkaloid galegine found in goat's rue (*Galega officinalis* L.) [12] and empagliflozin derived from the dihydrochalcone glucoside phlorizin found in apple bark (*Malus sylvestris* Mill.) [13]. Culinary herbs and spices, including chicory (*Chicorium intybus* L.; chlorogenic acids, sesquiterpene lactones) [14], fenugreek (*Trigonella foenum-graecum* L.; saponins) [15], cinnamon (*Cinnamomum cassia* (L.) J. Presl; cinnamaldehyde, type A procyanidins) [16], bitter melon (*Momordica charantia* L.; saponins, terpenes) [17], Japanese gentian (*Gentiana scabra* Bunge; iridoid glycosides), and wormwood (*Artemisia* spp.; sesquiterpene lactones, flavonoid glycosides) [18] have been extensively used to manage T2D hyperglycemia in different parts of the world. Moreover, despite wide differences in structure and metabolism [19], all major classes of phenolic compounds, including phenolic acids, anthocyanins, flavonoids, chalcones, stilbenes, lignans, and tannins, were shown to modulate glucose metabolism as summarized elsewhere [20,21]. The wide variety of aforesaid compounds have very few structural similarities, but strikingly, each of these compounds has a reported bitter taste.

Bitter taste is perceived by a dedicated class of type 2 receptors (TAS2Rs) found in gustatory tissues [22] and is classically thought to allow for avoidance of bitter tasting toxins in foods [23]. TAS2Rs are part of the G protein-coupled receptor (GPCR) family that, upon activation, transduce the signal via dissociation of Gα-gustducin from Gβ3 and Gγ13 subunits [24]. This activates phospholipase C (PLCβ2), releases Ca^{2+} from IP3-sensitive (InsP3R) calcium stores, induces Na+ influx through the TRPM5 channel, depolarizes the cell, and causes the release of neurotransmitter ATP in part through the calcium homeostasis modulator channels (CALHM1/3) [25]. About 25 human [26] and 35 mouse [27] TAS2Rs have different molecular receptive ranges in low μM concentrations with both broad (TAS2R10, R14, R43, R46) and narrow (TAS2R1, R3, R4, R5, R7, R13, R50) specificity that allow for possible discrimination among a multitude of TAS2R ligands [28]. Additionally, large populations of extra-oral TAS2R receptors are found in cell membranes throughout the body, including the entire gastrointestinal tract, lungs, heart, kidney, erythrocytes, and macrophages [29], where TAS2R function expands beyond taste sensation [30]. Remarkably, many bitter but nontoxic substances are agonistic to both human and non-human TAS2Rs, despite large variability in diets, thus providing additional evidence for further functionality.

Because extra-oral bitter TAS2Rs are found throughout the gastrointestinal epithelium, it is logical to assume that they sample the intestinal luminal content without our awareness [31]. TAS2Rs co-localize with enteroendocrine cells in the gastrointestinal tract similar to the TGR5 receptor that recognizes bitter bile acids [32]. Our earlier studies hypothesized that the intestinal TAS2Rs evolved to recognize the diverse bitter-plant-derived phytochemicals and modulate glucose metabolism in anticipation of the incoming carbohydrate load from plant-based foods [33], in part by inducing intestinal glucagon-like peptide-1 (GLP-1) release and reducing gastrointestinal glucose absorption [34]. The objective of this study was to confirm the ability of diverse classes of plant phytochemicals associated with bitter tastes to alleviate postprandial hyperglycemia in a C57BL/6J mouse model of diet-induced obesity, and to determine the effect of TAS2R activation on glucose absorption, GPCR signaling, and gastrointestinal hormone expression.

2. Materials and Methods

2.1. Reagents and Diets

All chemicals, solvents, and reagents were purchased from Sigma (St Louis, MO, USA) or Fisher Scientific (Pittsburg, PA, USA). Effective bitter concentrations and human TAS2R affinities of bitter substances were used as summarized in the BitterDB database, last updated in 2018 [35]. Cell culture materials were from Life Technologies (Carlsbad, CA, USA), unless otherwise specified. Low fat diet D12450J (LFD, 10 kcal % from fat, 3.85 kcal/g) and high fat diet D12492 (HFD, 60 kcal % from fat, 5.24 kcal/g) were from Research Diets (New Brunswick, NJ, USA). All animal diets were kept at −80 °C to ensure long-term storage and stability.

2.2. Animal Study

Male, 6-week-old C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and housed, four animals per cage, under controlled temperature (24 ± 2 °C) and light (12 h light–dark cycle, lights off at 7:00 am). Upon arrival, animals were allowed to adapt to new conditions for 7 days and handling of the animals was performed daily to reduce the stress of physical manipulation. Mice (n = 32) were then randomized into 2 groups with ad libitum access to LFD (n = 8) or HFD (n = 24) diet for 8 weeks. HFD mice were considered prediabetic with overnight fasting blood glucose levels of 125 mg/dL, and diabetic if greater than 200 mg/dL. For each treatment, animals were newly randomized into the respective treatment groups as indicated, and 2 oral glucose tolerance tests were performed weekly, separated by a 3- to 4-day washout period in between. After the last treatment, blood was collected by cardiac puncture after CO_2 inhalation. Gastrointestinal (stomach, duodenum, jejunum, ileum, cecum, and colon) and metabolic (liver, gastrocnemius muscle, and epididymal fat) tissues were collected and stored at −80 °C to determine the temporal sequence and signaling events responsible for the observed changes in physiology and metabolism. All animal experimental procedures were carried out at the DHMRI Center of Laboratory Animal Sciences, an AAALAC-accredited facility, and approved by the NC Research Campus IACUC.

2.3. Oral Glucose Tolerance with Diverse Classes of Plant Phytochemicals

All treatments were gavaged at the dose range of 3–300 mg/kg, 30 min prior to glucose challenge as listed in Table 1. For oral glucose tolerance test (OGTT), mice were fasted overnight and received oral gavage of D-glucose (1.5 g/kg body weight). Gavage volume of 20% glucose solution was calculated in µL for each animal as 7.5× fasted body weight (g). Blood glucose concentrations were measured at 0, 30, 60, and 120 min after glucose challenge in blood samples obtained from the tail-tip bleedings, using the True Result glucometer (Trivida, Fort Lauderdale, FL, USA). To identify animals with impaired glucose tolerance, area under the curve (AUC120) was determined using the linear trapezoid method and presented as arbitrary AUC units [36]. All HFD controls were gavaged with vehicle alone (0.1% DMSO in water).

Table 1. Diverse classes of plant bitter phytochemicals and synthetic compounds used in this study.

Chemical Class	Compound	Effective [1] Conc., µM	Human [2] TAS2R	Dose, [3] mg/kg	Dietary Sources
Synthetic ref.	Denatonium benzoate	0.03–300	4, 8, 10, 13, 39, 43, 46, 47	3	Bitter synthetic
	Metformin	n/a	n/a	100	Diabetes medicine
Alkaloids	Caffeine	300	7, 10, 14, 43, 46	30	Coffee
	Quinine	10	4, 7, 10, 14, 39, 40, 43, 46	100	Tonic water
	Yohimbine	300	1, 4, 10, 38, 46	30	Dietary supplement
Phenolic acids	Gallic acid	220	4, 14	300	Celery seed, cloves, tea, wine
	Protocatechuic acid	100	14, 30	300	Star anise, chicory greens
	Syringic acid	570	n/a	300	Sage, thyme, oregano
	Vanillic acid	1500	14	300	Chestnut, basil, oregano, sage
Anthocyanins	Cyanidin, delphinidin	30–250	14, 39	100	Elderberry, berries, grape, plum

Table 1. *Cont.*

Chemical Class	Compound	Effective [1] Conc., μM	Human [2] TAS2R	Dose, [3] mg/kg	Dietary Sources
Flavones	Luteolin	2	14, 39	100	Mint, sage, oregano, artichoke
Flavonols	Quercetin	1	14, 39	100	Capers, cloves, cumin, caraway, oregano, buckwheat, onion
	Dihydroquercetin (taxifolin)	60–120	14, 39	100	
	Quercetin-3-O-glucoside	28	n/a	100	
	Quercetin-3-O-rutinoside	n/a	n/a	100	
Flavanones	Naringenin	10	14	100	Oregano, mint, citrus
Flavanols	Catechin	n/a	n/a	100	Chestnut, dark chocolate, cocoa powder, apple, tea
	Epicatechin	1000	4, 5, 14, 39	100	
Tannins	Proanthocyanidin monomers	See flavanols		100	
	PAC oligomers (DP 2–10)	400–500	n/a	100	Chokeberry, rosehips, cranberry, blueberry
	PAC polymers (DP 11+)	n/a	n/a	100	
	Ellagic acid	n/a	n/a	100	Chestnut, raspberry, blackberry
Stilbenes	Rhaponticin	n/a	n/a	100	Rhubarb
Inhibitor ref.	Probenecid	–	16, 38, 43	3	Gout medicine

[1] Effective μM concentration for bitter recognition threshold as listed in [35]. [2] Previously reported interactions with human TAS2Rs as listed in [35]. [3] Dosing ranges used in present study.

2.4. Glucose Tolerance and Different Routes of Administration

Denatonium benzoate, one of the most bitter synthetic compounds [37], was pretreated 30 min prior to glucose challenge at the dose of 3 mg/kg in the form of either oral gavage (bypassing the bitter sensation in the oral cavity), mouth rinse (applied to the oral cavity only, then discarded), and intraperitoneal injection (whole body administration outside of the gastrointestinal tract). These treatments were performed with and without 3 mg/kg probenecid, a synthetic compound that acts as a nonselective allosteric inhibitor of the broad specificity TAS2R16, TAS2R38, and TAS2R43 bitter receptors [38]. Probenecid is a sulfonamide in which the nitrogen of 4-sulfamoylbenzoic acid is substituted with two propyl groups. Subsequent OGTT and AUC120 determinations were performed essentially as described in Section 2.3.

2.5. RNA Extraction, Purification, and cDNA Synthesis

The total RNA was isolated from liquid-nitrogen-preserved duodenum tissues using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. RNA was quantified using the Biotek SynergyH1/Take 3 plate (Agilent, Santa Clara, CA, USA). The cDNAs were synthesized on ABI GeneAMP 9700 using the high-capacity cDNA Reverse Transcription kit and 2 μg of RNA (Life Technologies).

2.6. Quantitative PCR Analysis

The resulting cDNA was amplified by real-time quantitative PCR using SYBR green PCR master mix (Life Technologies). The following primers were used: β-actin as a housekeeping gene (NM_007393.3), forward primer: 5′-AAC CGT GAA AAG ATG ACC CAG AT-3′, reverse primer: 5′-CAC AGC CTG GAT GGC TAC GT-3′; bitter receptor TAS2R108 (NM_020502.1), forward primer: 5′-GGT CAA CAG TCG CAG AAT TGC-3′, reverse primer: 5′-TGT CCT GGA GGG TAA GCA GC-3′; proglucagon GCG (NM_008100.4), forward primer: 5′-TGA AGA CCA TTT ACT TTG TGG CT-3′, reverse primer: 5′-CCA AGT GAC TGG CAC GAG AT-3′; GLP-1 receptor (NM_021332.2), forward primer: 5′-CAG GGC TTG ATG GTG GCT ATC-3′, reverse primer: 5′-CGC TCC CAG CAT TTC CG-3′; cholecystokinin CCK (NM_031161), forward primer: 5′-CAC GAC CCC TCG CTT CTA A-3′, reverse primer: 5′-GGC TGC ATT GCA CAC TCT GA-3′.

Quantitative PCR (qPCR) amplifications were performed on an ABI 7500 Fast real-time PCR (Life Technologies) using 1 cycle at 50 °C for 2 min and 1 cycle at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The dissociation curve was completed with 1 cycle of 1 min at 95 °C, 30 s at 55 °C, and 30 s at 95 °C. mRNA expression

was analyzed using the ΔΔCT method and normalized with respect to the expression of the β-actin housekeeping gene. Amplification of specific transcripts was confirmed by obtaining melting curve profiles. The evolutionary relationships between human and mouse TAS2R genes are summarized in Table 2.

Table 2. Evolutionary relationships between human and mouse TAS2Rs, summarized after [27,39].

Bitter Taste Receptors	**Clustering Based on Multiple Sequence Alignment** [1,2,3]																										
Broad specificity (*)								*		*								*	*		*					
Human hTAS2R [4,5]	1	3	4	5	7	8	9	10	13	14	16	38	39	40	41	42	43	44	45	46	47	48	49	50	60		
Mouse mTAS2R	119	137	108	X 6		130		104	102	103	118	138	139	144	126	131				120					135		
								105	121	109	134									122							
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										125																	
										129																	
										140																	

[1] Blue denotes TAS2R genes localized to human chromosome 5 and mouse chromosome 15. [2] Green denotes TAS2R genes localized to human chromosome 7 and mouse chromosome 2. [3] Yellow denotes TAS2R genes localized to human chromosome 12 and mouse chromosome 6. [4] Human TAS2R 2, 12 (26), 15, 18, 62, 63, and 64 are not listed due to nonfunctional pseudogene status, but see [40]. [5] Human TAS2R 44 (31), 47 (30), 48 (19, 23), 49 (20), and 50 (51) gene names are synonymous. [6] Human TAS2R5 does not seem to have an ortholog in mice. *, broad specificity.

2.7. Cell Culture and Glucose Uptake Measurements

The mouse neuroendocrine intestinal cell line STC-1 (CRL-3254) that acts as a model for glucose absorption and hormone secretion was obtained from ATCC (Manassas, VA, USA). The choice of STC-1 was driven by the fact that these cells originate from the same stem cells near the base of the intestinal crypt as enterocytes, they express many sugar transporters, they respond to lower glucose concentration by upregulating the expression of sugar transporters [41], and they express and secrete GLP-1 [42] which was important to test our hypothesis. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies), supplemented with 10% fetal bovine serum (Life Technologies), 100 IU/mL of penicillin, and 100 µg/mL of streptomycin (Fisher Scientific) at a density not exceeding 5×10^5 cells/mL. Cells were routinely passaged every 3–4 days in Nunc cell culture dishes (Nalge Nunc International, Rochester, NY) maintained at 37 °C and 5% CO_2 in a humidified Thermo Forma Series II incubator (Fisher Scientific).

STC-1 cells were sub-cultured into 24-well plates and, once confluent, changed to the induction medium that contained glucose-free DMEM supplemented with 2 mM sodium pyruvate to facilitate fluorescent glucose analog uptake (2-NBDG, Fisher Scientific). Treatments were administered for 2 h before cells were exposed to 10 µM 2-NBDG for 20 min, washed with 1x PBS, and quantified spectrophotometrically with excitation/emission set at 465/540 nm.

2.8. Calcium Influx Assays

STC-1 cells were plated in 96-well plates at a concentration of 20,000 cells/mL and grown overnight. The growth medium was removed from the adherent cell cultures and 100 µL of Fluo-4 NW dye loading solution (Carlsbad, CA, USA) was added to each well. Probenecid acts as a standard inhibitor of nonspecific anion transport in the Fluo-4 NW Calcium Assay kit; however, because of the compound's inhibitory activity on G-protein coupled receptors, we tested for differences in calcium flux in the absence of probenecid in this assay as recommended previously [43]. The plates were incubated at 37 °C for 30 min, then at room temperature for an additional 30 min. Next, treatments were added at the

dose ranges specified for each test article and changes in fluorescens were recorded with excitation/emission set at 494/516 nm.

2.9. Cell Viability Assays

Cell toxicity was also evaluated using STC-1 cells treated with denatonium benzoate and probenecid over the dose ranges used in this study (1–30 μM). Briefly, cells were treated with vehicle (0.1% DMSO), cytotoxic control (6.5% DMSO), and serial dilutions of test compounds for 24 h, and cell viability was quantified spectrophotometrically at 570 nm using the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye as the indicator [44].

2.10. Statistical Analysis

Statistical analyses were performed using Prism 9.0 (GraphPad Software, San Diego, CA) and expressed as means ± SEM. Two-tailed *t*-test or one-way ANOVA were applied at a significance level of $p < 0.05$. Post-hoc analyses of differences between individual experimental groups were made using the Dunnett's multiple comparison test.

3. Results

3.1. Prediabetic Cohort of Diet-Induced Obese C57BL/6J Mice

The study consisted of two groups of C57BL/6J mice fed either a standard (LFD) diet or high-fat (HFD) diet starting at 7 weeks of age to induce obesity and hyperglycemia. Body weights and food intake were monitored weekly, and fasting blood glucose was observed every 4 weeks until the majority of the HFD animals reached the prediabetic state with the fasting blood glucose values in the range of 120–200 mg/dL (Table 3). Although the average fasting glucose after 4 weeks on HFD was observed at 138 ± 8 mg/dL, some animals remained below the 120 mg/dL threshold, and treatment was extended to 8 weeks.

Table 3. Metabolic phenotyping of LFD and HFD mice to establish a prediabetic HFD cohort. Values are mean ± SEM, two-way ANOVA with a post-hoc Bonferroni multiple comparisons test, * $p < 0.05$, ** $p < 0.01$ versus the respective LFD controls.

Parameters				**LFD**				
Weeks	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Body weight, g	24.6 ± 0.4	25.2 ± 0.4	24.3 ± 0.4	26.9 ± 0.4	26.6 ± 0.4	27.3 ± 0.4	27.6 ± 0.5	28.1 ± 0.5
Body weight gain, g		0.6 ± 0.7	0.4 ± 0.7	2.3 ± 0.7	1.9 ± 0.7	2.6 ± 0.7	2.96 ± 0.7	3.5 ± 0.7
Food intake, g/mouse/d	2.32 ± 0.05	2.57 ± 0.05	2.56 ± 0.07	2.73 ± 0.05	2.67 ± 0.05	2.80 ± 0.06	2.78 ± 0.07	2.76 ± 0.04
Fasting glucose, mg/dL				79 ± 2				79 ± 2
				HFD				
Weeks	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Body weight, g	28.8 ± 0.3	29.9 ± 0.4	30.9 ± 0.6	31.8 ± 0.5	31.6 ± 0.5	32.7 ± 0.6	33.2 ± 0.5	33.9 ± 0.8
Body weight gain, g		1.1 ± 0.7	2.2 ± 0.7 **	3.0 ± 0.7 *	2.8 ± 0.7 **	3.9 ± 0.7 **	4.4 ± 0.7 **	5.1 ± 0.7 **
Food intake, g/mouse/d	3.96 ± 0.26 *	3.56 ± 0.39 *	4.46 ± 1.27 **	2.83 ± 0.14	3.97 ± 0.11 *	3.61 ± 0.33 *	4.51 ± 0.26 **	4.07 ± 0.48 *
Fasting glucose, mg/dL				138 ± 8 **				159 ± 5 **

3.2. Glucose Tolerance When Treated with Diverse Classes of Plant Phytochemicals

HFD mice showed impaired glucose tolerance at 8 weeks of the study, with the highest glucose levels observed at the 30 min time point of OGTT testing (227 ± 54 mg/dL versus 135 ± 31 mg/dL in LFD controls). A bitter biguanide derivative metformin, the main first-line medication for the treatment of hyperglycemia associated with T2D, was able to ameliorate this effect in the prediabetic HFD mice when administered orally at 100 mg/kg, 30 min prior to glucose challenge (Figure 1a). The total AUC120 showed

a 2.1-fold increase associated with prediabetes, and a respective 2.2-fold decrease when treated with metformin (Figure 1b).

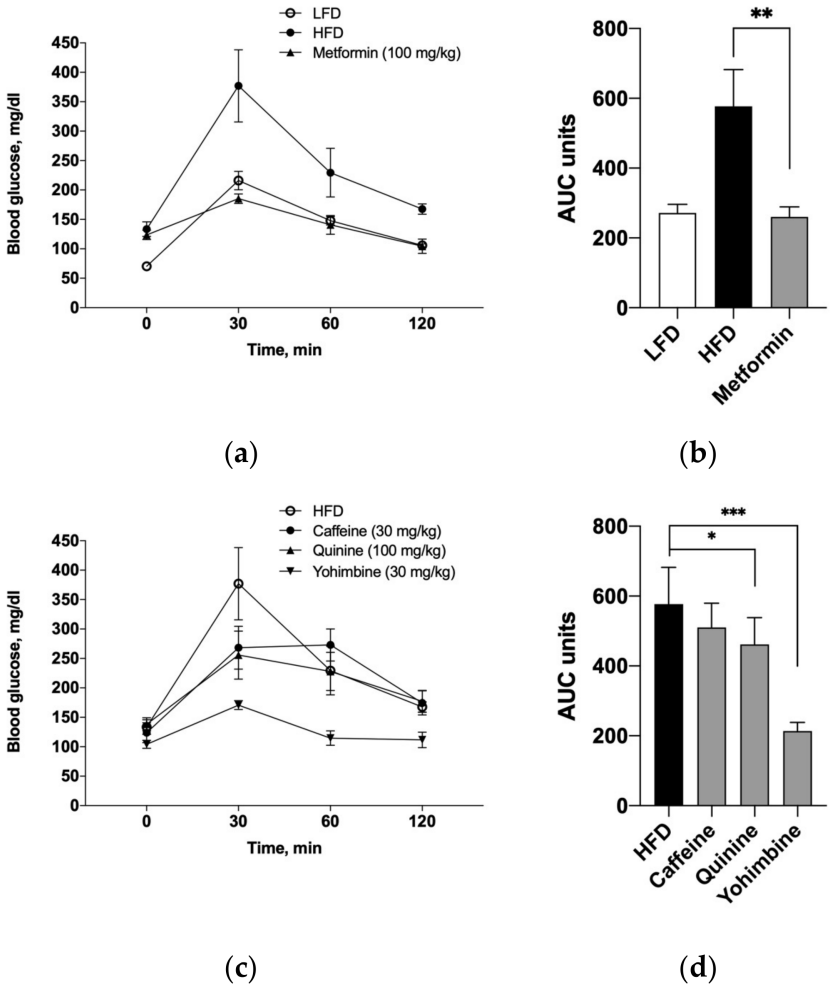

Figure 1. The modulation of OGTT parameters by a reference antidiabetic drug (metformin) and selected plant alkaloids (caffeine, quinine, and yohimbine). (**a**,**c**) OGTT curves were determined by sampling blood glucose at 0, 30, 60, and 120 min after the carbohydrate challenge. (**b**,**d**) The corresponding areas under the curve (AUC120). The results are expressed as means ± SD (n = 4). The data were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the respective LFD or HFD controls. The HFD controls were gavaged with the vehicle alone (0.1% DMSO in water).

Next, a series of OGTT assays were performed to determine the effects of diverse classes of bitter plant metabolites on glucose disposal in the prediabetic cohort of C57BL/6J mice. All substances were tested at dosing levels that reflected their expected bitterness, although data about the effective threshold concentrations and bitter receptor interactions are very fragmentary and unreliable (Table 1). Most alkaloids uniformly evoke a bitter taste, and administration of all three alkaloids tested in this study lead to significant improvements in peak blood glucose values recorded at 30 min (Figure 1c). Caffeine administration, however, extended the OGTT shoulder observed at 60 min that rendered the overall change in AUC120 glucose disposal nonsignificant versus the respective HFD control. Two other alkaloids showed significant decreases both in the OGTT and AUC120 values, up to 20.2% for quinine and 63.1% for yohimbine (Figure 1d).

Different classes of flavonoids, represented by naringenin (flavanones), luteolin (flavones), and quercetin (flavonols) aglycones, were tested as the same reference concentration of 100 mg/kg (Table 1). Both peak blood glucose values and overall AUC120 glucose ap-

pearances were significantly decreased by naringenin and quercetin, but not luteolin (Figure 2a,b). The lack of glucose-lowering activity observed in luteolin is not clear and warrants additional investigation into a set of related flavones such as apigenin or tricetin before further conclusions about the biological activity of flavones can be reached.

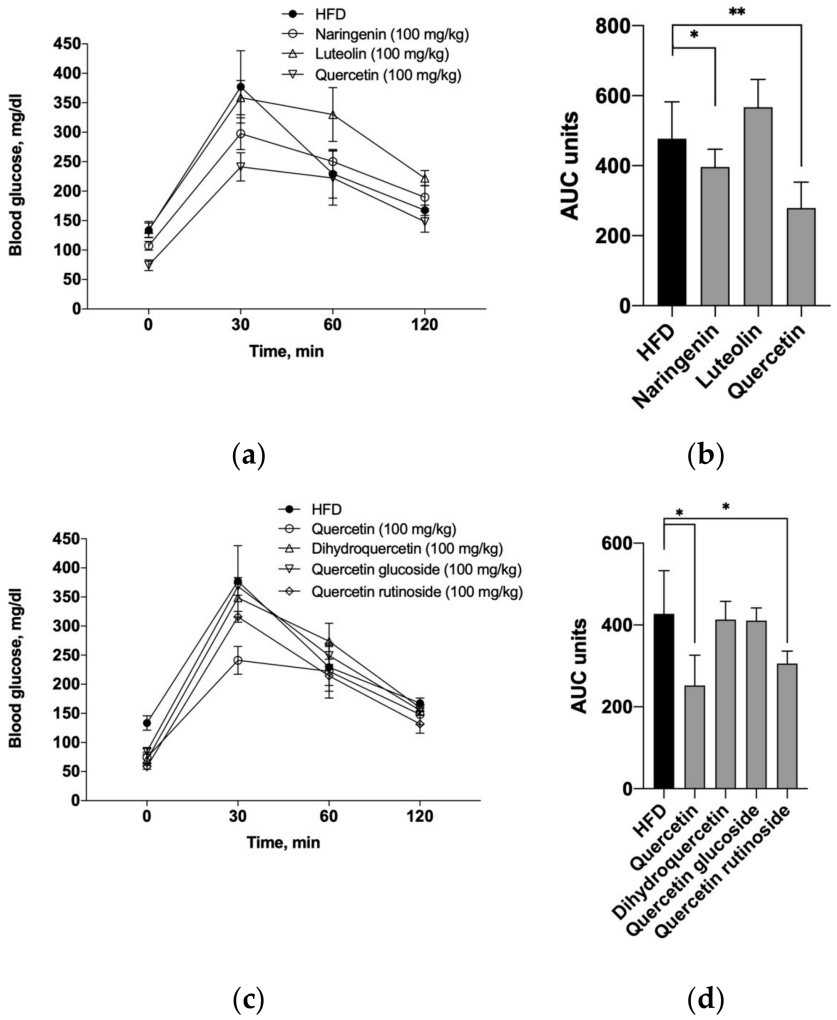

Figure 2. The modulation of OGTT parameters by different classes of flavonoids represented by naringenin, luteolin, and quercetin. The effects of saturation (dihydroquercetin) and glycosylation (mono- and diglycosides of quercetin) were also explored. (**a**,**c**) OGTT curves were determined by sampling blood glucose at 0, 30, 60, and 120 min after the carbohydrate challenge. (**b**,**d**) The corresponding areas under the curve (AUC120). The results are means ± SD (n = 4). The data were analyzed by one-way ANOVA and Dunnett's multiple comparisons, * $p < 0.05$, ** $p < 0.01$ versus HFD controls. HFD controls were gavaged with the vehicle alone (0.1% DMSO in water).

Both saturation and glycosylation change the hydrophobicity of the compounds and may affect their interactions with bitter TAS2R receptors. To explore this aspect of the study, saturated, mono-glycosylated (3-O-glucoside), and di-glycosylated (3-O-rutinoside or rhamnopyranosyl-(1→6)-β-D-glucopyranoside) versions of quercetin were tested at the same concentration of 100 mg/kg (Figure 2c). Saturation as well as glucosidation with one glucose molecule decreased the observed effects on the OGTT response significantly; however, quercetin rutinoside preserved the biological activity (−28.6%) similar to that of the aglycone (−40.9%) (Figure 2d).

3.3. Glucose Tolerance When Treated with Polymeric Phenolic Compounds

A diverse set of phenolic metabolites is naturally present in the form of monomers, oligomers, and polymers, including hydrolysable and condensed tannins that contain flavanols (catechin, epicatechin) and phenolic acids [19]. Procyanidin oligomers (average degree of polymerization DP 2–10 units) and polymers (DP 11 and higher) were tested at a 100 mg/kg dosing together with their monomeric units (Figure 3a). Both catechin and epicatechin showed a clear tendency to lower the peak blood glucose values; however, the extended the OGTT shoulder at the later timepoints resulted in a non-significant AUC120 glucose disposal effect (Figure 3b). The results remained the same when monomeric procyanidins were isolated from the procyanidin mixtures of various polymerization degrees (Figure 3c). The administration of procyanidin oligomers and polymers resulted in net decreases (−40.6% and −44.7%) in post-challenge glucose appearance in the blood (Figure 3d).

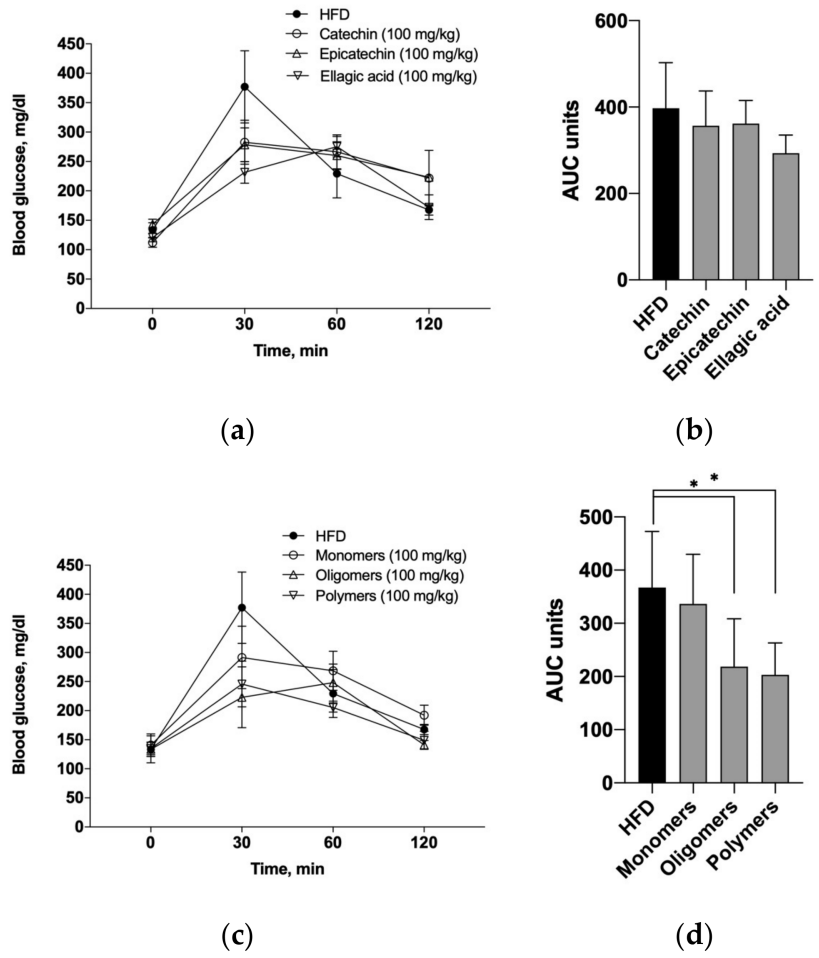

Figure 3. The modulation of OGTT parameters by monomeric (catechin, epicatechin), monomeric ellagitannins (ellagic acid), and polymeric procyanidins (condensed tannins) represented by different degrees of polymerization. (**a**,**c**) The OGTT curves. (**b**,**d**) The corresponding AUC120. The results are expressed as means ± SD (n = 4). The data were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons, * $p < 0.05$ versus HFD controls. HFD controls were gavaged with the vehicle alone (0.1% DMSO in water).

3.4. Glucose Tolerance When Treated with Anthocyanins, Their Metabolites, and Stilbenes

Two additional classes of phytochemicals were tested as mixtures due to limitations with sourcing pure compounds. Black currant anthocyanins (1:1 ratio of cyanidins and delphinidins) and rhubarb stilbenoids (2:1 ratio of rhaponticin and deoxyrhaponticin) were

tested at 100 mg/kg dose. Additionally, a series of phenolic acids including monohydroxy (4-hydroxybenzoic acid or 4HBA), dihydroxy (protocatechuic acid or 3,4DHBA), trihydroxy (gallic acid or 3,4,5THBA), and their methylated counterparts, vanillic acid (4H3MBA) and syringic acid (4H3,5DMBA), were tested at 300 mg/kg to account for their estimated higher bitterness thresholds (Table 1) (Figure 4).

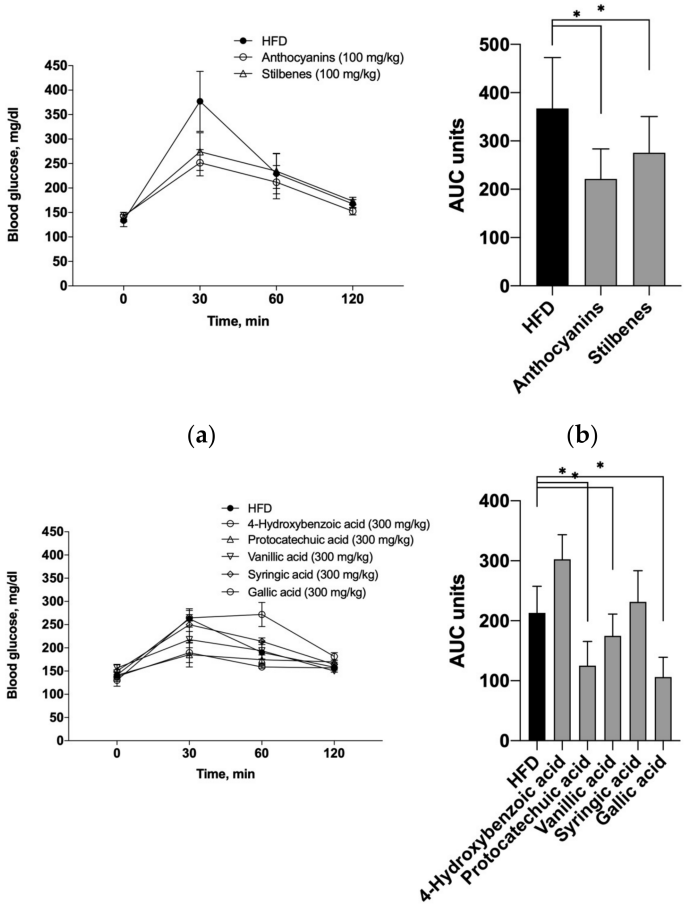

Figure 4. The modulation of OGTT parameters by mixed anthocyanins (black currant cyanidin and delphinidin), stilbenes (rhubarb rhaponticin and deoxyrhaponticin), as well as a series of benzoic phenolic acid metabolites of anthocyanins. (**a**,**c**) The OGTT curves. (**b**,**d**) The corresponding AUC120. The results are expressed as means ± SD (n = 4). The data were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons, * $p < 0.05$ versus HFD controls. HFD controls were gavaged with the vehicle alone (0.1% DMSO in water).

The administration of both anthocyanins and stilbenes resulted in net decreases (−39.8% and −25.1%) in post-challenge glucose appearance in the blood (Figure 3d). Treatment with individual phenolic acids from the benzoic series showed that hydroxylation was important for maintaining the post-challenge glucose excursion effect (−41.3% for protocatechuic and −50.2% for gallic acid), and the methylation progressively decreased this activity (−18.3% for vanillic and no decrease for syringic acid), while application of a monohydroxy benzoic acid showed an opposing effect (+41.8%) (Figure 4c,d).

3.5. Glucose Tolerance with a Model Bitter Compound

The diversity of phytochemical classes and pharmacophores that modulate glucose metabolism following the oral gavage (and thus bypassing the oral cavity and the classical gustatory taste receptors) suggests a shared molecular target in the gastrointestinal tract or subsequent to the gastrointestinal metabolism and absorption. It is very difficult to

envision a single molecular target such as a receptor, an ion channel, or an enzyme capable of recognizing a vast majority of the evaluated chemical moieties; however, a family of bitter TAS2R receptors is one of a few possible candidates. To test this hypothesis, the OGTT tolerance assays were performed with denatonium benzoate, a synthetic and extremely bitter chemical compound unrelated to natural phytochemicals [37]. Denatonium benzoate was administered at the concentration of 3 mg/kg to account for its high bitter threshold at three different application routes: (i) a mouth rinse that delivers the bitter compound to the oral cavity; (ii) an oral gavage that bypasses oral cavity and delivers the bitter compound to the gut; and (iii) an intraperitoneal injection that bypasses the gastrointestinal tract and delivers the bitter compound to the rest of the body. This was followed by a standard OGTT challenge with 1.5 g/kg glucose.

Denatonium benzoate reduced both the peak blood glucose values and total AUC120 when applied as a mouth rinse (148.5 ± 21.5 units, $p = 0.0417$) or oral gavage (146.1 ± 24.2 units, $p = 0.0467$) versus the HFD controls (228.9 ± 37.9 units), but not when injected into the peritoneal cavity, thus suggesting that the observed effect is localized to the gastrointestinal tract (Figure 5a,b). Additionally, when animals were gavaged with denatonium benzoate and challenged with the intraperitoneal glucose injection, the observed OGTT parameters remained similar to the HFD controls, once again localizing the post-challenge glucose excursion effect to the gastrointestinal tract. Moreover, when used in combination with 100 mg/kg p-(dipropylsulfamoyl)benzoic acid (probenecid), an allosteric inhibitor of a subset of TAS2R receptors [38], the observed OGTT effects were significantly diminished to the level of the HFD controls (Figure 5c,d). The mouth rinse with the vehicle alone (0.1% DMSO in water) and the i.p. injection with the vehicle alone (0.1% DMSO in saline) or probenecid alone (100 mg/kg in saline) had no effect of the OGTT response.

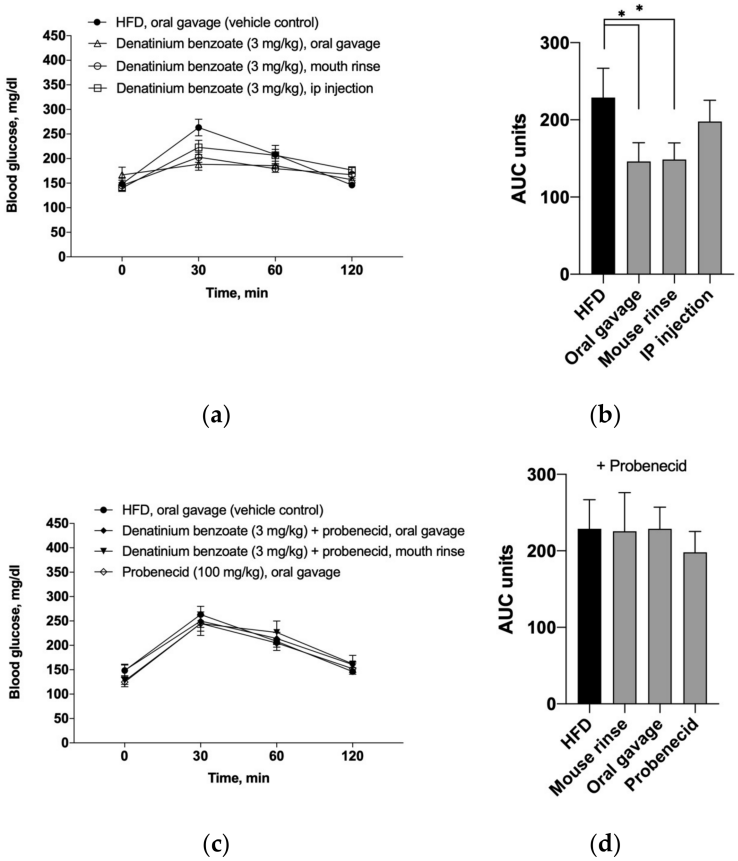

Figure 5. The modulation of OGTT parameters by a model bitter synthetic compound denatonium benzoate applied at 3 mg/kg as a mouth rinse, oral gavage, and intraperitoneal injection. (**a**,**c**) The

OGTT curves. (**b**,**d**) The corresponding AUC120. The OGTT assays were also performed in the presence of probenecid, a selective allosteric inhibitor of the subset of TAS2R receptors (**c**,**d**). The results are expressed as means ± SD (n = 4). The data were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons, * $p < 0.05$ versus HFD controls. HFD controls were gavaged with the vehicle alone (0.1% DMSO in water).

3.6. Co-Expression of TAS2R and Gastrointestinal Hormones in the Gut

In the next set of proof-of-concept studies, the gastrointestinal tissues of the animals treated with denatonium benzoate and challenged with oral glucose were collected and analyzed for co-expression of the mouse mTAS2R108 previously shown to be activated by denatonium benzoate in mouse tissues [45], gastrointestinal hormones cholecystokinin (CCK), proglucagon (GCG)-derived glucagon-like peptide-1 (GLP-1), and the glucagon-like peptide-1 receptor (GLP-1R). Both mTAS2R108 and GLP-1 were expressed in the same tissue, and their mRNA levels were upregulated in response to denatonium benzoate/glucose oral gavage intervention (Figure 6a,b). The GLP-1 receptor as well as CCK were also expressed in the same tissue, but did not change expression levels following the intervention (Figure 6c,d). Co-treatment with probenecid showed an opposing effect on mouse-rinse versus oral-gavage-induced mTAS2R108 expression (Figure 6a), and probenecid further upregulated GLP-1 expression independent of the application site (Figure 6b). The significance of the latter observation remains to be elucidated.

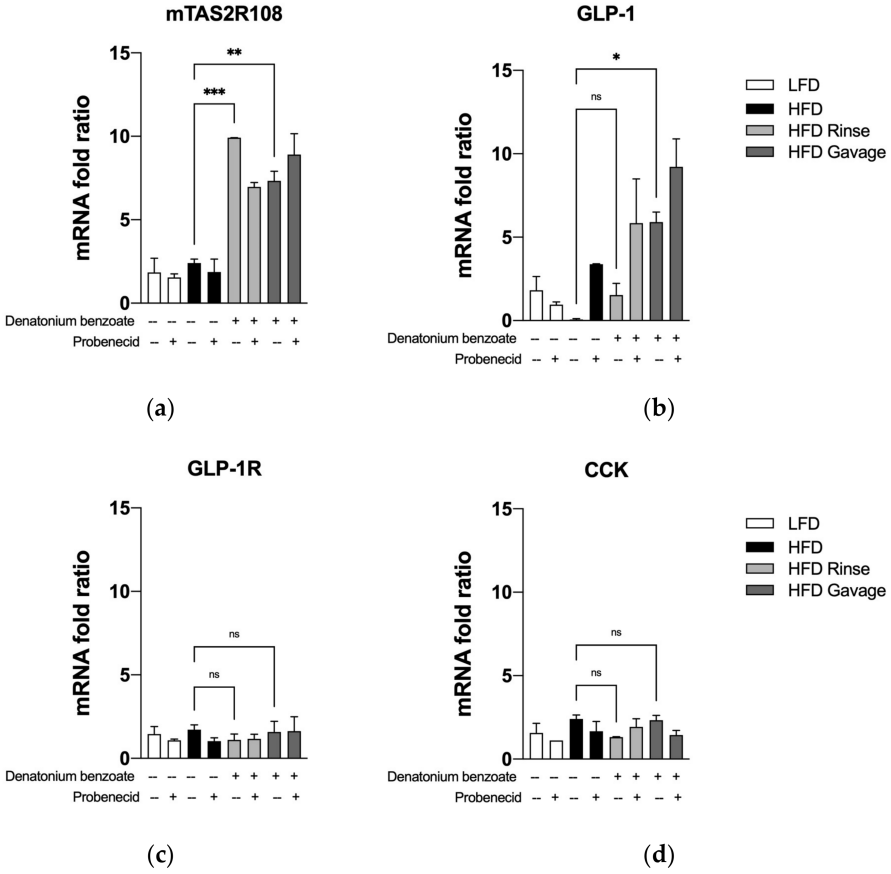

Figure 6. The expression profiles of (**a**) gastrointestinal bitter receptor mTAS2R108, (**b**) glucagon-like peptide-1 (GLP-1), (**c**) glucagon-like peptide-1 receptor (GLP-1R), and (**d**) cholecystokinin (CCK) in lean control animals (LFD), obese control animals (HFD), and HFD animals administered with 3 mg/kg denatonium benzoate in the form of mouse rinse or oral gavage. Co-treatment with the allosteric inhibitor probenecid is indicated with (+/–) signs. Fold changes in gene expression are reported as means relative to the HFD controls (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant).

3.7. Glucose Uptake and Ca^{2+} Signaling in Enteroendocrine STC-1 Cell Model

STC-1 cells established from the enteroendocrine duodenal tissues that harbor many features of native intestinal hormone producing cells, including expression and secretion of a wide range of gut hormones including CCK, GIP, PYY, and the proglucagon-derived GLP-1 [42], were used to evaluate glucose uptake in response to bitter agents (Figure 7).

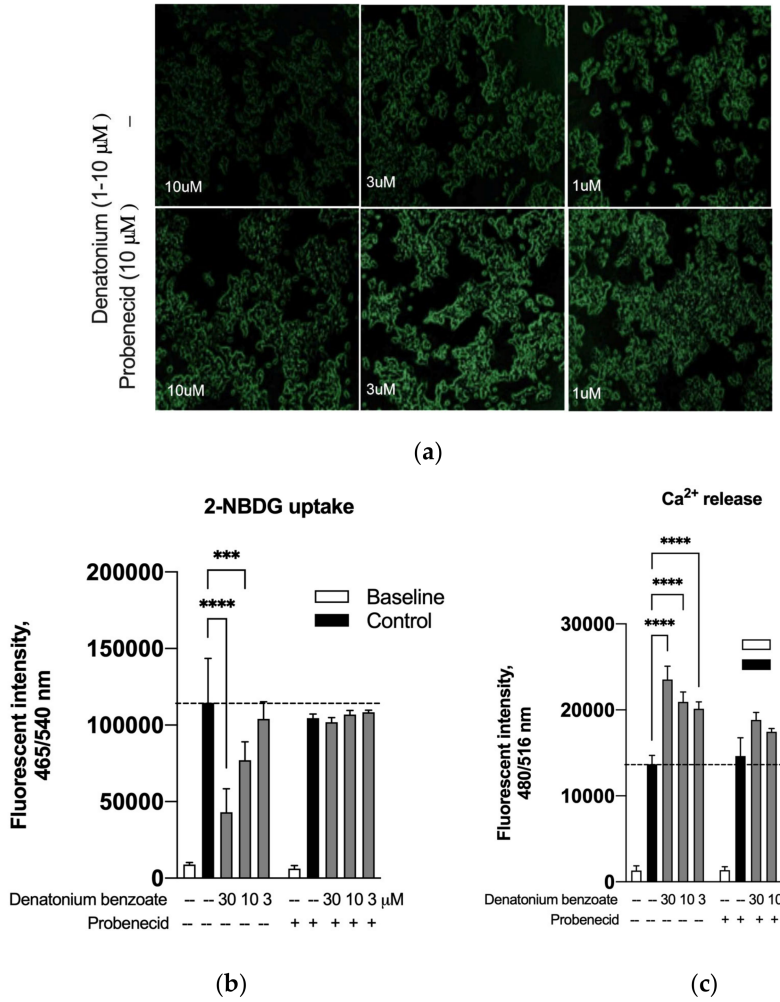

Figure 7. Involvement of bitter receptor signaling in glucose uptake and Ca^{2+} fluxes induced by treatment of STC-1 cells with denatonium benzoate. (**a**) Visualization of glucose uptake of fluorescent 2-NBDG glucose analog with exposure to increasing doses of denatonium benzoate (1–10 µM), and with or without allosteric inhibitor probenecid (10 µM). (**b**) Quantification of 2-NBDG glucose fluorescence at ex/em 465/540 nm after STC-1 cells were treated for 2 h as indicated and presented with 2-NBDG for 20 min. (**c**) Intracellular Ca^{2+} fluxes were quantified fluorometrically at ex/em 480/516 nm after STC-1 cells were treated as indicated. Results are expressed as means ± SD (n = 4). Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons, *** $p < 0.001$, **** $p < 0.0001$ versus dye-loaded controls.

The application of denatonium benzoate in the dose range of 1–30 µM decreased the net 2-NBDG glucose analog uptake by −9.1% to −62.4% that reached significance at 10 µM ($p = 0.0004$) and 30 µM ($p < 0.0001$) doses (Figure 7a,b). The denatonium benzoate response was concentration-dependent, and the co-application of the allosteric bitter receptor inhibitor probenecid (10 µM) abolished the observed effect at all concentrations tested (Figure 7b). STC-1 cells express components of the canonical bitter taste signaling cascade including an abundant mTAS2R108 bitter receptor, G protein subunits, and phospholipase C that generates inositol 1,4,5-trisphosphate (IP3) from phosphatidylinositol

4,5-bisphosphate to activate Ca^{2+} release from the endoplasmic reticulum. The administration of 3–30 μM denatonium benzoate increased intracellular [Ca^{2+}] by 47.1% to 71.9% in a concentration-dependent manner, and this effect was also blunted by co-application of 10 μM of probenecid (Figure 7c). The test compounds had no significant effects on STC-1 cell viability over the dose ranges used in this study (1–30 μM) as indicated by an MTT assay.

4. Discussion

From an early evolutionary perspective, it remains unclear whether bitter taste perception or wider chemoperception (including nutrient sensing) was the primary driver when the TAS2R gene family first appeared in bony but not in cartilaginous fishes 430 million years ago [46]. This development may have coincided with the evolution of terrestrial plants or insects, both of which originated in the Ordovician about 480 million years ago [47] and harbor bitter substances. The TAS2R family expanded to 74 loci in coelacanth and peaked at 50–136 loci in anuran frogs and 36–50 loci in lizards before contracting to 0–17 in birds and 0–35 in mammals, with both complete TAS2R losses associated with the readaptation of penguins and cetacea to aquatic environments [23]. Most primates maintain 18–26 intact TAS2R loci, with humans carrying 25 functional loci and 8 pseudogenes in clusters on chromosomes 5, 7, and 12 [48] (Table 2).

For hominids, daily foraging necessitated frequent encounters with bitter plants [49]. The early recorded history of humans using plant bitters includes Nikander (*Theriaca*, ca. 200 BC), Cornelius Celsus (*De Medicina*, ca. 25 BC-50 AD), and Scribonius Largus (*Compositiones*, ca. 1–60 AD) that describe bitter mithridate and theriaca remedies in the form of electuary aromatic honey blends, as well as Pedanius Dioscorides (*De materia medica*, ca. 40–90 AD), who advises steeping juniper berries in wine in an early form of gin [11]. Many digestive bitters and aperitives emerged subsequently from the herbal apothecary tradition such as Benedictine, Campari, Jägermeister, or Fernet, as well as those developed independently in the New World (Angostura, Peychaud's). In the last 100 years, however, the human relationship to bitter tastes was considered exotic and often undesirable [50], and bitter plant constituents have been actively eliminated from food products by selective breeding and industrial debittering [51].

Earlier, our group hypothesized that the gastrointestinal system could have evolved in part to recognize bitter phytochemicals in anticipation of plant-based, high-carbohydrate meals, as animal tissues are not bitter except when contaminated with bile and contain only small amounts of carbohydrates [33,34,52]. This hypothesis was further supported by multiple observations that diverse, unrelated classes of phytochemicals rapidly modulate glucose metabolism despite not having a commonly known molecular target [12–21].

Our current study confirms these observations in a standardized C57BL/6J prediabetic mouse model using 24 model phytochemicals, and highlights some differences based on their structural pharmacophores. While it was not possible to test the entire variety of bitter phytochemical classes in this study, we also used a potent synthetic bitter compound, denatonium benzoate, with a bitterness threshold of 0.05 ppm (100 nM) [53] to evaluate the post-challenge glucose excursion effects in the C57BL/6J animal model, and observed similar results. Our findings, therefore, suggest that dietary bitter substances modulate glucose metabolism in part by activating extraoral bitter taste receptors in the gut, somewhat similar to the bronchodilator effects described earlier in the airway smooth muscle [54]. Lower peak blood glucose values and improved postprandial glycemic responses (AUC120) in response to bitter receptor activation in the gastrointestinal system indicate that this pathway has both therapeutic and dietary supplementation relevance in managing disorders of carbohydrate metabolism.

Both the peak blood glucose values (glucose spikes) and whole glucose excursion (AUC120) obtained during the OGTT challenge provide critical information on glycemic variability considered harmful for blood vessels and directly associated with pathologies of cardiovascular and metabolic health. They are also used for calculating the glycemic index

of foods [55], evaluating the efficacy of hyperglycemia medications [56], dietary management of sports performance [57], as well as satiety and weight support [58]. The changes in the OGTT glucose curve shapes correlate with glucose tolerance status and depend on gastric emptying, intestinal motility, glucose absorption from lumen via Na(+)/glucose co-transporter (SGLT1), glucose transport across the basolateral membrane through glucose transporter 2 (SLC2A2), gastrointestinal incretin release, pancreatic β-cell function, glucose transport across the endothelial barrier tissues through glucose transporter 1 (SLC2A1), and insulin sensitivity of the metabolically active tissues through glucose transporter 4 (SLC2A4) [59]. The incretin-mediated insulin response is dependent on the ingested amount of carbohydrates with relative contributions estimated at 44% for glucose-dependent insulinotropic polypeptide (GIP), 22% for glucagon-like peptide-1 (GLP-1), and 33% for glucose alone [60]. The acute (within 30 min) changes in the glucose excursion observed in this study suggested the physiological effects of bitters on glucose metabolism were predominantly localized to the gastrointestinal tract, which was corroborated with the facts that intraperitoneal delivery of the bitters or nonselective allosteric inhibition of bitter receptor signaling with probenecid reduced these effects.

The insulinotropic action of GLP-1 is of particular interest, as our data suggested that the abundant mTAS2R108 bitter receptor, GLP-1 (proglucagon), and the GLP-1R receptor were co-expressed in the same region of the gastrointestinal tracts, as well as the fact that both mTAS2R108 and GLP-1 mRNAs were upregulated in response to intragastric administration of bitter substances in the animal model. Diet-induced upregulation of bitter taste receptors has been observed previously [61]. We also demonstrated that the application of model bitter substances led to an increase in intracellular [Ca^{2+}] in neuroendocrine intestinal STC-1 cells, a recognized model for glucose absorption and hormone secretion [42], and thus, were suitable agonists for managing this pathway through future modifications of therapeutic and dietary strategies.

Several concepts have been explored by other groups that support this hypothesis, as it applies to the activation of the gastrointestinal bitter receptors by endogenous bitter amino acids, peptides, and bile acids [62], as well as dietary supplementation with quinine [63]. It is interesting to note that the hypoglycemic effect of quinine administration was known for decades in association with the treatment of malaria [64], and it was attributed to a putative direct effect on insulin release from the pancreatic beta cells [65]. However, because of the multitude of GLP-1-related effects in the intestine and pancreatic beta cells [66], together with the current lack of understanding of the distribution, co-activation, and function of numerous TAS2R bitter receptor subtypes, this concept so far has yielded inconsistent outcomes when translated to humans [67]. It also remains unclear why probenecid treatment alone was associated with increases in GLP-1 and mTas2r108 expression. We think that the response to probenecid may be mediated in part by its ability to activate TRPV2 channels, as was shown previously for lysophosphatidylinositol [68], but this hypothesis needs to be confirmed in future studies.

Lastly, the study highlights that dietary bitter substances can modulate glucose metabolism through the activation of extraoral bitter taste receptors in the gastrointestinal tract. Activation of these receptors helps reduce the peak blood glucose levels after a meal, which is crucial in managing glycemic variability and preventing the harmful effects of glucose spikes on cardiovascular and metabolic health. These findings open possibilities for developing novel nutritional and supplementation strategies using bitter phytochemicals to manage carbohydrate metabolism disorders. This can be partly achieved by enhancing the natural insulinotropic response mediated by GLP-1 signaling, thus improving insulin sensitivity and glucose uptake. The depletion of bitter principles in food may also have negative consequences for metabolic health, as the gastrointestinal chemosensing mechanisms are not being adequately stimulated in modern diets.

5. Conclusions

Our current data suggest that the inhibition of glucose transport from the intestinal lumen to the blood by TAS2R bitter receptor signaling represents a common mechanism that is employed in the acute response to oral administration of bitter phytochemicals. We show that these effects occur dose-dependently within minutes, are associated with increased intracellular [Ca^{2+}] fluxes, and are attenuated in the presence of a nonselective allosteric TAS2R inhibitor.

Taken together, these findings contribute to understanding the general mechanisms by which bitter phytochemicals affect glucose tolerance, that in turn might also mediate their anti-diabetic effects as a part of dietary intervention or lifestyle modification strategies. The existence of physiologically active extraoral bitter receptors that do not carry gustatory function in the gut and other tissues also raises the question of the wider significance of TAS2R-mediated chemosensing in the body, and the ongoing loss of the baseline TAS2R signaling due to the depletion of bitter phytochemicals in the human diet.

Author Contributions: S.K. conceived the study and outlined the scope of work.; K.M.P.J. performed the animal and cell culture studies.; R.M. researched and summarized table data.; K.M.P.J. and S.K. wrote the manuscript.; S.K. edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported in part by the USDA National Institute of Food and Agriculture Hatch project #1023927 (S.K.). The bitter receptor work was supported in part by the unrestricted seed grant from the McCormick Science Institute to establish the Pharmacogenomics Research Fund #670120 (S.K.).

Institutional Review Board Statement: The animal study was carried out at the DHMRI Center of Laboratory Animal Sciences, an AAALAC-accredited facility, and approved by the NC Research Campus IACUC (protocol No. 12-018, approved on 10 April 2013).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data may be obtained from a corresponding author upon request.

Conflicts of Interest: The authors declare no conflicts of interest.

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