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Stable oxyntomodulin analogues exert positive effects on hippocampal neurogenesis and gene expression as well as improving glucose homeostasis in high fat fed mice

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ABSTRACT

The weight-lowering and gluco-regulatory actions of oxyntomodulin (Oxm) have been well-documented however potential actions of this peptide in brain regions associated with learning and memory have not yet been evaluated. The present study examined the long-term actions of a stable acylated analogue of Oxm, (DS^2)Oxm(K-γ-glu-Pal), together with parent (DS^2)Oxm peptide, on hippocampal neurogenesis, gene expression and metabolic control in high fat (HF) mice. Groups of HF mice (n = 12) received twice-daily injections of Oxm analogues (both at 25 nmol/kg body weight) or saline vehicle (0.9% wt/vol) over 28 days. Hippocampal gene expression and histology were assessed together with evaluation of energy intake, body weight, non-fasting glucose and insulin, glucose tolerance, insulin sensitivity and lipids. Oxm analogues significantly reduced body weight, improved glucose tolerance, glucose-mediated insulin secretion, insulin sensitivity, islet architecture and lipid profile. Analysis of brain histology revealed significant reduction in hippocampal oxidative damage (8-oxoguanine), enhanced hippocampal neurogenesis (doublecortin) and improved hippocampal and cortical synaptogenesis (synaptophysin) following treatment. Furthermore, Oxm analogues up-regulated hippocampal mRNA expression of MASH1, Synaptophysin, SIRT1, GLUT4 and IRS1, and down-regulated expression of LDL-R and GSK3β. These data demonstrate potential of stable Oxm analogues, and particularly (DS^2)Oxm(K-γ-glu-Pal) to improve metabolic function and enhance neurogenesis, synaptic plasticity, insulin signalling and exert protective effects against oxidative damage in hippocampus and cortex brain regions in HF mice.

1. Introduction

Following feeding, endocrine L-cells secrete the 37 amino acid gut peptide oxyntomodulin (Oxm) in parallel with the incretin hormone glucagon-like peptide-1 (GLP-1) (Bataille and Dalle, 2014). Both Oxm and GLP-1 are generated from pre-proglucagon by proteolytic processing in a tissue-specific manner (Holst, 1997). Oxm is specifically cleaved from pre-proglucagon by prohormone convertase 1/3 to the full amino acid sequence of glucagon with an additional C-terminal octapeptide extension (Bataille et al., 1981). Whilst initial research on Oxm focussed on its ability to modulate gastric acid secretion, current consideration is being directed towards understanding more fully its actions on body weight regulation and metabolic control. Indeed, Oxm has been shown to enhance beta-cell function in mice (Maida et al., 2008) and to differentially regulate murine food intake, energy expenditure and glucose metabolism in comparison to GLP-1 (Baggio et al., 2004; Du et al., 2012) making it a potentially attractive anti-obesity and/or anti-diabetic agent (Pocai, 2013). Unlike other well-characterised proglucagon-derived hormones, the precise mechanisms underlying the biological actions of Oxm have not been fully elucidated, due in part to the lack of a specific receptor. That being said, current understanding suggests that Oxm acts as a dual agonist binding both glucagon and GLP-1 receptors although with reduced affinity compared to native peptide hormones (Irwin and Flatt, 2013). To add further complexity, it appears that the glucagon and GLP-1 mediated actions of Oxm could differ from that of native glucagon and GLP-1 (Schepp et al., 1996).

Despite beneficial body weight lowering and metabolic effects, the clinical effectiveness of Oxm is limited due primarily to its short circulating half-life by degrading enzymes including DPP-4 (Zhu et al., 2003). Similar to incretin hormones, GLP-1 and glucose-dependent insulinotropic polypeptide (GIP), a series of peptidase-resistant Oxm analogues have been generated (Bianchi et al., 2013; Druce et al., 2009; Kerr et al., 2010; Liu et al., 2010; Lynch et al., 2014; Santoprete et al., 2011). Of the N-terminal modifications tested, replacing the naturally-occurring L-Ser amino acid at position 2 in Oxm with a D-isomer ((DS^2)Oxm) resulted in significantly improved peptidase resistance, glucose-lowering and insulin-releasing activity (Bianchi

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et al., 2013; Kerr et al., 2010; Lynch et al., 2014). Moreover, acylation of (DS²)Oxm with a C-terminal fatty acid adduct attached to a Lys residue via a linker molecule ((DS²)Oxm(K-γ-glu-Pal)) displayed a protracted biological action profile *in vivo* (Lynch et al., 2014). Thus, (DS²)Oxm(K-γ-glu-Pal) represents a promising candidate for future testing in long-term pre-clinical studies.

Parker et al. (2013) demonstrated that peripheral administration of glucagon or GLP-1 increases c-*fos* expression by activating similar appetite regulating centres in the brainstem and amygdala. This, together with the fact that Oxm has been shown to cross the blood–brain barrier, indicates that Oxm-mediated actions are not limited to the pancreas and gut (Wynne and Bloom, 2006). Recent studies have shown that GLP-1 receptors are present in brain regions associated with learning and memory such as the hippocampus and cortex (Alvarez et al., 1996; Campos et al., 1994; Dunphy et al., 1998; Hamilton and Hölscher, 2009; Merchenthaler et al., 1999). Indeed, administration of GLP-1 receptor agonists and DPP-4 inhibitors have been shown to enhance memory and learning in animal models of obesity-diabetes and other forms of cognitive impairment including Alzheimer's disease and Parkinson's disease (Garcia-Casares et al., 2014; Gault et al., 2015; Hölscher, 2014; Patrone et al., 2014; Talbot, 2014). Improvements in cognitive parameters following GLP-1 and/or DPP-4 inhibitor therapy in models of obesity-diabetes have been shown to be coupled with enhanced synaptic plasticity, neurogenesis, insulin signalling and reduced oxidative stress within the hippocampus and cortex (Gault et al., 2010, 2015; Isacson et al., 2011; Kosaraju et al., 2013; Lennox et al., 2013, 2014a, 2014b; Pintana et al., 2013; Pipatpiboon et al., 2013; Porter et al., 2010, 2013).

Whilst there is little evidence for beneficial effects of glucagon receptor activation in the hippocampus, glucagon binding sites have been detected in the hippocampus as well as olfactory tubercle, anterior pituitary, amygdala, septum, medulla, thalamus, olfactory bulb and hypothalamus (Dunphy et al., 1998; Hoosein and Gurd, 1984). Activation at these sites would primarily regulate glucagon-mediated anorectic function and maintenance of overall energy balance. In the present study, we have assessed whether chronic administration of the stable oxyntomodulin analogues (DS²)Oxm and (DS²)Oxm(K-γ-glu-Pal), which target receptors for glucagon and GLP-1, exert positive effects on hippocampal neurogenesis, synaptogenesis, oxidative stress and expression of a panel of important genes implicated in cognitive decline in HF mice.

2. Materials and methods

2.1. Peptides

Native Oxm, (DS²)Oxm and (DS²)Oxm[K-γ-glu-Pal] were purchased from GL Biochem Ltd. (Shanghai, China). (DS²)Oxm was synthesised based on the amino acid sequence of native Oxm with the exception of substitution of L-isomer of Ser at position 2 with a D-isomer. Similarly, (DS²)Oxm[γ-glu-Pal] contained D-isomer of Ser at position 2 and an additional C16 palmitic acid moiety attached at the C-terminal to an additional Lys residue via a γ-glu linker (Lynch et al., 2014). Experimental masses were evaluated in-house by MALDI-ToF MS and corresponded closely with theoretical values (in parentheses): Oxm - 4450.4 Da (4449.1 Da); (DS²)Oxm - 4450.4 Da (4449.9 Da); and (DS²)Oxm[K-γ-glu-Pal] - 4944.8 Da (4945.6 Da).

2.2. Animals

Male NIH mice (aged 8–10 weeks; Harlan Ltd., Blackthorn, UK) were housed individually in an air-conditioned room (22 ± 2 °C) with artificially controlled 12:12 h dark/light cycles (08:00–20:00 h). Groups of mice (n = 12) had free access to high fat (HF) diet (45% AFE Fat; Product Code 824053; Special Diet Services, Witham, UK; total energy 26.15 kJ/g) containing lard and soya oil for 13 weeks prior to experimentation. A group of normal mice (n = 12) had free access to standard rodent chow (10% fat, 30% protein and 60% carbohydrate; percent of total energy of 12.99 kJ/g; Trouw Nutrition, Cheshire, UK). Consumption of HF diet resulted in body weight gain (54.9 ± 3.0 g *vs* 48.5 ± 2.1; $p < 0.001$), hyperglycaemia (15.0 ± 2.4 mmol/L *vs* 5.0 ± 1.4 mmol/L; $p < 0.001$) and hyperinsulinemia (5.4 ng/ml *vs* 2.4 ng/ml; $p < 0.001$) when compared to aged-matched controls. Mice had free access to drinking water at all times. All experiments were 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) under appropriate project and personal licenses.

2.3. Study protocol

Groups of HF mice (n = 12) received twice-daily *i.p.* injections of saline vehicle (0.9% wt/vol), (DS²)Oxm and (DS²)Oxm[K-γ-glu-Pal] (both at 25 nmol/kg body weight) at 09:00 h and 17:00 h for 28 days. Lean mice also received twice-daily *i.p.* injections of saline vehicle (0.9% wt/vol). Energy intake, body weight, non-fasting blood glucose and non-fasting plasma insulin concentrations were measured (prior to 09:00 h injections) at regular intervals 5 days prior to commencement of the study (run-in period where all animals received twice-daily *i.p.* injections of saline vehicle) and during the 28 day treatment period. Glucose tolerance (18 mmol/kg body weight; *i.p.*) and insulin sensitivity (25 U/kg body weight; *i.p.*) were determined in 12 hr-fasted and non-fasted mice, respectively at the end of study. Blood samples were collected from the tail vein of conscious mice into fluoride/heparin micro-centrifuge tubes (Sarstedt, Numbrecht, Germany) at the times indicated in the figures. Samples were immediately centrifuged using a Beckman micro-centrifuge (Beckman Instruments, UK) for 30 s at 13,000 *g* and resulting plasma was stored at –20 °C prior to further analysis. Plasma insulin was determined by radioimmunoassay as described previously (Flatt and Bailey, 1981). Total plasma cholesterol, triglycerides and HDL-cholesterol were estimated using appropriate ILab Kits (Instrumentation Laboratories, Cheshire, UK) and samples read using ILab 600 (Instrumentation Laboratories, Cheshire, UK). Blood glucose was measured using a hand-held Ascencia Microfill Blood Glucose Meter (Bayer Healthcare, UK). Following measurement of metabolic parameters, mice were humanely euthanised (exposure to isoflurane, followed by cervical dislocation) and subjected to dual energy x-ray absorptiometry (PIXImus DEXA System; GE Lunar, Madison, Wisconsin) to determine body fat, lean mass, bone mineral density and bone mineral content. Tissues were then collected for histology and gene expression as will be described later.

2.4. Pancreatic insulin content and islet morphology

Pancreatic tissues were harvested and half snap-frozen in liquid nitrogen for measurement of insulin content with the remainder fixed for islet histology. Insulin content was determined from pre-weighed pancreatic tissue which was washed thoroughly in ice-cold PBS, homogenised in chilled acid ethanol solution and further incubated at 4 °C for 12-h prior to measurement of insulin by radioimmunoassay (Vasu et al., 2013). For histology, pancreatic tissues were fixed (4% (w/v) paraformaldehyde in PBS), embedded in paraffin wax, sections of 7 μm prepared (Thermo Scientific Shandon Finesse 352 Microtome, Denmark) and placed on poly-L-lysine coated glass slides (VWR International, PA, USA). Tissues were then permeabilised by heating in citrate buffer–Tween 20 (98 °C for 20 min) prior to blocking with 2% w/v BSA in PBS for 30 min, after which Tween 20 was removed by washing twice with PBS. Immunohistochemistry was performed as described previously (Moffett et al., 2015; Vasu et al., 2013). The following primary antibodies were