Electronic Supplementary Information

"Grape powder attenuates the negative effects of GLP-1 receptor antagonism by exendin-3 (9-39) in a normoglycemic mouse model"

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Supplementary Results

Serum GLP-1 and insulin quantification (Study 2)

Serum GLP-1 and serum insulin levels were quantified at 10 and 30 minutes after treatments were administered in Study 2 (**Figure S7)**. Serum GLP-1 at the 30 min time point slightly, but not significantly, increased in the grape treatment versus the sugar treatment (**Figure S7**); however, due to the small number of data points in these sets (n=3 and n=6 for sugar-matched control and grape powder, respectively), it is difficult to establish significance of these data. Serum insulin increased in the grape treatment versus sugar-matched control at both the 10 and 30 minute time point (not significantly), indicating a trend towards increased insulin secretion.

Impact of acute oral grape administration on glycemic response in normoglycemic and prediabetic mice (Study 3)

The goal of Study 3 was to observe and analyze how consumption of freeze dried grape powder affects post-prandial circulating blood glucose levels in the context of normoglycemia as well as prediabetes/hyperglycemia. Normoglycemic and prediabetic mice were used for this study to observe the acute effects of grape consumption in both physiological states. The mice of the prediabetic group display a phenotype of elevated fasting blood glucose levels as well as impaired tolerance to oral glucose consumption. Both groups were orally gavaged with a grape powder/glucose solution in order to determine how grape powder affects the blood glucose response to oral glucose consumption. A sugar solution (1:1 fructose to glucose) was used as a sugar-matched control for the grape powder as above.

Glycemic responses of the acute grape powder administration can been seen in **Figure 5A-E**. Blood glucose time series paired by treatment or phenotype can be seen in **Figure 5B-E.** Predictably, the PD mice have significantly higher blood glucose levels throughout the excursions as compared to NG mice (**Figure 5D-E**). Blood glucose AUC and excursion values can be seen in **Figure 5F-G**; no significant differences were found between grape powder and sugar-matched treatments in these metrics.

Impact of long-term grape consumption on glycemic control in prediabetic and normoglycemic mice (Study 4)

Study 4 was the long-term feeding study featuring an 8-week feeding period with grape powder or sugar-matched control incorporated into high fat diets (60% kcal from fat) in both prediabetic and normoglycemic models. The test diet had grape powder incorporated at 5% (w/w) and the control diet that had fructose/glucose incorporated at 4.5% (w/w, to match the amount of sugar present in the grape powder). After 8 weeks of feeding, insulin tolerance (ITT) and glucose tolerance (GTT) tests were performed (separated by a one week recovery period) to determine how chronic grape feeding affects glycemic response and insulin sensitivity. This study was designed to show the efficacy of grape powder to ameliorate prediabetes in mice with the preexisting condition; additionally this work was designed to show if the grape powder can prevent the onset of prediabetes in normoglycemic mice after switching to a high fat diet. Study 4 differed from the other 3 studies in that the GTT was not co-administered with grape powder in order to isolate beneficial effects of chronic grape consumption rather than effects of acute grape consumption.

Blood glucose profiles and AUCs for the i.p. GTT and ITT can be found in **Figure S5A-D**. As also seen in Study 3, the PD mice had significantly higher blood glucose levels throughout the time series but there was not a significant difference within the PD group between the grape powder and sugar-matched diets in the glucose tolerance test. In both GTT and ITT, the S group displayed better glycemic control than all high fat diet groups (**Figure S5C-D**). Weight and fat gains can be seen in **Figure S5E-G.** All mice fed any high-fat diet (NG *and* PD groups) experienced similar weight gain over the course of the study, regardless of the addition of grape or sugar to the diet (**Figure 7E-G**); the LF group gained less weight and fat during the study.

Supplementary Discussion

In study 2, grape powder solution administered i.p. caused a significant increase in blood glucose AUC compared to both the oral grape powder solution and the sugar matched control intraperitoneal administration (all treatments provided equivalent qualitative and quantitative sugar profiles) (**Figures S3 and S4)**. The purpose of Study 2 was to determine if grape powder flavanols exhibit glucose-regulating effects through mechanisms in the GI track or mechanisms following absorption into the bloodstream (such as reducing skeletal muscle insulin resistance). Grape powder was suspended in water for i.p. injection; however, the powder did not fully dissolve in solution. Grape constituents likely remained insoluble in the peritoneal cavity, potentially interfering with

glucose clearance. This caused an extended spike in blood glucose compared with all other treatment and control groups; these insoluble constituents may have impaired blood glucose clearance or may have caused endogenous glycemic release. This is not representative of typical delivery of grape constituents to the bloodstream via oral consumption, as all constituents that reach the bloodstream would be soluble. We employed water as the vehicle to avoid introducing deleterious effects of better solvents for grape flavan-3-ols (e.g. DMSO). However, future experiments designed to elucidate the relative contributions of grape components in the gut vs. peripheral tissues will likely need to utilize these solvents in order to ensure complete solubilisation to be physiologically relevant. Therefore, the results of study 2 are inconclusive at best. However, results from the oral portion of this study (oral grape powder/OGTT administration vs. oral sugar-matched control/OGTT administration) were compared as these results were not affected by these insolubility issues. Serum insulin was quantified at 10 and 30 minutes; insulin in the grape treatment group was slightly higher than the sugar-matched control group. This indicates that the grape consumption may have slightly raised insulin levels under normoglycemic conditions, although no significant differences were seen in glycemic metrics.

In study 3, a simple experiment was performed to assess whether grape powder exerted distinct protective effects in normoglycemic vs. prediabetic mice. However, grape did not improve glycemic control compared to sugar match in either phenotype (**Figure 5**). While this was expected for the normoglycemic mice due to similar results seen in Study 2, the lack of protection in prediabetic mice was unexpected.

The long-term feeding study (Study 4) displayed significant increase in weight gain in all mice fed high fat diets compared to the low fat diet (**Figure S5**). There were no significant differences in the glycemic challenges (ITT, GTT) between the grape powder and the sugar-matched control diets for both normoglycemic and prediabetic mice. Previous work had shown that grape powder diet supplementation reduced overall AUC of GTT administered at 5 weeks of feeding compared with high fat control diet[5]. We had hypothesized that grape consumption would reduce the glycemic response to glucose tolerance test and insulin tolerance test. Additionally, we hypothesized that decreased fasting glucose levels, improved insulin sensitivity and decreased weight gain would be observed; grape consumption did not improve these biomarkers of glycemic control.

Supplementary Tables

Table S1. Detailed test diet composition information for all studies. Diets were purchased from Open Source Diets (Research Diets) and stored frozen until use.

	D12450J		D12492		D14090308		D14090309	
Macronutrient	**gm %**	**kcal %**	**gm %**	**kcal %**	**gm %**	**kcal %**	**gm %**	**kcal %**
Protein	19	20	26	20	26	20	26	20
Carbohydrate	67	70	26	20	27	20	26	20
Fat	4	10	35	60	35	60	35	60
Total		100		100		100		100
kcal/gram	3.8		5.2		5.2		5.2	
Ingredient	**gm %**	**kcal %**	**gm %**	**kcal %**	**gm %**	**kcal %**	**gm %**	**kcal %**
Casein, 80 Mesh	200	800	200	800	200	800	200	800
L-Cystine	3	12	3	12	3	12	3	12
Corn Starch	506.2	2025	0	0	0	0	0	0
Maltodextrin 10	125	500	125	500	125	500	125	500
Sucrose	68.8	275	68.8	275	33.96	136	33.96	136
Cellulose, BW200	50	0	50	0	50	0	50	0
Soybean Oil	25	225	25	225	25	225	25	225
Lard	20	180	245	2205	245	2205	245	2205
Mineral Mix S10026	10	0	10	0	10	0	10	0
Dicalcium Phosphate	13	0	13	0	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0	5.5	0	5.5	0
Potassium Citrate, 1 H2O	16.5	0	16.5	0	16.5	0	16.5	0
Grape Powder	0	0	0	0	38.7	139	0	0
Glucose/Fructose Mix (1:1)	0	0	0	0	0	0	34.83	139
Vitamin Mix V10001	10	40	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0	2	0

FD&C Yellow Dye #5	0.04	0	0	0	0	0	0.025	0
FD&C Red Dye #40	0	0	0	0	0.025	0	0.025	0
FD&C Blue Dye #1	0.01	0	0.05	0	0.025	0	0	0
TOTAL	1055.05	4057	773.85	4057	777.71	4057	773.84	4057

Table S2. Bodyweight, weight gain and feed intake after 8 weeks of feeding treatment diets in C57BL/6J in Study 4. Starting phenotypes and diet type are indicated in the column headers.

Parameter	**Treatment group (phenotype/diet)**				
	Normoglycemic standard fat	Normoglycemic sugar-matched control	Normoglycemic grape powder	Prediabetic sugar-matched control	Prediabetic grape powder
Initial bodyweight	25.85 ± 0.65 g	26.09 ± 0.42 g	25.89 ± 0.84 g	35.83 ± 1.26 g	35.61 ± 1.26 g
Final bodyweight	28.16 ± 0.87 g	33.59 ± 1.36 g	33.91 ± 0.95 g	45.89 ± 1.24 g	47.10 ± 1.03 g
Weight gain	2.31 g	7.50 g	8.03 g	10.06 g	11.49 g
Average feed intake (g/day)	3.22 g/day	2.66 g/day	2.76 g/day	2.87 g/day	2.91 g/day
Feed conversion efficiency	78.06	19.86	19.25	15.98	14.18

Supplementary Figures

Figure S1(a)

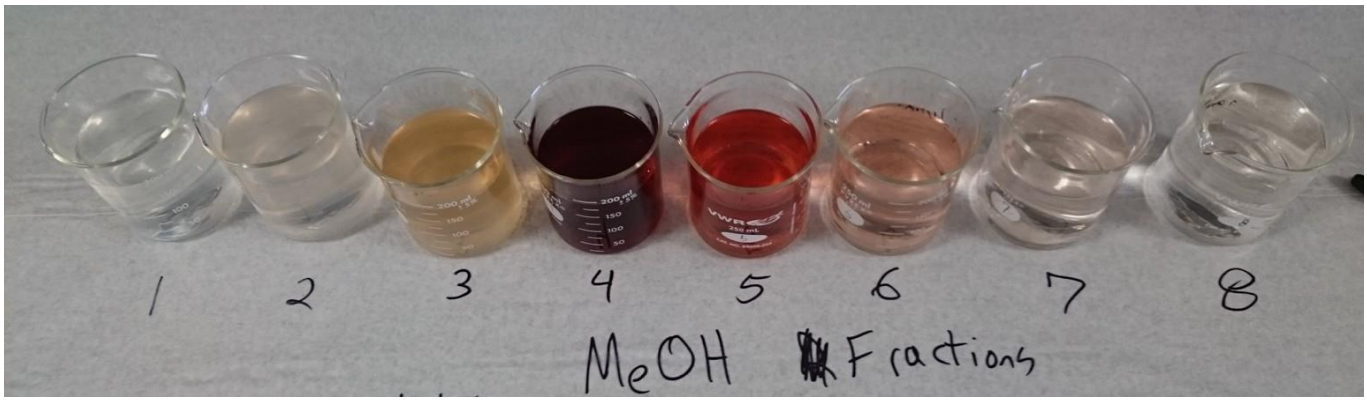


Figure S1(b)

Figure S1(c)

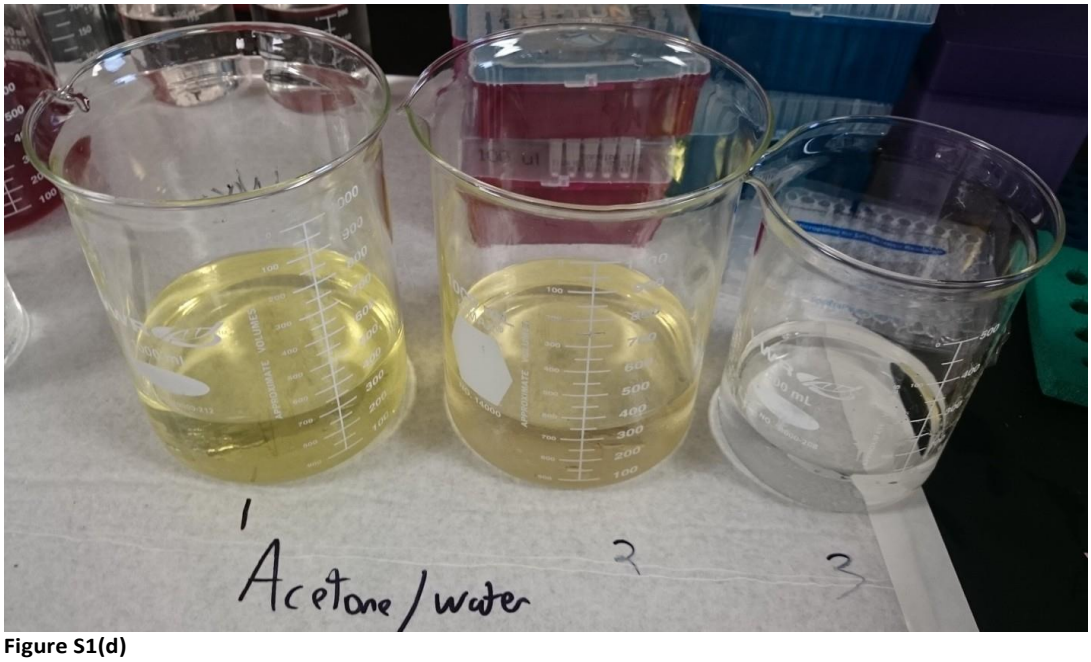


Figure S1(d)

Figure S1. Photos from the extraction of polyphenolic compounds from the freeze dried grape powder. (**a**) initial extraction of compounds from grape powder; from left to right, the beakers are: water extraction, methanol extraction, acetone/water/acetic acid extraction (**b**) after polar compounds were eluted off the open chromatography column with water, methanol was applied to the column and this photo represents the fractions collected. Beaker 1 is the first MeOH fraction collected and beaker 8 is the last MeOH fraction collected. (**c**) After methanol, acetone was applied to the column to remove any residual compounds. This photo shows the first three acetone fractions collected, and (**d**) shows the final 3 acetone fractions collected.

Study schematics (Figure S2)

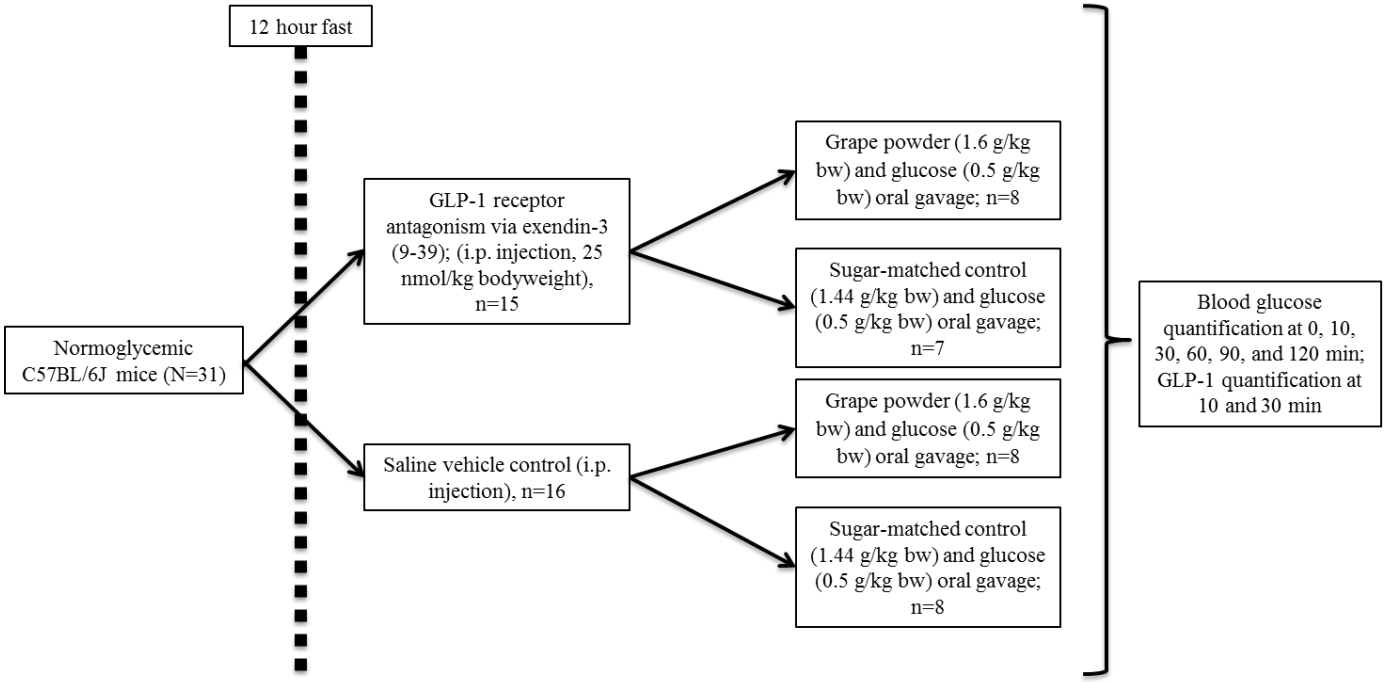
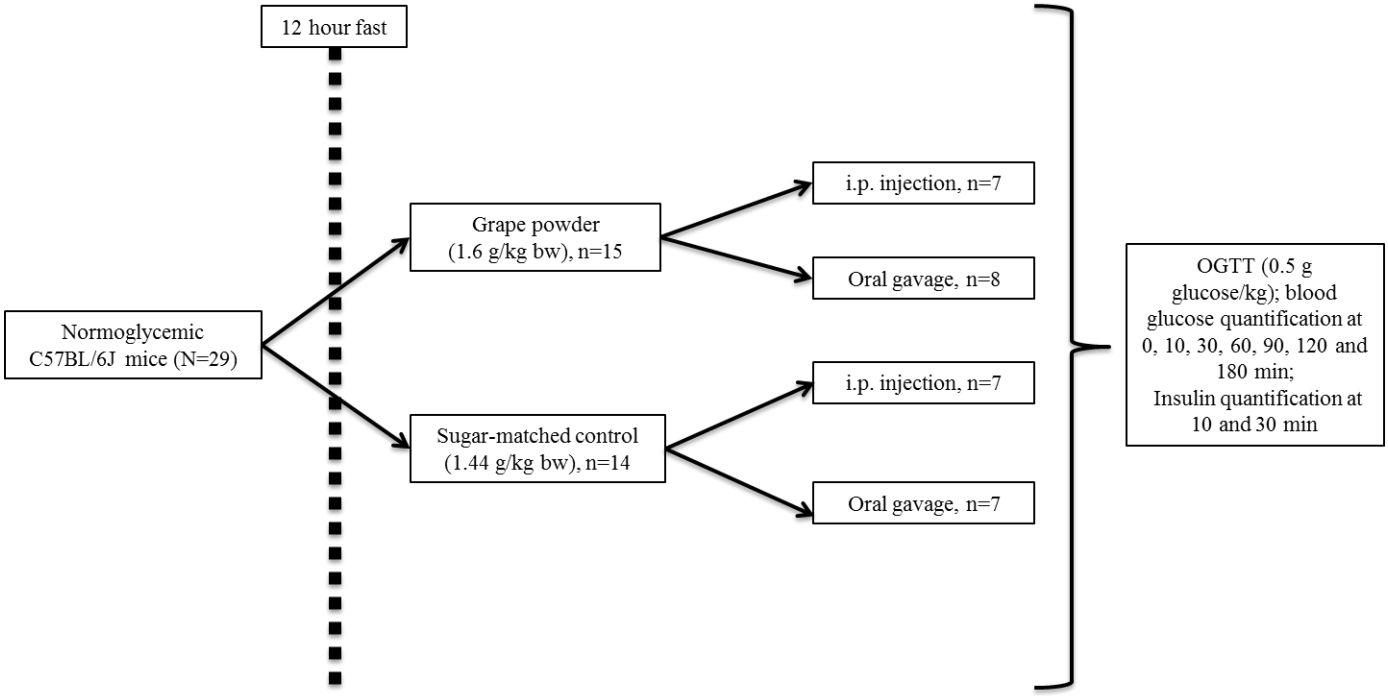


Figure S2(a)



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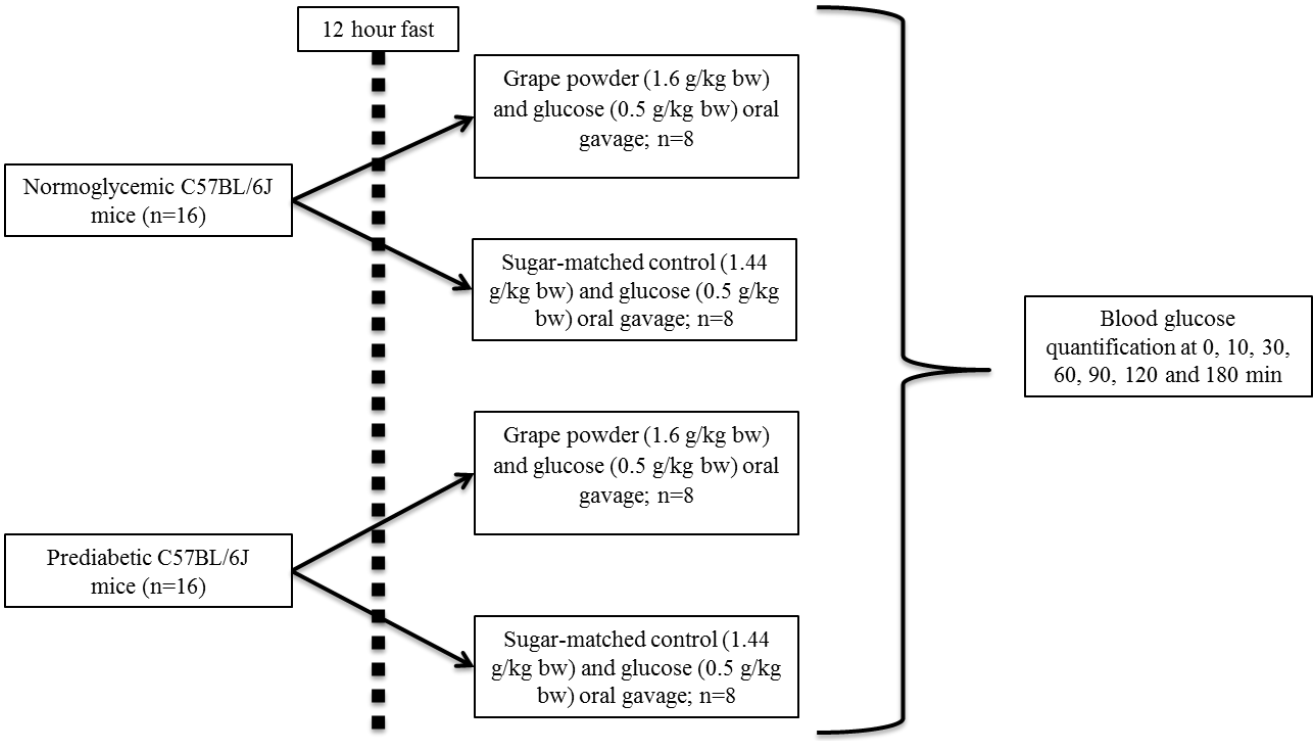

12 hour fast
Normoglycemic C57BL/6J mice (n=16)
Grape powder (1.6 g/kg bw) and glucose (0.5 g/kg bw) oral gavage; n=8
Sugar-matched control (1.44 g/kg bw) and glucose (0.5 g/kg bw) oral gavage; n=8
Prediabetic C57BL/6J mice (n=16)
Grape powder (1.6 g/kg bw) and glucose (0.5 g/kg bw) oral gavage; n=8
Sugar-matched control (1.44 g/kg bw) and glucose (0.5 g/kg bw) oral gavage; n=8
Blood glucose quantification at 0, 10, 30, 60, 90, 120 and 180 min

Figure S2(c)

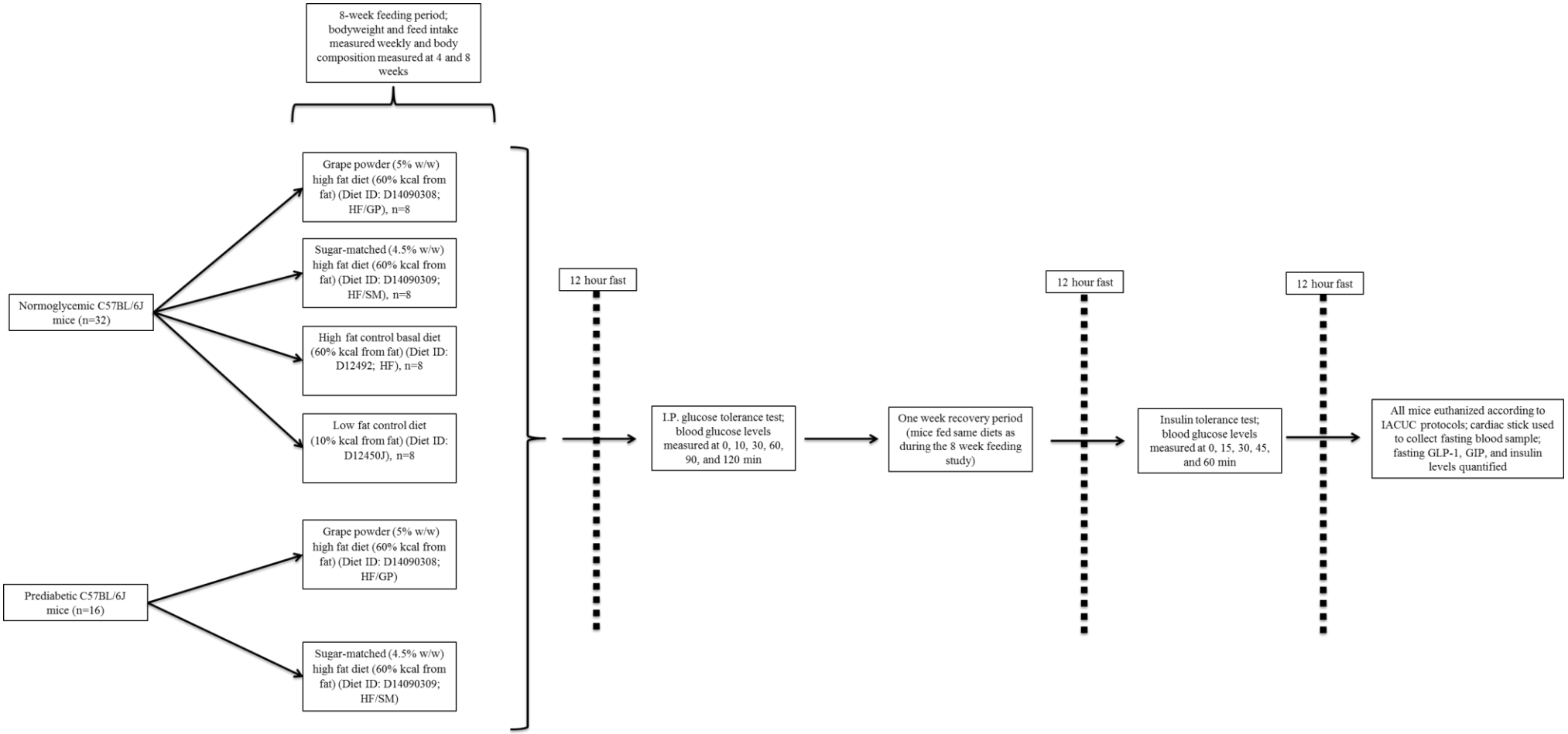


Figure S2(d)

Figure S2. Study schematics of all mouse experiments performed in the present study (**a**) Study 1 – GLP-1 receptor antagonism with concurrent grape powder administration (**b**) Study 2 – differing routes of administration of grape powder (**c**) Study 3 – acute effects of grape powder consumption in normoglycemic vs. prediabetic mouse models (**d**) Study 4 – long term effects on glycemic control in high-fat fed normoglycemic and prediabetic mice

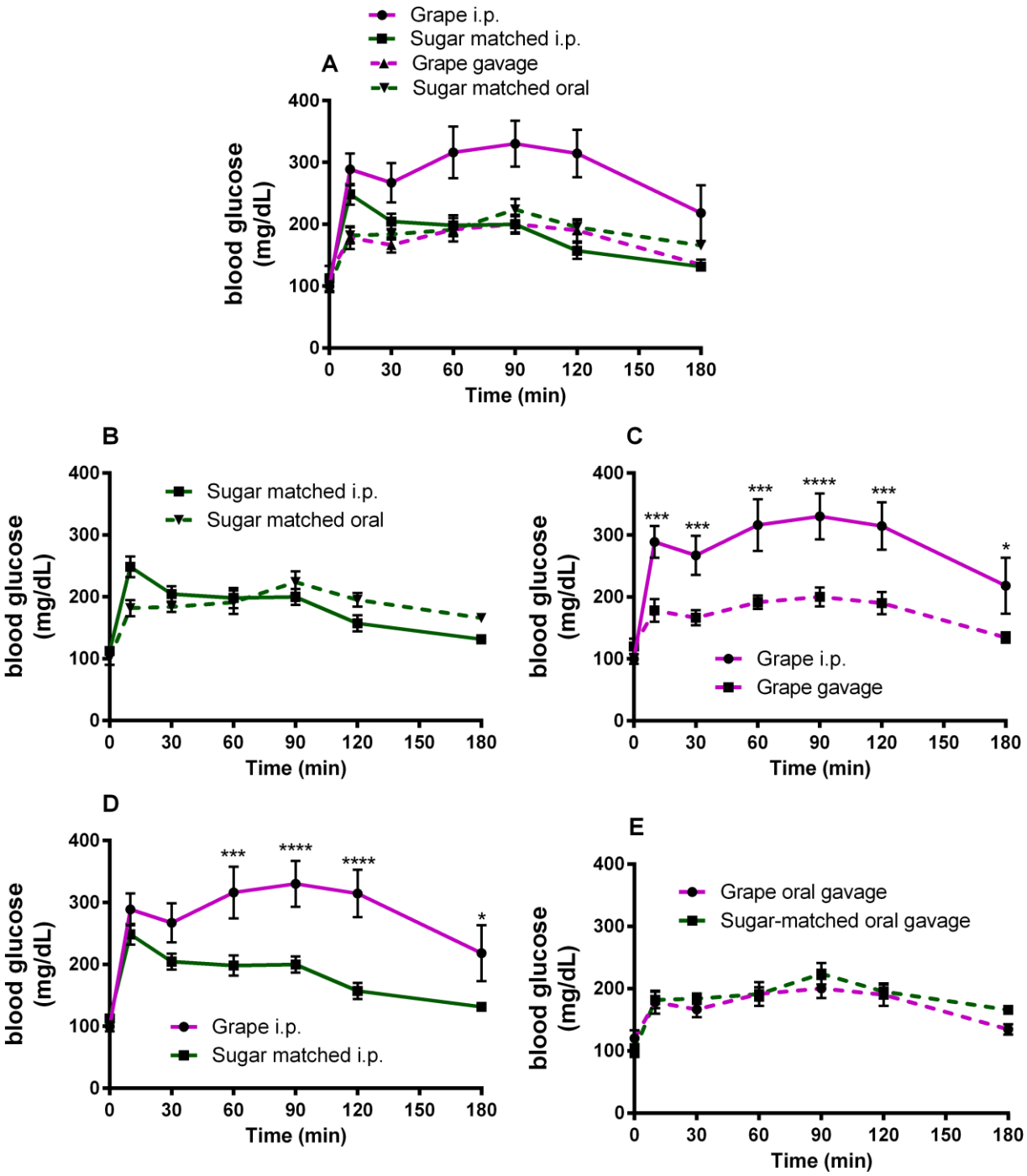


Figure S3. Blood glucose response curves after oral glucose tolerance test following administration of grape powder or sugar matched control via interperitoneal (i.p.) injection or intragastric gavage. (**A**) All treatments, (**B**) sugar match: treatments, i.p vs. oral, (**C**) grape powder, i.p vs. oral, (**D**) i.p., grape powder vs. sugar (**E**) oral, grape powder vs. sugar match. Values are mean ± SEM (*n*=8). *p<0.05 **p<0.01 ***p<0.001 indicate significant difference between two treatment means at the specified time point as indicated by two-way ANOVA with Tukey's HSD post-hoc test (significance is indicated only on graphs with paired curves for ease of interpretation).

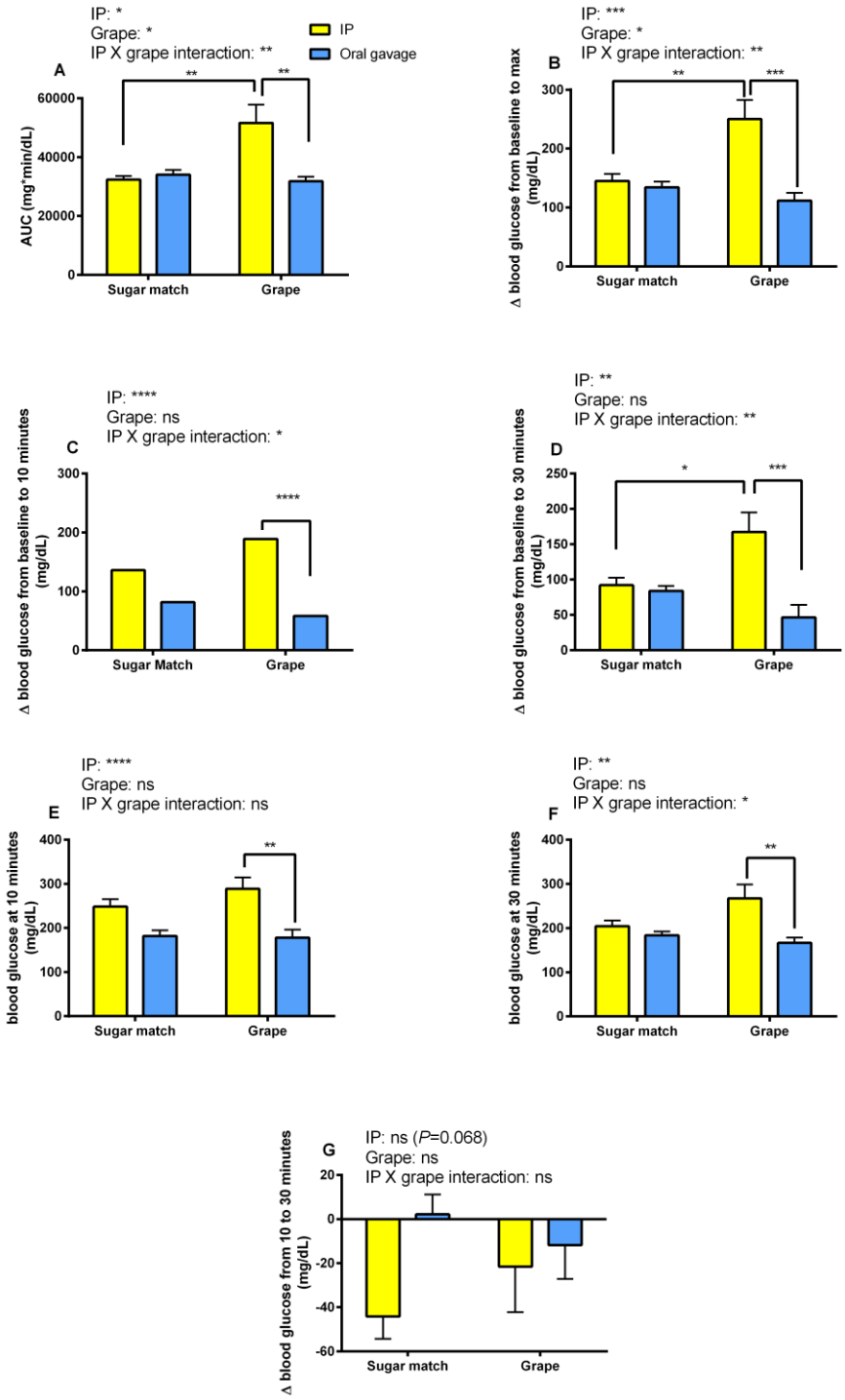
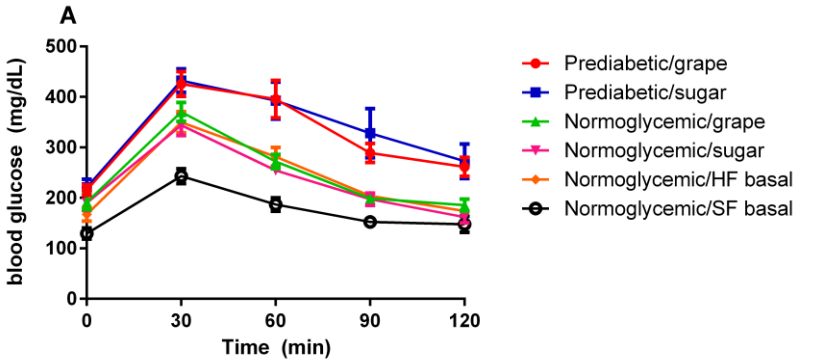
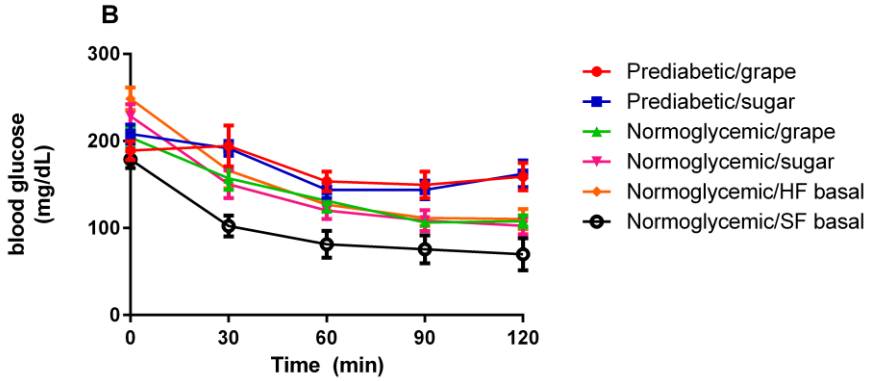
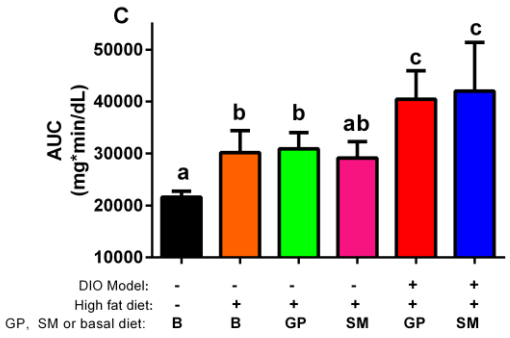
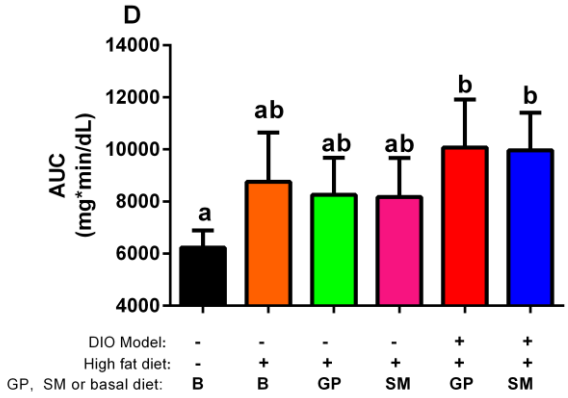


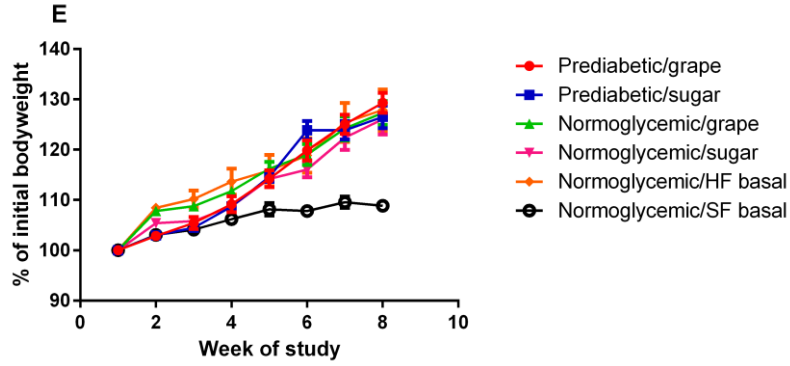
Figure S4. Glycemic response parameters after oral glucose tolerance test following administration of grape powder or sugar matched control via interperitoneal (i.p.) injection or intragastric gavage. (**A**) blood glucose area under the curve (AUC), (**B**) blood glucose excursion (maximum value minus baseline value), (**C**) baseline to 10 min change in blood glucose, (**D**) baseline to 30 min change in blood glucose, (**E**) blood glucose level at 10 minute, (**F**) blood glucose level at 30 min, (**G**) change in blood glucose from 10 to 30 min. Values are mean ± SEM (*n*=8). Legends above individual graphs indicate treatment main effects as determined by two-way ANOVA. *p<0.05 **p<0.01 ***p<0.001 indicate significant difference between treatment means as indicated by two-way ANOVA with Tukey's HSD post- hoc test.

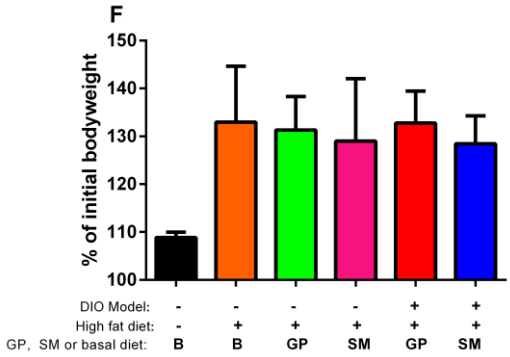

A
blood glucose (mg/dL)
500
400
300
200
100
0
0
30
60
90
120
Time (min)
Prediabetic/grape
Prediabetic/sugar
Normoglycemic/grape
Normoglycemic/sugar
Normoglycemic/HF basal
Normoglycemic/SF basal


B
blood glucose (mg/dL)
300
200
100
0
0
30
60
90
120
Time (min)
Prediabetic/grape
Prediabetic/sugar
Normoglycemic/grape
Normoglycemic/sugar
Normoglycemic/HF basal
Normoglycemic/SF basal


C
AUC (mg*min/dL)
50000
40000
30000
20000
10000
a
b
b
ab
c
c
DIO Model: - - - - + +
High fat diet: - + + + + +
GP, SM or basal diet: B B GP SM GP SM


D
AUC (mg*min/dL)
14000
12000
10000
8000
6000
4000
a
ab
ab
ab
b
b
DIO Model: - - - - + +
High fat diet: - + + + + +
GP, SM or basal diet: B B GP SM GP SM


E
% of initial bodyweight
140
130
120
110
100
90
0
2
4
6
8
10
Week of study
Prediabetic/grape
Prediabetic/sugar
Normoglycemic/grape
Normoglycemic/sugar
Normoglycemic/HF basal
Normoglycemic/SF basal


F
% of initial bodyweight
150
140
130
120
110
100
DIO Model: - - - - + +
High fat diet: - + + + + +
GP, SM or basal diet: B B GP SM GP SM

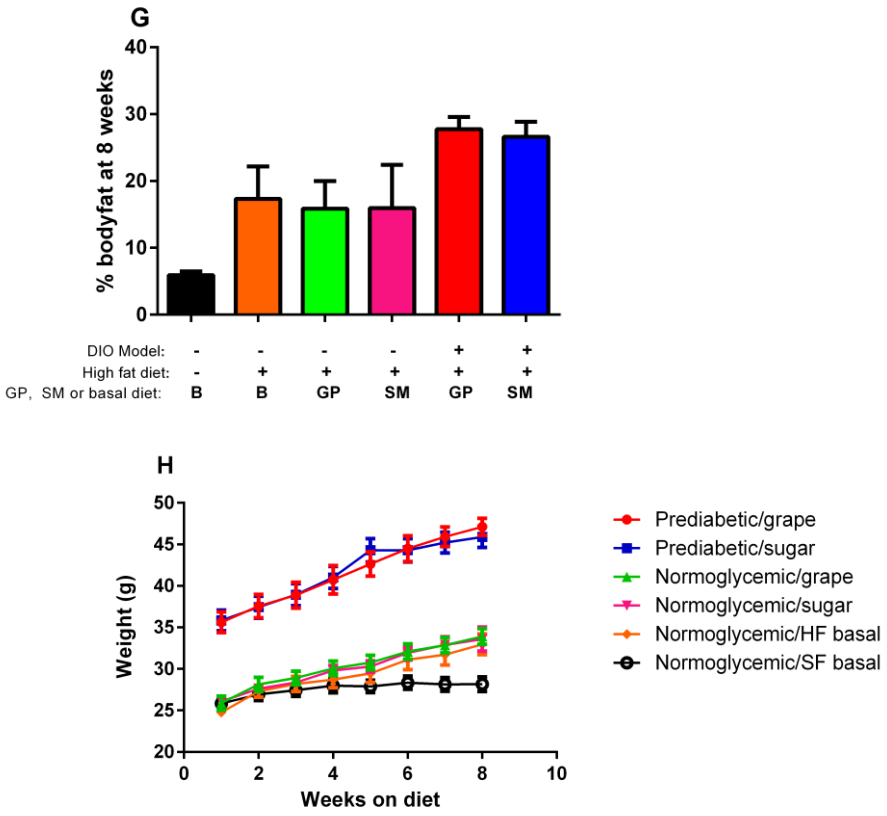


Figure S5. Physiological changes and outcomes during the long-term feeding study. (**A**) i.p. glucose tolerance test (GTT) blood glucose profiles, (**B**) i.p. insulin tolerance test (ITT) blood glucose profiles, (**C**) i.p. GTT blood glucose area under the curve (AUC), (**D**) i. p. ITT blood glucose AUC, (**E**) weight gain over time as a percentage of initial bodyweight, (**F**) total weight gain compared by initial bodyweight at the beginning of the study versus the end of the study, (**G**) body fat content at week 8 (**H**) bodyweight from week 0 to week 8 of feeding period. Values are mean ± SEM (*n*=7/8). For C, D, F and G, values with different superscripts are significantly different as indicated by one-way ANOVA with Tukey's HSD post-hoc test ($p<0.05$).

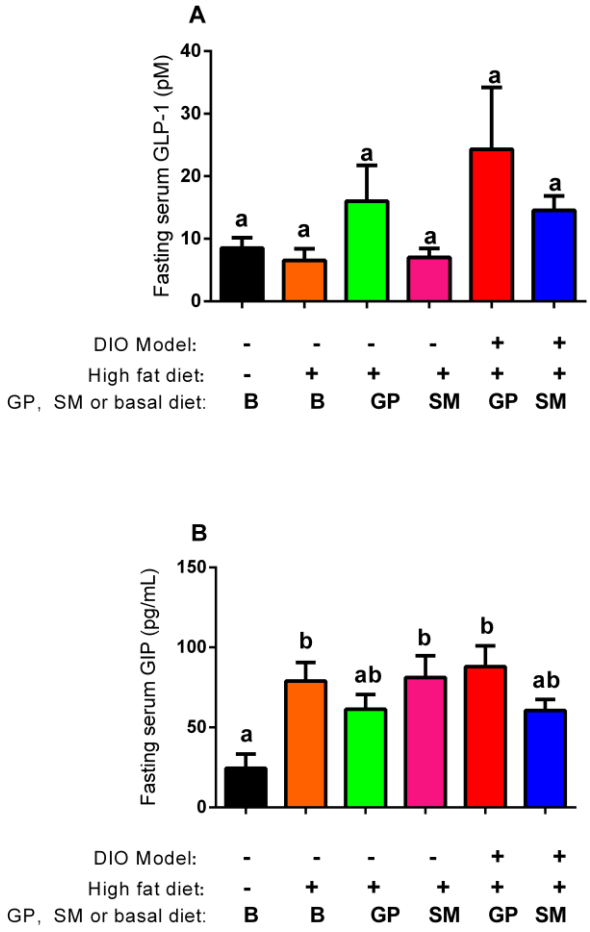


Figure S6. Fasting serum levels of (**A**) GLP-1 (**B**) GIP; blood samples were taken via cardiac puncture immediately after sacrifice following a 12 hr fast; values are mean ± SEM. These hormone levels were measured via ELISA assays. Superscripts indicate significant differences as indicated by one-way ANOVA with Tukey's HSD post-hoc test ($p<0.05$).

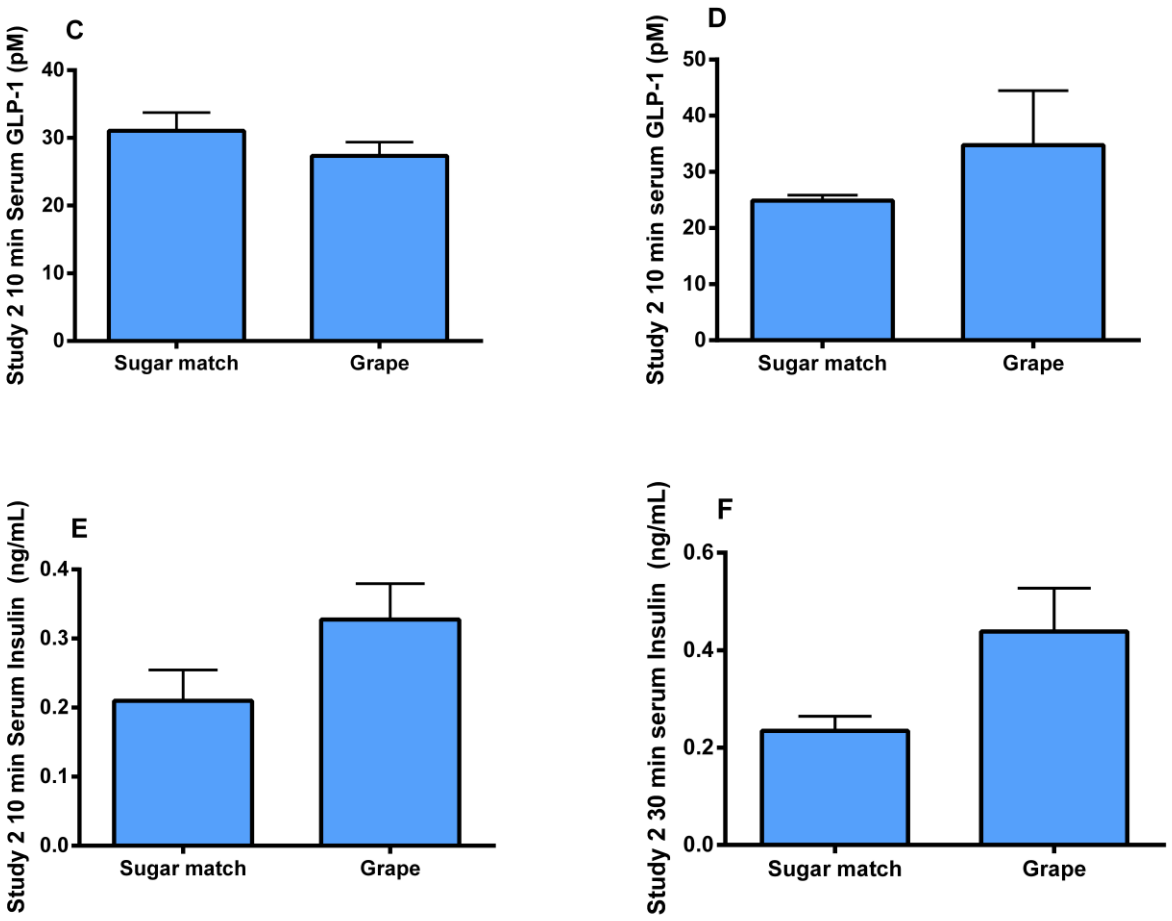


Figure S7. Serum insulin and GLP-1 levels as quantified by ELISA assays. (C) serum GLP-1 levels at 10 min in Study 2 oral grape powder vs. sugar-matched control administration, (D) serum GLP-1 levels at 30 min in Study 2 after oral grape powder vs. sugar-matched control administration, (E) serum insulin levels at 10 min in Study 2 after oral grape powder vs. sugar-matched control administration, (F) serum insulin levels at 30 min in Study 2 after oral grape powder vs. sugar-matched control administration. Values are mean ± SEM; significance was tested with two-tailed t-test.