Glucose homeostasis is differentially affected by dietary Maillard Reaction Products and macronutrients.

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Running title*: Heat treated diets and glucose homeostasis*

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Abstract

Maillard reaction products (MRPs) are generated when protein-rich foods are subjected to intensive heat during cooking. Overconsumption of a Western diet, high in MRP has been identified as a major risk factor for diabetes; yet precisely how MRPs contribute to defects in glucose homeostasis independent of consumption of other macronutrients remains unclear. Eight-week old male Sprague Dawley rats were randomized to feeding with one of six semi-pure diets: control, heat processed (high MRPs), high protein, high dextrose, high in saturated fat (of plant origin), or high in saturated fat (of animal origin). After feeding for 24 weeks body composition was determined by bioelectrical impedance spectroscopy and glucose homeostasis was assessed. When compared to the high MRP diet, excess consumption of the diet high in saturated fat (from an animal source) increased body weight and fat mass, and impaired insulin sensitivity, as defined by impaired skeletal muscle insulin signaling and insulin hypersecretion in the context of increased circulating glucagon-like peptide (GLP-1). Compared to the control diet, chronic consumption of the high MRP diet increased fasting glucose, decreased fasting insulin and insulin secretory capacity. It also resulted in lower GLP-1 and an increase in urinary 15-isoprostane F_{2t}, a sensitive marker of oxidative stress status. These data suggest that excessive consumption of heat-treated foodstuffs can impair glucose homeostasis and pancreatic function in rodents independent of excesses in other macronutrients. These data provide a link between over-consumption of processed foods and the development of diabetes.

Introduction

Diabetes prevalence, now estimated as 171 million people worldwide, is expected to double within the next 20 years (1). Diabetes is characterized by both hyperglycemia and a relative deficiency in insulin secretion, required before development of overt disease. In type 2 diabetes this is seen in the context of reduced insulin sensitivity, whereas in type 1 diabetes, autoimmune destruction of the pancreatic beta cells leads to absolute insulin deficiency. Reducing the global burden of diabetes is a high priority for the WHO (1).

The global increase in diabetes has arisen in parallel with the increasing popularity of Western-style diets, so that it has been argued that dietary factors and diabetes are closely associated (2-5). The adverse effects of the Western diet are most often attributed to its high energy density and poor nutrient profile with large amounts of saturated and *trans* fatty acids and poor quality carbohydrate. Yet other adverse features that derive from modern methods of food processing need also to be considered, one of which is the high generation of Maillard reaction products (MRPs) (6). MRPs, also known as advanced glycation end products (AGEs), are formed through the non-enzymatic irreversible modification of free amino groups within proteins and amino acids by reducing sugars and reactive aldehydes and can increase the shelf-life and taste of manufactured foods (7). Once ingested, 10 to 30% of dietary MRPs are thought to become absorbed into the circulation (8, 9) where they can form deleterious cross-linkages with many body tissues before excretion into the urine via the kidneys

(9). Some MRPs can also arise endogenously under physiologic conditions within tissues particularly in people with diabetes (7).

Recent studies in rodent models indicate that the restriction of dietary MRP intake not only improves insulin sensitivity, but can also extend the lifespan (10, 11). Moreover, other rodent studies suggest an association between AGEs and type 1 diabetes (12-14). There remains a need however, to distinguish the effects pertaining to MRPs from effects arising from other adverse dietary factors, particularly in relation to glucose homeostasis, insulin sensitivity and pancreatic function. In this study, undertaken in healthy rats, comparisons have therefore been made between the effect of a highly processed, heat-treated rodent diet (high in MRPs) with unheated rodent diets that are high in either saturated fatty acids, dietary protein or refined carbohydrates.

Methods and Materials

Rodents

All animal experiments were performed in accordance with the Alfred Medical Research and Education Precinct Animal Ethics Committee. Rats were housed in groups of three per cage with a 12 h light/dark cycle and *ad libitum* access to food and water. Healthy male 8-week-old Sprague Dawley rats, weighing 250 to 300g, were randomized into groups (n=10/group) and given one of the following diets: a control (C) diet (unbaked AIN93G (15)); a baked diet high in MRP (MRP diet) (AIN93G baked at 160°C for 1 h); a high protein (Pr) diet with 48% of total energy (%E) as protein; a high glucose (Glu) diet (with 636 g dextrose/kg); a high saturated fat diet of plant origin (Pla Fat) (40%E from hydrogenated coconut oil) or a high saturated fat diet of animal origin (Ani Fat) (40%E from clarified butter, ghee) and followed for a period of 24 weeks.

All diets were semi-pure formulations manufactured by Specialty Feeds (Western Australia, Australia). Unlike the high MRP diet, the control, protein, dextrose, and high fat diets were not heat treated (i.e., were kept raw) and were not dehydrated and formed into pellets. The MRP diet thus had a five times higher MRP content than the control diet, as determined by an ELISA specific to the AGE carboxymethyllysine (CML) (16). CML was chosen as a surrogate marker of all MRPs because it is present in tissues and serum from humans and rodents and correlates with other MRPs and oxidants (17).

At 23 weeks after feeding, rats were placed individually in metabolic cages (Tecniplast, VA, Italy) to collect a single 24-hour urine sample and to measure water and food intake. After 24 weeks, rats were anaesthetized with pentobarbitone sodium (50

mg/kg body weight) and perfused via the abdominal aorta with 0.1 mol/L PBS for 1-2 min to remove circulating blood. The liver, gastrocnemius skeletal muscle, fat pads and pancreas were removed, frozen in liquid nitrogen and stored at -80°C. Glycated hemoglobin was determined by HPLC as previously described (18).

Bioelectrical impedance spectroscopy (BIS)

At 23 weeks and after feeding, bioelectrical impedance spectroscopy was performed in rats anaesthetized with 2.5% isofluorane in 1.75 L/min of oxygen delivered via nose cone using a bioelectrical impedance analyzer (ImpSFB7, Impedimed, Brisbane, Australia) as previously described (19, 20).

Intravenous glucose tolerance testing (IVGTT)

After 24 weeks of feeding, intravenous glucose tolerance testing was performed (21). In brief, rats (n=6/group) were anaesthetized and the left carotid artery cannulated. After equilibration and a bolus glucose injection of 1 g/kg, 0.5 ml blood samples were taken at 2, 5, 10, 15, 30 and 45 min for the measurement of plasma glucose (glucose oxidase method using an autoanalyser, Beckman Coulter LX20PRO) and plasma insulin by radioimmunoassay (Rat Sensitive RIA, Linco Research, MO, USA). Whole blood was reconstituted in saline and returned to the rats after plasma was extracted. Area under the curve (AUC) was calculated by the trapezoidal rule (GraphPad Prism, GraphPad Software, San Diego, CA, USA).

Intra-peritoneal insulin tolerance testing (ipITT)

ipITT was performed after 23 weeks of feeding. After a fasting blood sample was collected, a 0.5 U/kg insulin bolus (Humalog, Insulin Lispro, Eli Lilly, USA) was injected intra-peritoneally into rats and blood samples were taken at 15, 30, 60 and 120 min post-bolus. Plasma glucose was measured as described above.

Homeostatic model assessment of insulin resistance (HOMA-IR)

HOMA-IR was used calculated to determine the relative insulin sensitivity (22) using the formula (insulin (μU/ml) x glucose (mmol/L)) divided by 22.5.

pAKT/AKT immunoblotting

Western immuno-blotting was used to determine the ratio of phosphorylated Akt (phosphoAkt) to total Akt as a marker of insulin signaling in both liver and skeletal muscle. Thirty μg of protein (liver or gastrocnemius skeletal muscle) was reduced with 2% β-mercaptoethanol and proteins were separated using polyacrylamide gel electrophoresis (Bio-Rad Laboratories, Gladesville, Australia). Separated protein bands were transferred onto a Hybond-P PVDF membrane (Millipore, Maryland, USA) using a semi-dry blotting apparatus (Bio-Rad Laboratories, Gladesville, Australia). After transfer, membranes were blocked with 5% skim milk powder diluted in a 1M Tris buffered saline solution with 0.05% Tween-20 (TBS-T) for 1 h. After blocking, membranes were washed in 1M TBS-T solution for 10 min before incubating overnight with either Akt or phospho-Akt primary antibodies (rabbit anti-rat S473, Cell Signaling

Technologies, Massachusetts, USA, Akt antibody at a dilution of 1/10,000 and pAkt antibody 1/5000). Akt and phosphoAkt membranes were washed six times in 1M TBS-T solution before incubating for 1 h at room temperature with an anti rabbit, HRP-labeled polymer secondary antibody (Dako, California, USA). Membranes were probed with Chemiluminescent Peroxidase Substrate-3 (Sigma-Aldrich, St.Louis, USA) for 3 min. Light emission was captured on CL-XPosure film (Thermo Scientific, Rockford, IL, USA). The density of each band was quantitated using Adobe Photoshop. Results were expressed as a ratio of phosphoAkt to Akt.

GLP-1, glucagon and Urinary 15-isoprostane F_{2t}

Plasma GLP-1 and glucagon were determined using ELISA kits from Wako (Osaka, Japan). Urinary 15-isoprostane F_{2t} was measured using an EIA kit specifically designed to assay urine samples (Oxford Biomedical Research, Rochester Hills, MI, USA).

Statistical analysis

All statistical computations were performed using GraphPad Prism version 4.0a for Mac OS X (GraphPad Software, San Diego, California, USA). Values for experimental groups are given as mean, with bars showing the SEM, unless otherwise stated. One-way ANOVA with Tukey's post-test analysis, or two-way ANOVA with Bonferroni post-test analysis was used to determine statistical significance. Where appropriate, two-tailed *t* tests were performed. A probability of $P < 0.05$ was considered to be statistically significant.

Results

To determine the effects of excess consumption of macronutrients and heat treated foodstuffs (MRPs) on glucose homeostasis and pancreatic function, healthy Sprague Dawley rats were fed one of the following diets for 24 weeks: a MRP (baked; MRP) or control (unbaked; C) diet, a high protein (Pr) diet, a high dextrose (Glu) diet, or a high fat diet in saturated fat from either a plant (hydrogenated coconut oil; Pla Fat) or animal fat (clarified butter; Ani Fat) source. The nutrient and energy content of each diet are presented in Table 1. All diets were isoenergetic but differed in specific macronutrients. The MRP content, specifically carboxymethyllsine (CML), was 5-fold higher in the MRP diet than in the unbaked control diet (101.9 versus 20.9 nmol/mol lysine/100 mg, respectively).

Body composition

After 24 weeks of chronic feeding, mean body weight was lower in rats that consumed high protein (Pr) diet (Figure 1A) than in controls. Conversely, body weight was significantly increased in rats fed the high saturated fat diet of animal origin (Ani Fat) (15% increase, $P < 0.05$). Total visceral adipose tissue was also increased in these rats (Figure 1B). In contrast, both the high protein (Pr) and high glucose (Glu) diets resulted in a smaller accumulation of total visceral adipose tissue. Consumption of the high saturated fat diet of animal origin (Ani fat) led to significant increases in both absolute and relative (% of body weight) fat mass as determined by BIS, (28%, $P < 0.05$ and 10%, $P < 0.05$, Figures 1C and 1D respectively). Whereas both absolute and

relative fat mass was lower in rats consuming the high glucose diet (22%, $P < 0.05$ and 21%, $P < 0.05$, Figure 1C and 1D respectively) compared to those fed the control diet. Relative fat mass was also lower in the MRP and high protein diet groups (Figure 1D). Although absolute fat free mass was greater in rats fed high glucose diets and both high fat diets (Figure 1E), when expressed as a % of body weight, fat free mass was increased in MRP, protein and glucose-fed rats, and not significantly altered in the high fat-fed rats (Figure 1F).

Assessment of glucose homeostasis and insulin sensitivity

Fasting plasma glucose was increased in rats that consumed excess MRPs (10%, $P < 0.05$, Figure 2A) or saturated fat from plant (18%, $P < 0.05$, Figure 2A) or animal sources (19%, $P < 0.05$, Figure 2A). Fasting plasma insulin was lower in rats fed the high MRP diet or the high glucose diet and also showed a tendency to increase in rats fed a diet high in animal fat (not of statistical significance) (Figure 2B). The diet high in animal fat, however, increased HOMA-IR, a surrogate measure of insulin resistance, (Figure 2C) and compromised long-term glucose control as reflected by the increase in glycated hemoglobin (Figure 2D). Compared with the control group, rats consuming the diet high in animal fat had lower insulin sensitivity, as plasma glucose did not normalize to control levels over 120 minutes post-insulin injection (Figure 2E), confirmed by the increased AUC calculated in this group (Figure 2F). Rats consuming all other diets had normal insulin sensitivity.

Circulating GLP-1, a gut hormone responsive to macronutrient intake, which stimulates pancreatic insulin secretion, was decreased in rats that consumed diets high

in excess MRP and glucose, whilst GLP-1 increased in rats consuming the high saturated fat animal diet (Figure 3A). Consumption of both high fat diets led to a decrease in circulating glucagon levels compared to the control diet (Figure 3B), an effect not observed in other groups.

The ratio of phospho-AKT to total AKT protein in the insulin target tissues, liver and skeletal muscle was also determined. AKT is a key protein of the insulin signaling pathway and a decrease in the ratio of phospho-AKT to total AKT indicates impaired insulin signaling. While there was no change in the ratio of phospho-AKT to total AKT in liver (Figure 3C), in gastrocnemius skeletal muscle (Figure 3D), chronic consumption of the high fat diet of animal origin led to a decrease in this ratio. Interestingly, urinary 15-isoprostane F_{2t}, a sensitive marker of oxidative stress status, was increased in rats that consumed the high MRP, high protein or high glucose diets, but not the high fat diets (Figure 3E).

Determination of pancreatic function

To test the insulin secretory capacity of the pancreas, IVGTTs were performed after 24 weeks of feeding. After glucose challenge, there were no differences in plasma glucose concentrations over time between diet groups (Figure 4A). Plasma insulin, however, during IVGTT, was reduced in rats that consumed the high MRP, high protein or high glucose diets and this was confirmed by a decrease in total AUC for insulin in these three groups (Figure 4C). In contrast, consumption of the high fat diet of animal origin led to an increase in plasma insulin at 2, 5 and 10 min post-glucose injection (Figure 4B), reflected by the elevated first phase AUC insulin (Figure 4D).

Discussion

While many studies now support the concept that dietary factors are involved in the development of diabetes, controversy exists as to the relative contribution of single dietary elements to disease pathogenesis. Before the development of agriculture, dietary choices were limited to minimally processed plant and animal foods. With advancing technology, and particularly since industrialization, original nutrient characteristics have changed (23), so that highly processed foods now dominate the typical western diet. In the current study, we examined the effects of raw unbaked diets predominating in different macronutrients as compared with a processed diet subjected to high heat to determine effects on pancreatic function, glucose homeostasis and insulin sensitivity in healthy rodents.

Data obtained in this study indicate that in rodents, heat-treated food high in MRPs can impair glucose homeostasis and pancreatic function independent of other macronutrient excesses. These findings provide a clear association between overconsumption of highly processed food and the development of diabetes. Indeed, overt diabetes does not develop without pancreatic islet dysfunction (24). We found that excess consumption of a heat treated diet (AIN93G baked at 160°C for 1 h), baked to increase the content of MRPs, led to a decrease in relative fat mass and an increase in fasting glucose in parallel with a decrease in fasting insulin concentrations when compared to consumption of an unbaked diet (AIN93G, control). Further investigation using an IVGTT revealed a defect in glucose-induced insulin secretion with chronic consumption of a diet high in MRPs. The defects elicited by the high MRP diet appear

similar to those occurring in patients prior to the onset of type 1 diabetes. This is in line with previous studies that have suggested that dietary MRPs may have direct effects on beta cell function. Indeed, AGEs, formed by heat treatment, have been implicated to mediate defects in insulin secretion in pancreatic beta cell lines (25, 26) and in rodent models (10, 12-14, 28).

The delivery of nutrients from the stomach into the duodenum and the subsequent interaction of these nutrients with the small intestine to stimulate incretin hormone release are considered key determinants of acute insulin secretion in response to food (29). The incretin effect has been attributed to the secretion of glucagon-like peptide-1 (GLP-1) from cells in the intestinal epithelium with GLP-1 enhancing insulin secretion (30). In the current study, it was interesting to note that plasma GLP-1 levels were suppressed in rats that consumed diets high in either MRPs or glucose, both diets that also elicited defective insulin secretion. Conversely, consumption of the high fat diet of animal origin, which caused insulin hypersecretion, also resulted in an increase in GLP-1 in the circulation. Other studies in rodents have demonstrated an increase in GLP-1 secretion in response to high fat feeding (31). These data are consistent with the view that GLP-1 plays a key role as a modulator of insulin secretion in response to dietary intake. GLP-1 also strongly inhibits glucagon secretion (30) and it was noteworthy that a decrease in plasma glucagon was observed in rats that consumed the high fat plant or animal diets. These data suggest that further examination of the direct effects of MRPs and saturated fats on gut incretins should be a focus of future studies.

Consumption of high protein or high glucose diets led to variable metabolic responses, including lower accumulation of fat mass. Even though *ad libitum* feeding

was used, the effect of the high protein diet on smaller body weight and fat mass could not be attributed to a decreased energy intake. Similarly, the increase in body fat mass in the rats fed a high fat diet of animal origin was not accompanied by higher energy intake. Differences in fat accumulation seem rather to relate to differences in macronutrient metabolism and energy expenditure. Fasting plasma insulin was decreased after 24 weeks of high glucose feeding in parallel with reduced plasma GLP-1 concentrations. Impaired insulin secretion was also seen in rodents that consumed high glucose or high protein diets. Interestingly, the diets that suppressed insulin secretion, namely those high in MRP, protein or glucose, also increased urinary excretion of 15-isoprostane F_{2t}, a biomarker of oxidative stress, suggesting that oxidative stress may be a key mediator of diet-induced pancreatic dysfunction. Indeed, there is a large body of evidence to implicate reactive oxygen species in beta cell dysfunction, albeit in other contexts (32-34).

In the current study, we found that excess consumption for 24 weeks, of an unbaked and unprocessed high saturated fat diet derived from clarified butter led to increased body weight and fat mass, insulin resistance and an elevation in plasma glucose and glycated haemoglobin. Although the high saturated fat diet derived from hydrogenated coconut oil also increased fasting plasma glucose, it did not impair glucose and insulin sensitivity, consistent with previous studies (35). It is also clear that animal and coconut-sourced saturated fats exert differential effects on insulin sensitivity and type 2 diabetes risk in humans. Polynesian islanders following a traditional diet with a high proportion of total energy intake from coconut-sourced saturated fat (approximately 40% of total energy) have very low prevalence rates of type 2 diabetes.

In contrast, Polynesians who migrate to countries which consume western style diets, consume less saturated fat in total (<30% of total energy), but a larger quantity of that saturated fat is derived from animal sources. In turn, their prevalence rate of type 2 diabetes is much higher (4, 36-38). It is possible that the differences in fatty acid composition observed between plant and animal-sourced saturated fats, in addition to the different metabolic fates of these fatty acids, may be responsible. For example, it is known that long and medium chain saturated fatty acids undergo different pathways of hydrolysis, absorption, storage, and oxidation (39, 40). However, although both high fat diets were not heat processed, the clarified butter diet contained cholesterol. Our study is unique, in assessing these effects in an unbaked diet where they are not confounded by the introduction of MRPs, as would be the case in other studies where conventional heat-treated rodent diets have been used.

In conclusion, data obtained from this study indicate that consumption of heat-treated food can in itself impair glucose homeostasis and pancreatic function in susceptible rodents. Further studies are now warranted to explore potential synergistic effects between high dietary MRPs and other macronutrients, particularly simple sugars and saturated fat, in the promotion of risk factors for diabetes.

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Figure Legends

Figure 1

Body weights (*A*), absolute total visceral depot fat pad weights (*B*), absolute fat mass (*C*), relative fat mass (*D*), absolute fat free mass (*E*) relative fat-free mass (*F*) were measured in rats after 24 weeks feeding of control (C), MRP, protein (Pr), glucose (Glu), saturated plant fat (Pla Fat) or saturated animal fat (Ani Fat) diets. Data are mean±SEM. *p<0.05 compared to control, n=10 rats/group.

Figure 2

Fasting glucose (*A*), fasting insulin (*B*), HOMA-IR (*C*), glycated Hb (GHb) (*D*), plasma glucose during ipITT (*E*), and corresponding AUC glucose (mmol/l) (*F*) were measured in rats after 24 weeks feeding of control, MRP, protein (Pr), glucose (Glu), saturated plant fat (Pla Fat) or saturated animal fat (Ani Fat) diets. Data are mean±SEM. *p<0.05 compared to control, n=6-10 rats/group.

Figure 3

Plasma GLP-1 (*A*), plasma glucagon (*B*), liver phospho-AKT to AKT ratio (*C*), gastrocnemius phospho-AKT to AKT ratio (GHb) (*D*), urinary excretion of 15-isoprostane F_{2t} (*E*) were measured in rats after 24 weeks feeding of control, MRP, protein (Pr), glucose (Glu), saturated plant fat (Pla Fat) or saturated animal fat (Ani Fat) diets. Data are mean±SEM. *p<0.05 compared to control, n=10 rats/group.

Figure 4

Plasma glucose during IVGTT (*A*), plasma insulin during IVGTT (*B*), total AUC insulin (ng/ml) (*C*), first phase AUC insulin (ng/ml) (*D*), were measured in rats after 24 weeks feeding of control, MRP, protein (Pr), glucose (Glu), saturated plant fat (Pla Fat) or saturated animal fat (Ani Fat) diets. Data are mean±SEM. *$p<0.05$ compared to control, n=6 rats/group.

Table 1. Nutrient content of rodent diets, macronutrient and energy intake

	Control	MRP	Protein	Dextrose	Fat	
					Hydrogenated Coconut Oil	Clarified Butter
Protein, *% of total energy*	19.3	19.3	48.0	19.3	19.3	19.3
Fat, *% of total energy*	16.4	16.4	16.4	16.4	40	40
Carbohydrate, *g/kg*	100	100	100	636	340	340
Digestible Energy, *MJ/kg*	16.1	16.1	18.2	16.7	19.5	19.4
Energy intake, *KJ/24h*	350±68	357±63	440±26	385±97	368±67	397±50
Protein, *g/24h*	4.3±0.8	4.3±0.8	12.8±0.8[2]	4.5±1.1	3.7±0.7	4.0±0.5
Fat, *g/24h*	1.5±0.3	1.5±0.3	1.7±0.1	1.6±0.4	4.0±0.7[2]	4.3±0.5[2]
Carbohydrate, *g/24h*	2.2±0.4	2.2±0.4	2.4±0.2	14.7±3.7[2]	6.4±1.2	6.9±0.9

[1]24 h intake data are mean±SD, n=10 rats per group.
[2]*P*<0.05 compared to control diet

Figure(s)

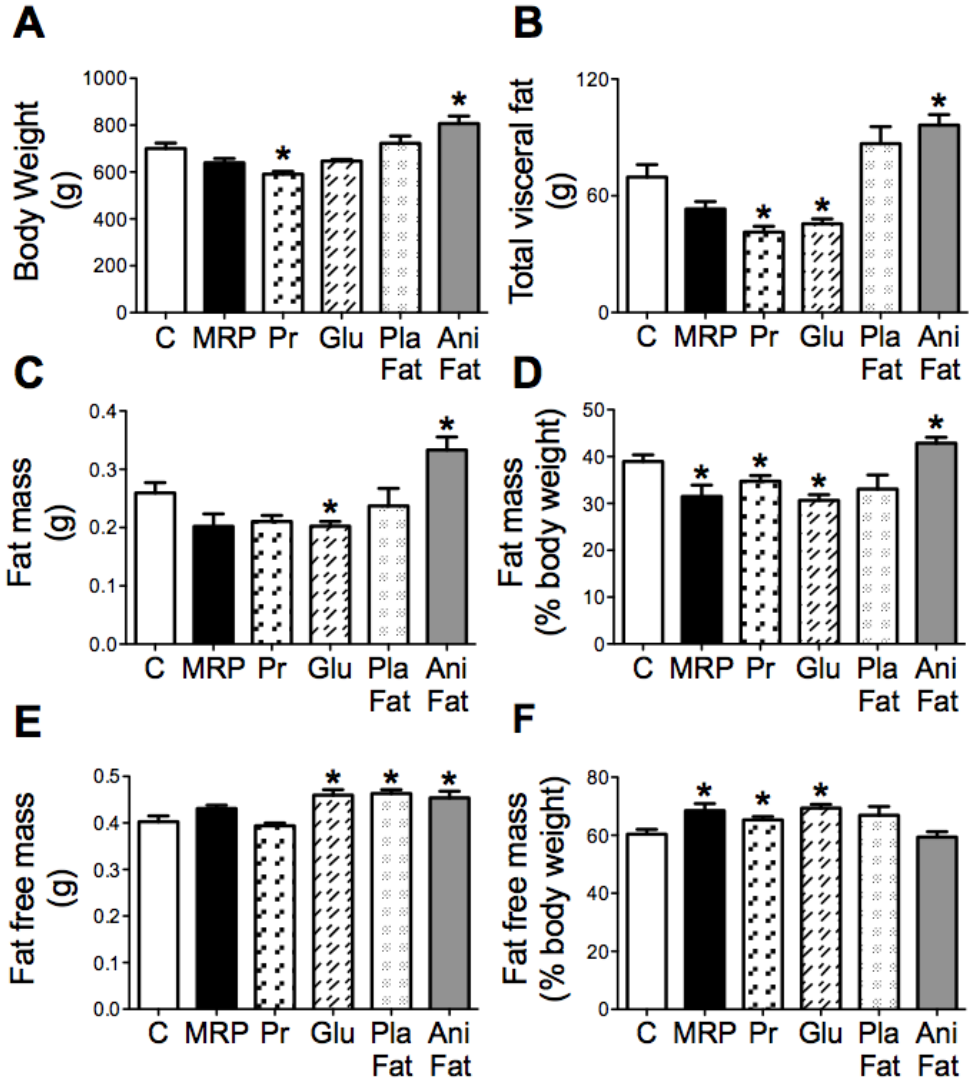

Figure 1

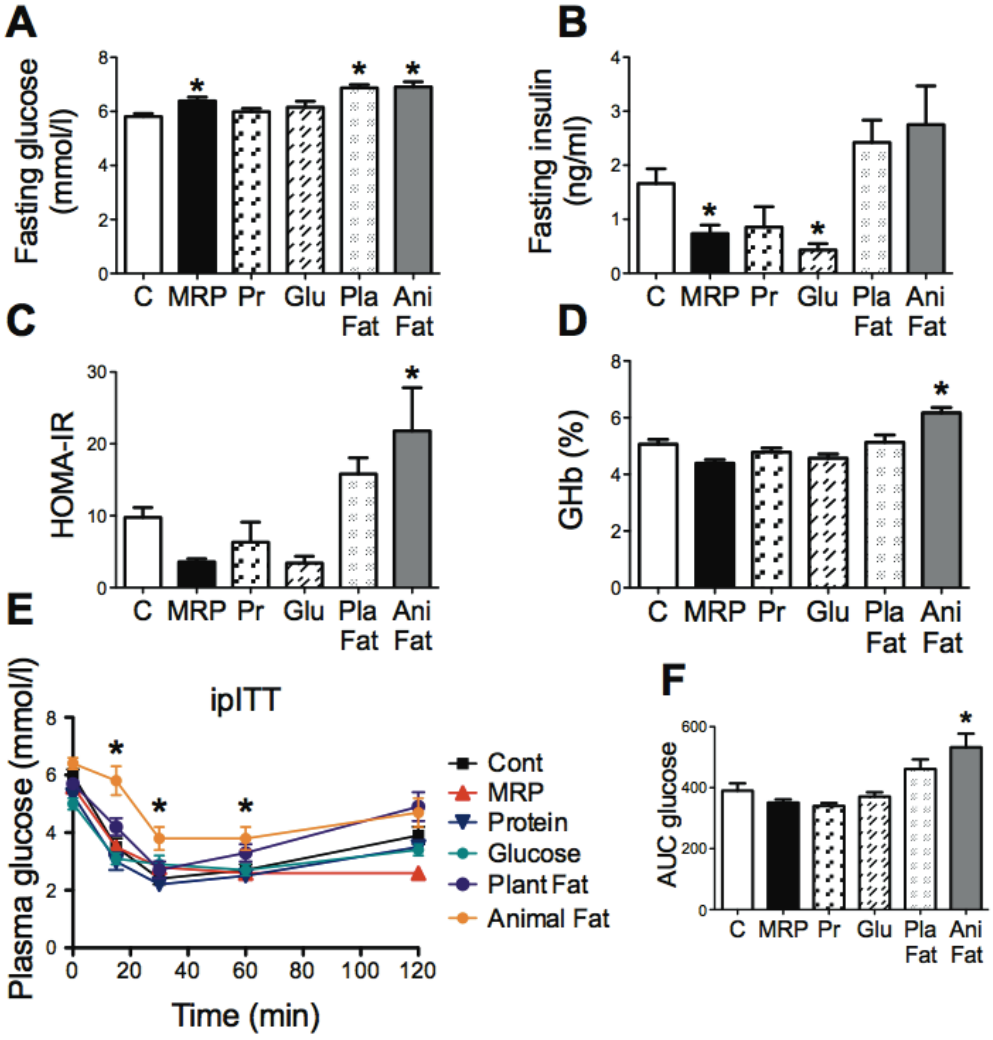
A
Fasting glucose (mmol/l)
C MRP Pr Glu Pla Fat Ani Fat
B
Fasting insulin (ng/ml)
C MRP Pr Glu Pla Fat Ani Fat
C
HOMA-IR
C MRP Pr Glu Pla Fat Ani Fat
D
GHb (%)
C MRP Pr Glu Pla Fat Ani Fat
E
ipITT
Plasma glucose (mmol/l)
Time (min)
Cont
MRP
Protein
Glucose
Plant Fat
Animal Fat
F
AUC glucose
C MRP Pr Glu Pla Fat Ani Fat

Figure 2

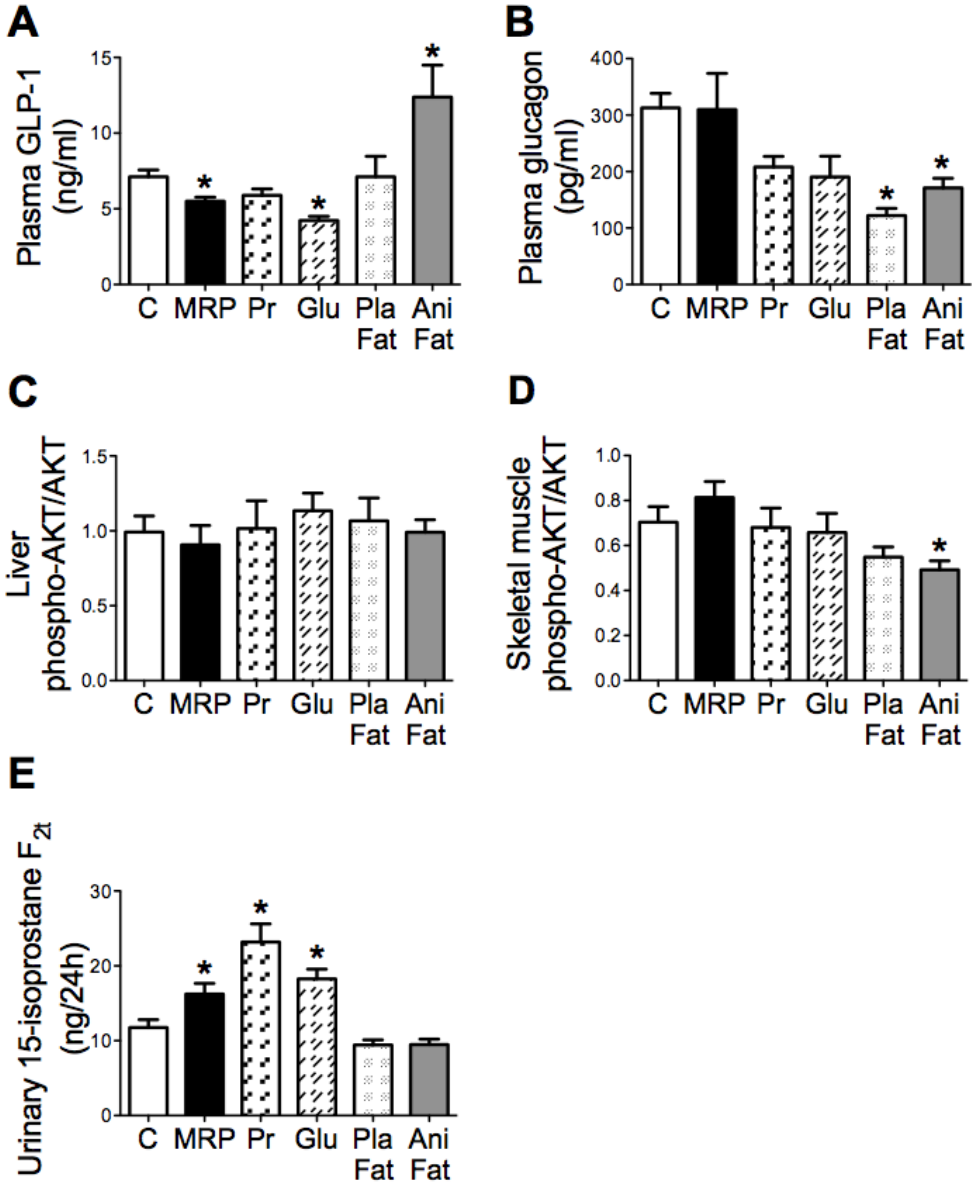
A
Plasma GLP-1 (ng/ml)
15
10
5
0
C MRP Pr Glu Pla Fat Ani Fat
B
Plasma glucagon (pg/ml)
400
300
200
100
0
C MRP Pr Glu Pla Fat Ani Fat
C
Liver phospho-AKT/AKT
1.5
1.0
0.5
0.0
C MRP Pr Glu Pla Fat Ani Fat
D
Skeletal muscle phospho-AKT/AKT
1.0
0.8
0.6
0.4
0.2
0.0
C MRP Pr Glu Pla Fat Ani Fat
E
Urinary 15-isoprostane F2t (ng/24h)
30
20
10
0
C MRP Pr Glu Pla Fat Ani Fat

Figure 3

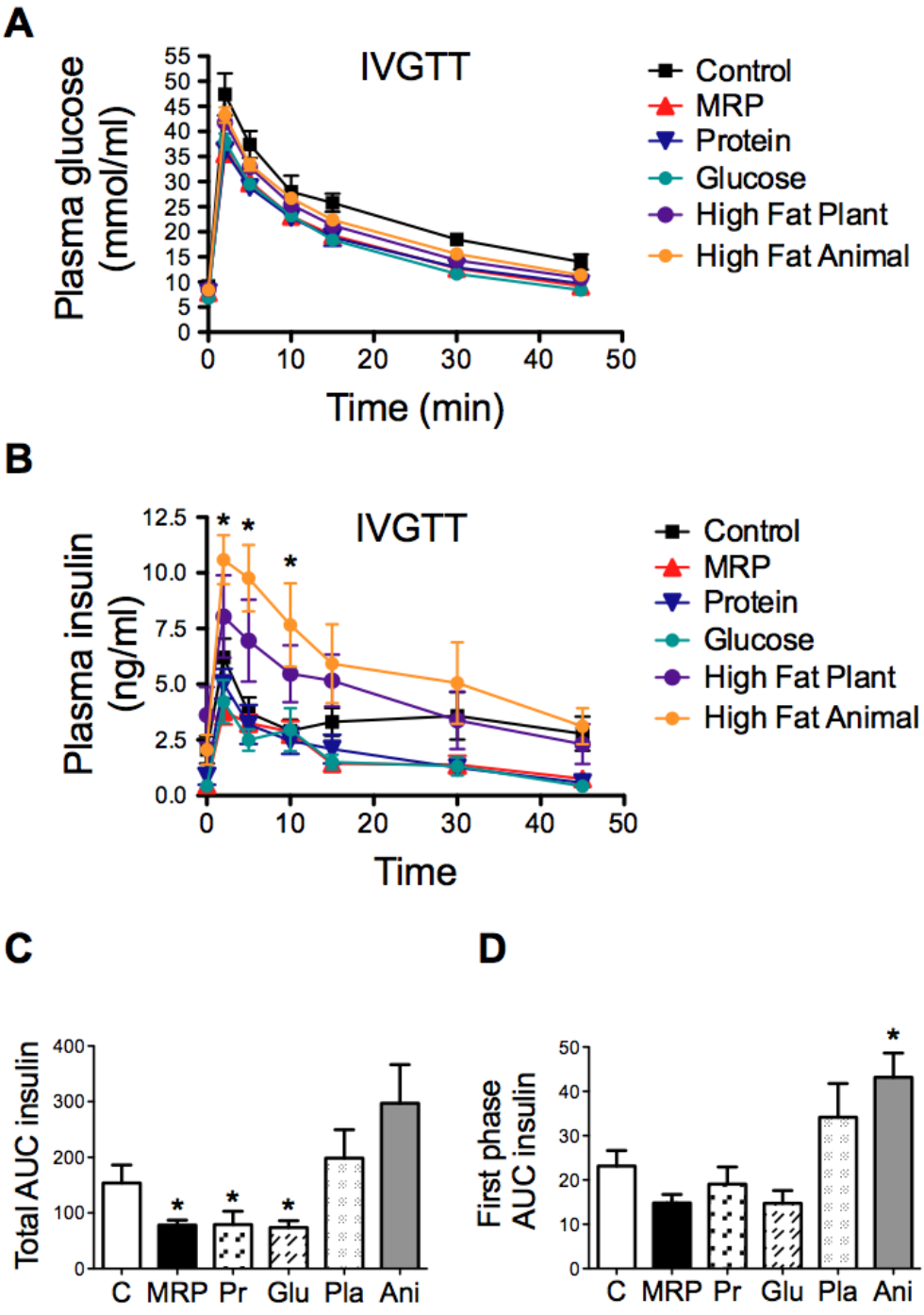
A
IVGTT
Plasma glucose (mmol/ml)
Time (min)
Control
MRP
Protein
Glucose
High Fat Plant
High Fat Animal
B
IVGTT
Plasma insulin (ng/ml)
Time
C
Total AUC insulin
C MRP Pr Glu Pla Ani
D
First phase AUC insulin
C MRP Pr Glu Pla Ani

Figure 4