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Beneficial metabolic effects of dietary epigallocatechin gallate alone and in combination with exendin-4 in high fat diabetic mice

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ABSTRACT

Objective: Significant attempts are being made to generate multifunctional, hybrid or peptide combinations as novel therapeutic strategies for type 2 diabetes, however this presents key challenges including design and pharmaceutical development. In this study, we evaluated metabolic properties of oral nutritional supplement epigallocatechin gallate (EGCG) in combination with GLP-1 agonist exendin-4 in a mouse model of dietary-induced diabetes and obesity.

Methods: EGCG, exendin-4 or combination of both were administered twice-daily over 28 days to high fat (HF) mice on background of low-dose streptozotocin. Energy intake, body weight, fat mass, glucose tolerance, insulin sensitivity, lipid profile, biochemical and hormone markers, and islet histology were examined.

Results: All treatment groups exhibited significantly reduced body weight, fat mass, circulating glucose and insulin concentrations, and HbA1c levels which were independent of changes in energy intake. Similarly, there was marked improvement in glycaemic control, glucose-stimulated insulin release, insulin sensitivity, total cholesterol and triglycerides, with most prominent effects observed following combination therapy. Circulating corticosterone concentrations and 11β-hydroxysteroid dehydrogenase type1 (11β-HSD1) staining (in pancreas) were beneficially decreased without changes in circulating interleukin 6 (IL-6), alanine transaminase (ALT) and glutathione reductase. Combination therapy resulted in increased islet area and number, beta cell area, and pancreatic insulin content. Generally, metabolic effects were much more pronounced in mice which received combination therapy.

Conclusions: EGCG alone and particularly in combination with exendin-4 exerts positive metabolic properties in HF mice. EGCG may be useful dietary adjunct alongside GLP-1 mimetics in treatment of diabetes and related disorders.

1. Introduction

Glucagon-like peptide-1 (GLP-1) is an amidated 30-amino acid gut peptide secreted from enteroendocrine L-cells in response to nutrient ingestion (Reimann and Gribble, 2016). Upon secretion, GLP-1 binds to the GLP-1 receptor which is highly expressed in pancreatic beta cells and other extra-pancreatic tissues including the brain, heart, kidney, lung, enteric and peripheral nervous systems (Yamada et al., 2016). The most widely recognised and characterised physiological action of GLP-1 is its ability to stimulate glucose-induced insulin secretion (Tudurí et al., 2016). However, GLP-1 also inhibits glucagon release, delays gastric emptying, lowers body weight and induces satiety (Tahrani et al., 2016). Furthermore, research in animal models of diabetes-obesity and neurodegenerative disease show that GLP-
improves cognition, synaptic plasticity and enhances hippocampal neurogenesis, further highlighting GLP-1 as an extremely attractive therapeutic agent (Gault et al., 2010; Gengler et al., 2012; Porter et al., 2013; Cai et al., 2016). Yet, like numerous gut peptides, native GLP-1 is quickly degraded in the circulation by the enzyme dipeptidylpeptidase-4 (DPP4) and, to circumvent this, DPP4 inhibitors and stable GLP-1 receptor agonists have been developed. The first approved GLP-1 receptor agonist, exenatide (Byetta®), was isolated (as exendin-4) from the salivary gland of the Gila monster (Heloderma suspectum) (Eng et al., 1992). Exendin-4 is a 39-amino acid peptide which has 53% sequence homology with human GLP-1, and contains glycine at position 2 instead of alanine, and an additional C-terminal amino acid sequence of PSSGAPPSS (Raufman, 1996). These modifications protect exendin-4 against DPP4 degradation and promote formation of secondary structure, leading to enhanced pharmacokinetic properties, longer circulating half-life and extended duration of action (Lovshin and Drucker, 2009).

Even though a range of GLP-1 receptor agonists are now available, it has become increasingly evident that successful management of patients with type 2 diabetes requires the further development of safe and more effective mono and/or combination therapies with complementary mechanisms of action. At present, considerable effort is being made to generate multifunctional, hybrid or peptide combinations as novel therapeutic strategies (Henderson et al., 2016; Trevaskis et al., 2015; Dalbøge et al., 2014; Finan et al., 2015; Clemmensen et al., 2014; Irwin et al., 2015; Gault et al., 2013; O’Harte et al., 2016; Bhat et al., 2013). However, this approach poses several key challenges especially with regards to designing an appropriate and balanced pharmaceutical entity and moreover, likely requirements for the patient to increase the frequency and/or number of injections. One alternative possibility is to combine the powerful effects of a GLP-1 receptor agonist such as exenatide with a widely available oral nutritional supplement such as epigallocatechin gallate (EGCG) which is an antioxidant polyphenol found in green tea (Suzuki et al., 2016).

EGCG is an ester of epigallocatechin and gallic acid and is the most abundant catechin found in green tea (Peter et al., 2016). It has been shown to offer a range of beneficial effects against cancer, obesity, atherosclerosis and infection (Suzuki et al., 2016; Niedzwiecki et al., 2016; Chowdhury et al., 2016). There is also evidence to link EGCG with body weight loss, release of endocrine pancreatic hormones, appetite suppression, improvement in glucose tolerance, augmentation of glucose-stimulated insulin secretion and enhanced insulin sensitivity (Nishiumi et al., 2010; Yang et al., 2016; Kao et al., 2000; Wolfram et al., 2006; Ortsäter et al., 2012). While the precise underlying cellular, biochemical and molecular mechanisms by which EGCG ameliorates metabolic disease are not fully understood,
emerging data point to EGCG as an inhibitor of the microsomal enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) which decreases local cortisol concentrations and alleviates insulin resistance (Hintzpeter et al., 2014). Indeed, several 11β-HSD1 inhibitors have been developed and data suggests that they improve glycaemic control, lipid profile and blood pressure, with moderate body weight loss (Anderson and Walker, 2013). Thus, we hypothesised that combination of exendin-4 with the oral dietary supplement EGCG would provide improved metabolic outcomes. High fat diabetic mice were treated with EGCG alone, exendin-4 alone and a combination of both over a 28-day period. Effects on body weight, glycaemic control, insulin secretion and action, lipids, circulating biomarkers, and islet histology were assessed.

2. Materials and methods

2.1. Chemicals

Exendin-4 (molecular mass: 4186.6 Da) was purchased from GL Biochem Ltd. (Shanghai, China). (-)-Epigallocatechin gallate purified from green tea (EGCG; molecular weight 458.37 g/mol) and streptozotocin were purchased from Sigma-Aldrich (Poole, Dorset, UK). All other chemicals were obtained from standard sources and of the highest purity available.

2.2. Animals

For acute in vivo studies obese diabetic db/db mice (BKS.Cg-+Lepr<sup>db</sup>/+Lepr<sup>db</sup>/OlaHsd) were purchased from Harlan Ltd. (Blackthorn, UK) aged 12-14 weeks and maintained on standard rodent chow (10% fat, 30% protein and 60% carbohydrate; percent of total energy 12.99 kJ/g; Trouw Nutrition, Cheshire, UK). For long-term in vivo studies, male NIH mice (aged 8-10 weeks; Harlan, UK) were placed on high fat diet (composed of 45% Fat; Special Diet Services, Witham, UK; total energy 26.15 kJ/g) containing lard and soya oil for 45 days prior to experimentation. Separate group of lean mice (control) had free access to standard rodent chow. All mice had free access to drinking water, remained on their respective diets for the duration of the study and were housed in an air-conditioned room (22±2°C) with artificially controlled 12 h light/12 h dark cycle (08:00-20:00h). All experiments performed according to Principles of Laboratory Animal Care (NIH publication no. 86-23, revised 1985) and UK Home Office Regulations (UK Animals Scientific Procedures Act 1986).

2.3. Experimental protocols
For acute studies, glucose and insulin concentrations were determined in db/db mice that received glucose alone (18 mmol/kg bw; i.p.) or together with EGCG (50 mg/kg; p.o.), exendin-4 (25 nmol/kg; i.p.), or a combination of both. Prior to the start of the long-term study, high fat mice were injected with STZ (prepared in citrate buffer) on day -14 (50 mg/kg; i.p.) and day -7 (75 mg/kg; i.p.) as described previously (Millar et al., 2016). Consumption of high fat diet and combination of low-dose STZ resulted in increased body weight (38.5±1.9 vs 30.1±1.7g; P<0.001), hyperglycaemia (18.4±1.8 vs 3.8±1.1 mmol/l; P<0.001) and hyperinsulinaemia (4.9±0.12 vs 2.2±0.11 ng/ml; P<0.001). At day 0, groups of mice (n=8) received twice-daily saline vehicle (0.9% wt/vol; i.p.), exendin-4 (25 nmol/kg; i.p.), EGCG (50 mg/kg; p.o.) or EGCG (50 mg/kg; p.o.) plus exendin-4 (25 nmol/kg; i.p.) at 09:00 and 17:00 over 28 days. Lean mice received twice-daily injections of saline vehicle (0.9% wt/vol; i.p.).

2.4. Measurement of metabolic parameters
Energy intake, body weight, non-fasting glucose and insulin concentrations were measured at regular intervals. Glucose tolerance (18 mmol/kg; p.o. in 12 hour fasted mice) and insulin sensitivity (25 U/kg bovine insulin; i.p. in non-fasted mice) were carried out at end of the study. Blood glucose was determined using Ascencia Contour glucose meter (Bayer Healthcare, Newbury, UK). For analysis of plasma samples, blood was collected from tail vein of conscious mice into fluoride/heparin micro-centrifuge tubes (Sarstedt, Numbrecht, Germany) and centrifuged in a Beckman micro-centrifuge (Beckman Instruments, UK) for 30 s at 13,000g with plasma stored at -20°C prior to further analysis. Insulin was determined by RIA as described previously (Flatt and Bailey, 1981). HbA1c was determined with commercial kit (HB3058, Chirus Limited, Watford, UK). Lipids (total cholesterol – CH200; and triglycerides – TR210), ALT (AL1205) and glutathione reductase (GR2368) were measured using enzymatic kits from Randox Laboratories (Crumlin, UK). Plasma corticosterone (ab100712) and IL-6 (ab108821) were measured using kits from Abcam (Cambridge, UK) and analysed with SOFTMAX PRO Software Version 5.2 on Flexstation 3 (Molecular Devices, Sunnyvale, CA, USA). Body fat and lean mass were measured using dual-energy x-ray absorptiometry (DEXA) densitometry (Piximus Densitometer, USA) as described previously (Millar et al., 2016).

2.5. Immunohistochemistry and image analysis
Whole pancreata were excised for immunohistochemistry and measurement of insulin content by radioimmunoassay. For histology, tissues were fixed in paraformaldehyde (4% w/v) for 48 hours at 4°C, processed using automated tissue processor (Leica TP1020 Semi-Enclosed Benchtop Tissue Processor, Leica Microsystems, Nussloch, Germany) and embedded in paraffin wax. Immunohistochemistry was performed as described previously (Moffett et al., 2015). The following primary antibodies were used: anti-insulin antibody (ab6995, 1:1000 dilution, Abcam); anti-glucagon antibody (PCA2/4, 1:400 dilution, raised in-house); and anti-HSD11B1, 1:250 dilution, ab109554, Abcam). Secondary antibodies were used as appropriate: Alexa Fluor 594 goat anti-mouse IgG (1:400) and Alexa Fluor 488 goat anti-guinea pig IgG (1:400). Quantification and image analysis was performed in a blinded manner using bright field microscopy and CellF image analysis software (Olympus Soft Imaging Solutions, GmbH). Islet parameters including islet number, islet area, beta cell area, and percentage area stained for $11\beta$HSD1 were determined. Islet size distribution (small islets: < 10,000 $\mu m^2$; medium islets: >10,000 and < 25,000 $\mu m^2$; and large islets: >25,000 $\mu m^2$) was also determined.

2.6. Statistical analysis

Results were analysed using PRISM (San Diego, CA, USA) and data expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using ANOVA followed by Student-Newman-Keuls post-hoc test. For immunohistochemistry, statistical analyses were carried out by unpaired Student’s t test (non-parametric, with two-tailed P values and 95 % confidence interval) and one-way ANOVA with Bonferroni post-hoc test. Groups of data were considered to be significantly different if P<0.05.

3. Results

3.1. Acute glucose-lowering and insulin-releasing actions of EGCG alone and in combination with exendin-4 in db/db mice

Administration of EGCG and exendin-4 alone or in combination resulted in significantly reduced (20-24%; P<0.05-P<0.01) blood glucose levels at 60 and 105 minutes post-injection (Fig. 1A). This was corroborated by a marked reduction (24-39%; P<0.05-P<0.001) in glucose AUC$_{105}$ values (Fig. 1A). Combination therapy reduced glucose concentrations more than either drug administered alone (P<0.001 vs P<0.01; Fig. 1A), although not significantly compared to exendin-4 alone. All drug treatments had a tendency to increase insulin concentrations but these did not reach significance in terms of individual time-points or overall AUC values (Fig. 1B).
3.2. Effects of EGCG alone and in combination with exendin-4 on body weight, energy intake, glucose, insulin, fat mass, and HbA1c in high fat fed mice

Treatment with EGCG alone and in combination with exendin-4 resulted in a progressive decrease (9-10%; P<0.05-P<0.001) in body weight compared to high fat controls (Fig. 2A). In addition, mice treated with combination therapy displayed superior reduction in body weight (P<0.05) compared to mice treated with exendin-4 alone (Fig. 2A). Furthermore, body weights for mice given combination therapy were similar (P>0.05) to lean controls. No changes in energy intake were observed in any of the high fat groups (P>0.05; Fig. 2B). Circulating glucose concentrations were significantly reduced (17-61%; P<0.05-P<0.001) in all treatment groups from day 5 onwards (Fig. 2C). On day 28, glucose concentrations in mice on combination therapy were significantly lower (P<0.05) compared to EGCG alone. Changes in glucose were accompanied by significantly reduced (22-34%; P<0.05) insulin concentrations (Fig. 2D). As expected, high fat control mice displayed significantly increased (1.6-fold; P<0.001) fat mass compared to lean controls (Fig. 2E). Moreover, mice treated with EGCG, exendin-4 and combination therapy exhibited reduced (20-31%; P<0.05-P<0.01) fat mass compared to high fat controls (Fig. 2E). No effect on lean mass was observed in any of the treatment groups (P>0.05; data not shown). All groups of high fat mice displayed significantly increased (1.2-1.3-fold; P<0.001) HbA1c values compared to lean counterparts, which were significantly reduced in all treatment groups (10-19%; P<0.05-P<0.01; Fig. 2F).

3.3. Effects of EGCG alone and in combination with exendin-4 on glucose tolerance, insulin secretion and insulin sensitivity in high fat fed mice

Glucose concentrations were significantly reduced (9-39%; P<0.05-P<0.01) in mice treated with combination therapy compared to high fat controls following oral glucose challenge (Fig. 3A). Furthermore, glycaemic response of combination therapy was superior to EGCG alone at 105 minutes post-injection. These observations were corroborated by significantly reduced (50%; P<0.05) AUC_{105} glucose values compared to high fat controls, EGCG and exendin-4 alone (Fig. 3A). All treatment groups displayed an increase (2.2-3.8 fold; P<0.01-P<0.001) in glucose-stimulated insulin release compared to high fat controls (Fig. 3B). Furthermore, mice receiving combination therapy displayed significantly increased (1.5-fold; P<0.01) insulin concentrations compared to EGCG alone (Fig. 3B). All treatment groups augmented (1.6-1.8-fold; P<0.05-P<0.001) the glucose-lowering action of exogenous insulin (Figure 3C). Combination therapy resulted in significantly improved insulin sensitivity (P<0.05) compared to exendin-4 as judged by glucose AUC_{60} values (Fig. 3C).
3.4.  **Effects of EGCG alone and in combination with exendin-4 on lipids, ALT and glutathione reductase in high fat fed mice**

All treatment groups exhibited significant reductions in total cholesterol (13-38%; P<0.05-P<0.001; Fig. 4A) and triglycerides (25-78%; P<0.05-P<0.001; Fig. 4B) compared to high fat controls. Furthermore, mice treated with EGCG alone and combination therapy displayed significantly reduced (31-66%; P<0.05-P<0.001) total cholesterol and triglycerides compared to exendin-4 and EGCG alone (Fig. 4A-B). No significant differences (P>0.05) were observed in plasma ALT levels in any of the groups (Fig. 4C). Plasma glutathione reductase activity was significantly reduced (21%; P<0.05) in high fat control mice but levels were similar to lean in all treatment groups (Fig. 4D).

3.5.  **Effects of EGCG alone and in combination with exendin-4 on islet histology and insulin content in high fat fed mice**

Exendin-4, EGCG and combination therapy resulted in significantly increased (P<0.05-P<0.001) islet number, islet area, beta cell area and medium-to-large sized islets compared to high fat controls (Fig. 5A-D). Of all groups, combination therapy induced the most prominent effects on islet morphology with significant increase (P<0.05-P<0.01) in islet number and beta cell area compared to exendin-4 and EGCG alone (Fig. 5A and 5C) and an increase (P<0.01) in islet area compared to EGCG alone (Fig. 5B). Mice treated with EGCG alone and combination therapy exhibited significantly increased (1.1 to 1.3-fold; P<0.05) pancreatic insulin content compared to high fat controls (Fig. 5E).

3.6.  **Effects of EGCG alone and in combination with exendin-4 on corticosterone, IL-6 and 11βHSD1 activity in high fat fed mice**

High fat control and exendin-4 treated mice had significantly increased (1.2 to 1.4-fold; P<0.05-P<0.01) circulating plasma corticosterone concentrations compared to lean controls (Fig. 6A). In contrast, mice treated with EGCG alone and combination therapy displayed reduced (16-28%; P<0.05-P<0.01) corticosterone concentrations compared to high fat and exendin-4 alone groups (Fig. 6A). No significant differences (P>0.05) were noted in IL-6 concentrations in any of the treatment groups (Fig. 6B). Consistent with enzyme inhibition, both EGCG alone and combination therapy resulted in significantly decreased 11βHSD1 staining in the pancreas (26-41%; P<0.05-P<0.01; Fig. 6C) compared to high fat control and exendin-4 groups.
4. Discussion

Glucagon-like peptide-1 (GLP-1) receptor agonists are frequently used for the treatment of type 2 diabetes although a significant proportion of patients do not achieve optimal glycaemic control. A number of approaches are actively being pursued to enhance their therapeutic efficacy (Henderson et al., 2016; Trevaskis et al., 2015; Dalbøge et al., 2014; Finan et al., 2015; Clemmensen et al., 2014; Irwin et al., 2015; Gault et al., 2013) but improved multiple-acting peptides have several drawbacks particularly in relation to pharmaceutical development and patient compliance. Therefore, rather than developing another injectable, this study utilised a complementary therapeutic approach based on an oral dietary adjunct derived from green tea. *Camellia sinensis* (Green tea) is a widely consumed herbal beverage and recent research has demonstrated that the predominant biologically active component is the polyphenolic catechin, (-)-epigallocatechin-3-gallate (EGCG) (Suzuki et al., 2016). Studies in rodents and humans suggest that EGCG may improve hyperglycaemia, insulin resistance, oxidative stress, dyslipidaemia and enhance glucose tolerance in type 2 diabetes (Fukino et al., 2008; Roghani and Baluchnejadmojarad, 2010). Thus, we examined biological actions of combining EGCG with the GLP-1 receptor agonist exenin-4 in dietary-induced obese diabetic mice.

Based on acute studies in *db/db* mice, we chose to administer EGCG and exenin-4 in a chronic study in diabetic mice employing a twice-daily treatment regimen (Gault et al., 2010; Gault et al., 2013; Wolfram et al., 2006). Following 28-day therapy, all treated mice exhibited decreased glucose and HbA1C, and significantly improved glucose tolerance, glucose-mediated insulin secretion and insulin sensitivity. These findings reproduced the classical metabolic actions of GLP-1 but also confirmed the glucoregulatory and insulinotropic actions of EGCG (Wolfram et al., 2006). In both groups receiving EGCG, the effects were accompanied by reductions in body weight and percentage fat mass (Kao et al., 2000). Even though exenin-4 treatment exhibited a trend towards body weight reduction, statistical significance was not achieved. To some extent this is unexpected given the well-documented effects of GLP-1 receptor signalling on weight loss in humans (Lovshin and Drucker, 2009), nevertheless, it confirms previous studies in diabetic rodents (Moffett et al., 2015; Irwin et al., 2009; Rolin et al., 2002; Irwin et al., 2007). Circulating insulin concentrations were also reduced following EGCG therapy suggesting that improvements in hyperglycaemia and associated metabolic effects could be due to benefits of beta cell function as well as insulin signalling. Indeed, pancreatic histology revealed that EGCG treatment increased islet area and number, beta cell area and insulin content indicating that positive
actions of combination therapy are not due solely to additional weight loss over single agent alone. These positive actions of EGCG plus exendin-4 therapy on glucose homeostasis are important and may have implications for combination therapy for T2DM.

In harmony with previous studies, treatment with EGCG or exendin-4 significantly reduced total cholesterol and triglycerides (Sun et al., 2015; Raederstorff et al., 2003). GLP-1 receptor agonists reduce dyslipidaemia via several mechanisms including reduction of very-low-density lipoprotein-triglyceride production rate from liver, decrease in hepatic triglyceride content, and inhibition of de novo lipogenesis and beta-oxidation (Patel et al., 2014). Interestingly, EGCG and combination therapy gave rise to powerful lipid-lowering actions to the extent that total-cholesterol and triglycerides were returned to levels observed in lean controls. Importantly, the metabolic changes were not associated with detrimental effects on the liver, as witnessed by unchanged alanine transaminase (ALT) concentrations. Similarly, circulating concentrations of the pro-inflammatory cytokine, interleukin 6 (IL-6), were not altered in any of the treatment groups, indicating lack of toxicity at the dose and regimen selected. Consistent with antioxidant properties, reduced levels of plasma glutathione reductase activity in diabetic mice were decreased by EGCG, exendin-4 and combination therapy. The lack of toxicity and beneficial effects of EGCG plus exendin-4 therapy on lipid metabolism are significant and further highlight potential of combination therapy.

Circulating plasma corticosterone concentrations were significantly elevated in diabetic mice but EGCG alone or combination with exendin-4 reversed this effect. Corticosterone (or cortisol in humans), plays a pivotal role in many physiological processes including stress response, metabolism, immunity and cognitive function (Atanasov and Odermatt, 2007). Furthermore, studies show that exposure to elevated levels of glucocorticoids induces insulin resistance and exerts negative effects on hippocampal function (Stranahan et al., 2008a; Stranahan et al., 2008b). Although glucocorticoids are secreted from the adrenal cortex, glucocorticoid production and effects can be controlled at the local cellular level by the actions of 11beta-hydroxysteroid dehydrogenase type 1 (11β-HSD1) (Stranahan et al., 2008a). Recent data suggest that part of the beneficial metabolic actions of EGCG may be as a consequence of inhibition of 11β-HSD1 activity (Hintzpeter et al., 2014). In the present study, 11β-HSD1 staining in the pancreas was actually increased in diabetic control mice (Duplomb et al., 2004). Treatment with exendin-4 had no effect on 11β-HSD1 activity, whereas EGCG treatment (alone and in combination with exendin-4) markedly reduced 11β-HSD1 staining. Although not examined in the present study, measurement of 11β-HSD1 in adipose tissue would be interesting. A similar pattern of change in 11β-HSD1 staining was also observed in the hippocampus. Taken together, these findings are consistent with previous
studies highlighting therapeutic action of negatively modulating 11\(\beta\)-HSD1 in pathophysiology of type 2 diabetes and obesity (Morton et al., 2004).

In summary, oral administration of the green tea polyphenol EGCG exerts positive metabolic effects in high fat diabetic mice. When administered together with exendin-4, there is a more noticeable improvement in metabolic parameters compared to monotherapy. Overall, these data highlight a novel approach to the treatment of diabetes and related disorders using complementary therapy with a GLP-1 receptor agonist and an oral dietary adjunct.

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Author contributions
NMP, PJBM and VP contributed to conduct/data collection, data analysis and writing of the manuscript. PRF and VAG contributed to study design, analysis and writing of the manuscript. All authors approved the final version of the manuscript.

Conflict of interest
None

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**Figure Legends**

**Fig. 1** Acute effects of EGCG administered alone and in combination with exendin-4 on glucose and insulin concentrations in *db/db* mice. (A) Glucose and (B) insulin concentrations were measured prior to and after administration of glucose alone (18 mmol/kg; i.p.) or in combination with EGCG (50 mg/kg; p.o.), exendin-4 (25 nmol/kg; i.p.), or a combination of the two. Glucose and insulin AUC values for 0-105 min post-injection are also shown. Values are mean ± SEM (n=6 mice). *P<0.05, **P<0.01 and ***P<0.001 compared to glucose alone.

**Fig. 2** Effects of twice-daily administration of EGCG alone and in combination with exendin-4 on (A) body weight, (B) energy intake, (C) glucose, (D) insulin, (E) fat mass, and (F) HbA1c in high fat fed mice. Mice received saline vehicle (0.9% wt/vol; i.p.), exendin-4 (25 nmol/kg; i.p.), EGCG (50 mg/kg; p.o.), or a combination of the two twice-daily for 28 days. Metabolic parameters were measured every 2-4 days (A-D) or at the end of the study period (E-F). Lean control mice received twice-daily injections of saline vehicle. Values are mean ± SEM (n=8 mice). *P<0.05, **P<0.01 and ***P<0.001 compared with lean controls. ∆P<0.05, ∆∆P<0.01 and ∆∆∆P<0.001 compared with high fat controls. ψP<0.05 compared with exendin-4 alone. θP<0.05 compared with EGCG alone.

**Fig. 3** Effects of twice-daily administration of EGCG alone and in combination with exendin-4 on (A) oral glucose tolerance, (B) insulin response to oral glucose, and (C) insulin sensitivity in high fat fed mice. Mice received saline vehicle (0.9% wt/vol; i.p.), exendin-4 (25 nmol/kg; i.p.), EGCG (50 mg/kg; p.o.), or a combination of the two twice-daily for 28 days. Lean control mice received twice-daily injections of saline vehicle. (A and B) Glucose and insulin concentrations were measured prior to and after administration of glucose (18 mmol/kg; p.o. in 12-hour fasted mice). AUC values for 0-105 minutes post-injection are also included. (C) Glucose concentrations were measured prior to and after administration of insulin (25 U/kg; i.p. in non-fasted mice). AAC values for 0-60 minutes post-injection are also included. Values are mean ± SEM (n=8 mice). *P<0.05, **P<0.01 and ***P<0.001 compared with lean controls. ∆P<0.05, ∆∆P<0.01 and ∆∆∆P<0.001 compared with high fat controls. ψP<0.05 compared with exendin-4 alone. θP<0.05 compared with EGCG alone.

**Fig. 4** Effects of twice-daily administration of EGCG alone and in combination with exendin-4 on (A) total cholesterol, (B) triglycerides, (C) ALT and (D) glutathione reductase in high fat fed mice. Mice received saline vehicle (0.9% wt/vol; i.p.), exendin-4 (25 nmol/kg; i.p.), EGCG (50 mg/kg; p.o.), or a combination of the two twice-daily for 28 days. Lean control mice received twice-daily injections of saline vehicle. Values are mean ± SEM (n=8 mice). *P<0.05, **P<0.01 and ***P<0.001 compared with lean controls. ∆P<0.05, ∆∆P<0.01 and ∆∆∆P<0.001 compared with high fat controls. ψP<0.05 compared with exendin-4 alone. θP<0.05 compared with EGCG alone.

**Fig. 5** Effects of twice-daily administration of EGCG alone and in combination with exendin-4 on (A) islet number, (B) islet area, (C) beta cell area, (D) islet size distribution and (E) pancreatic insulin content in high fat fed mice. Mice received saline vehicle (0.9% wt/vol; i.p.), exendin-4 (25 nmol/kg; i.p.), EGCG (50 mg/kg; p.o.), or a combination of the two twice-daily for 28 days. Lean control mice received twice-daily injections of saline vehicle. Values are mean ± SEM (n=8 mice). *P<0.05, **P<0.01 and ***P<0.001 compared with lean controls. ∆P<0.05, ∆∆P<0.01 and ∆∆∆P<0.001 compared with high fat controls. ψP<0.01 and ψψP<0.01 compared with exendin-4 alone. θP<0.05 compared with EGCG alone.

**Fig. 6** Effects of twice-daily administration of EGCG alone and in combination with exendin-4 on (A) corticosterone, (B) IL-6 and (C) pancreatic 11βHSD1 staining in high fat fed mice. Mice received saline vehicle (0.9% wt/vol; i.p.), exendin-4 (25 nmol/kg; i.p.), EGCG (50
mg/kg; p.o.), or a combination of the two twice-daily for 28 days. Lean control mice received twice-daily injections of saline vehicle. Values are mean ± SEM (n=8 mice). **P<0.01 and ***P<0.001 compared with lean controls. ∆P<0.05 and ∆∆P<0.01 compared with high fat controls. ℰP<0.01 and ℰℰP<0.01 compared with exendin-4 alone.
Figure 1

A

Blood glucose (mmol/l)

Time (min)

Glucose alone
Exendin-4
EGCG
EGCG + Exendin-4

B

Plasma insulin (ng/ml)

Time (min)

Glucose alone
Exendin-4
EGCG
EGCG + Exendin-4

AUC

Blood glucose AUC (mmol/l min)

Plasma insulin AUC (ng/ml min)
Figure 2

**A**
- Lean control
- High fat control
- Exendin-4
- EGCG
- EGCG + Exendin-4

**B**
- Lean control
- High fat control
- Exendin-4
- EGCG
- EGCG + Exendin-4

**C**
- Lean control
- High fat control
- Exendin-4
- EGCG
- EGCG + Exendin-4

**D**
- Lean control
- High fat control
- Exendin-4
- EGCG
- EGCG + Exendin-4

**E**
- Lean control
- High fat control
- Exendin-4
- EGCG
- EGCG + Exendin-4

**F**
- Lean control
- High fat control
- Exendin-4
- EGCG
- EGCG + Exendin-4
Figure 3

A

- Lean control
- High fat control
- Exendin 4
- EGCG
- EGCG + Exendin 4

B

- Lean control
- High fat control
- Exendin 4
- EGCG
- EGCG + Exendin 4

C

- Lean control
- High fat control
- Exendin 4
- EGCG
- EGCG + Exendin 4

Blood glucose (%) basal

Blood glucose AUC

Plasma insulin (ng/mL min)

Time (min)

Blood glucose AUC (mmol/L min)

Plasma insulin AUC (ng/mL min)

Blood glucose (%) basal

Time (min)
Figure 4

A. Total cholesterol (mmol/l)

B. Triglycerides (mmol/l)

C. Alanine aminotransferase (U/L)

D. Glutathione reductase (U/L)
Figure 5

(A) Number of islets (per mm$^2$ of pancreas)

(B) Islet area ($\mu$m$^2$)

(C) Beta cell area ($\mu$m$^2$)

(D) Islet size distribution (% total islets)

(E) Pancreatic insulin content (µg/g tissue)
Figure 6

A

- Lean control
- High fat control
- Exendin-4
- EGCG
- EGCG + Exendin-4

Corticosterone (ng/ml)

B

- Lean control
- High fat control
- Exendin-4
- EGCG
- EGCG + Exendin-4

IL-6 (pg/ml)

C

- Lean control
- High fat control
- Exendin-4
- EGCG
- EGCG + Exendin-4

11β-HSD1 (% pancreas stained)
Highlights

Pathak, Millar, Pathak, Flatt and Gault
Beneficial metabolic effects of dietary epigallocatechin gallate alone and in combination with exendin-4 in high fat diabetic mice

- EGCG and exendin-4 combination therapy exerted powerful glucose-lowering and body weight reduction
- Combination therapy markedly decreased circulating cholesterol and triglycerides
- Combination therapy resulted in increased islet and beta cell number and insulin content