Chapter 2

Oxyntomodulin ameliorates glucose intolerance in mice fed a high-fat diet

Edwin T. Parlevliet*, Annemieke C. Heijboer*1,2, Janny P. Schröder-van der Elst1, Louis M. Havekes1,2,3,4, Johannes A. Romijn5, Hanno Pijl1, and Eleonora P.M. Corssmit*

*both authors contributed equally

1 Department of Endocrinology and Metabolic Diseases, Leiden University Medical Center, Leiden, the Netherlands
2 Organization of Applied Scientific Research-Quality of Life, Gaubius Laboratory, Leiden, the Netherlands
3 Department of General Internal Medicine, Leiden University Medical Center, Leiden, the Netherlands
4 Department of Cardiology, Leiden University Medical Center, Leiden, the Netherlands

Abstract

We evaluated the acute effects of oxyntomodulin (OXM), a proglucagon derived peptide, on glucose metabolism in diet-induced insulin resistant male C57Bl/6 mice. To determine the effects on glucose tolerance, mice were intraperitoneally injected with OXM (0.75, 2.5, or 7.5 nmol) or vehicle prior to an intraperitoneal glucose tolerance test. 0.75 nmol/h OXM or vehicle was infused during a hyperinsulinemic euglycemic clamp to quantify insulin action on glucose production and disposal. OXM dose-dependently improved glucose tolerance as estimated by the area under the curve (AUC) for glucose (OXM, 7.5 nmol: 1564 ± 460, p<0.01; 2.5 nmol: 1828 ± 684, p<0.01; 0.75 nmol: 2322 ± 303, p<0.05; control: 2790 ± 222 mmol/l/120 min). Insulin levels in response to glucose administration were higher in 7.5 nmol OXM-treated animals compared to controls (AUCinsulin: 343 ± 113 vs. 231 ± 39 ng/ml/120 min, p<0.05). In basal clamp conditions, OXM increased endogenous glucose production (82.2 ± 14.7 vs. 39.9 ± 5.7 µmol/min/kg, p<0.001). During insulin infusion, insulin levels were twice as high in OXM-treated mice compared to controls (10.6 ± 2.8 vs. 4.4 ± 2.2 ng/ml, p<0.01). Consequently, glucose infusion rate (118.6 ± 30.8 vs. 38.8 ± 26.4 µl/h, p<0.001) and glucose disposal (88.1 ± 13.0 vs. 45.2 ± 6.9 µmol/min/kg, p<0.001) were enhanced in mice that received OXM. In addition, glucose production was more suppressed during OXM infusion (35.7 ± 15.5 vs. 15.8 ± 11.4% inhibition, p<0.05). However, if these data were expressed per unit concentration of circulating insulin, OXM did not affect insulin action on glucose disposal and production. These results indicate that OXM beneficially affects glucose metabolism in diet-induced insulin resistant C57Bl/6 mice. It ameliorates glucose intolerance, most likely because it elevates glucose-induced plasma insulin concentrations. OXM does not appear to impact on insulin action.
Introduction

Posttranslational processing of proglucagon in the intestine and the central nervous system (CNS) yields glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2), glicentin, and oxyntomodulin (OXM)\(^1\). OXM contains 37-amino acids, including the complete glucagon sequence with a basic octapeptide carboxyterminal extension\(^4\). OXM is released from intestinal L-cells into the blood in response to food ingestion in amounts proportional to caloric content\(^5, 6\). It potently inhibits meal-stimulated gastric acid and enzyme secretion in rodents and man\(^7\).

OXM appears to be an effective regulator of appetite and body weight. Intravenous (i.v.) infusion of OXM suppresses appetite and reduces food intake in humans during a buffet meal\(^8\). Furthermore, both intracerebroventricular and intraperitoneal (i.p.) administration of OXM reduces food intake and body weight gain during refeeding in fasted rats\(^9, 10\). OXM most likely causes these anorexigenic effects by modulating neuronal activity in the arcuate nucleus (ARC) of the hypothalamus\(^10\). OXM-like immunoreactivity has been found in the CNS, particularly in the hypothalamus\(^11, 12\), and i.p. administration of OXM inhibits neuronal activity in the ARC as detected by magnetic resonance imaging\(^13\). A distinct receptor for OXM has not been identified to date. However, OXM can bind to both the glucagon and GLP-1 receptor, which is to be expected on the basis of its molecular structure\(^4, 14\). Its anorexigenic effect requires the (central) GLP-1 receptor only\(^15\). Thus, OXM probably impacts on food intake via neural routes in the ARC, which are similarly engaged by other gut-derived peptides modulating appetite, like GLP-1, ghrelin, and peptide YY\(^3\)\(_{3-36}\) (PYY\(^3\)\(_{3-36}\))\(^16\)\(^-\)\(^20\).

Gut-hormones are important for the regulation of glucose metabolism. We recently showed that ghrelin and PYY\(^3\)\(_{3-36}\) affect insulin sensitivity via mechanistic routes that are independent of food intake and body weight\(^21, 22\). Also, recent evidence suggests that GLP-1 ameliorates insulin resistance and hyperglycemia in diabetic ob/ob mice\(^23\). Therefore, we speculated that OXM could impact on insulin sensitivity in insulin resistant mice. The aim of this study was to explore the effects of systemic OXM administration on glucose tolerance and insulin sensitivity in diet-induced insulin resistant C57Bl/6 mice. To this end, we injected OXM intraperitoneally (i.p.) prior to an i.p. glucose tolerance test (IPGTT), and administered OXM intravenously (i.v.) during a hyperinsulinemic euglycemic clamp in high-fat-fed, insulin resistant C57Bl/6 mice.

Methods

Animals and diet. Male C57Bl/6 mice (12 weeks old) (Charles River, Maastricht, the Netherlands) were housed in a temperature and humidity-controlled environment and
were fed a high-fat diet (44 energy% fat derived from bovine fat, Hope Farms, Woerden, The Netherlands) with free access to water for 16-20 weeks to induce insulin resistance. All animal experiments were approved by the Animal Ethics Committee from the Leiden University Medical Center, Leiden, The Netherlands.

**Drugs.** OXM (human, mouse, rat; molecular weight = 4449, 90 g/mol) was purchased from Phoenix Pharmaceuticals (Belmont, USA).

**Intraperitoneal glucose tolerance test.** After 16 weeks on diet, mice were fasted for 10 hours with food withdrawn at 23:00 p.m. the day prior to the study. At 9:00 a.m. the experimental day, mice were given an i.p. injection of OXM dissolved in PBS (0.75, 2.5, or 7.5 nmol) or PBS alone in a volume of 100 µl. The doses were based on a previous report in which OXM 0.75 nmol reduced food intake in rats. Fifteen minutes after injection blood was taken, immediately followed by i.p. injection of a 2 g/kg 20% D-glucose solution (t=0). Additional blood samples were taken via tail bleeding at 5, 15, 30, 45, 60, and 120 minutes after glucose injection for measurement of plasma glucose and insulin levels.

**Hyperinsulinemic euglycemic clamp study.** The same cohort of mice was used for the clamp study as for the IPGTT with 4 weeks of intermission. Mice were fasted for 16 hours with food withdrawn at 17:00 p.m. the day prior to the study. Hyperinsulinemic euglycemic clamp studies started at 9:00 a.m. and were performed as described earlier. During the experiment, mice were sedated with 6.25 mg/kg acepromazine (Alfasan, Woerden, The Netherlands), 6.25 mg/kg midazolam (Roche, Mijdrecht, The Netherlands), and 0.3125 mg/kg fentanyl (Janssen-Cilag, Tilburg, The Netherlands). Vehicle or OXM was administered in a primed (0 and 0.45 nmol respectively) continuous (0 and 0.75 nmol/h respectively) i.v. infusion during the whole experiment. First, basal rate of glucose turnover was determined by giving a primed (p) continuous (c) i.v. infusion of $^{14}$C-glucose (p: 0.7 µCi; c: 1.2 µCi/h, GE Healthcare, Little Chalfont, U.K.) for 60 minutes. Subsequently, insulin was administered in a primed (4.5 mU) continuous (6.8 mU/h) i.v. infusion for 90 minutes to attain steady state circulating insulin levels of ~4 ng/ml. A variable i.v. infusion of a 12.5% D-glucose solution was used to maintain euglycemia as determined at 10 min intervals via tail bleeding (< 3 µl) (Accu-chek, Sensor Comfort, Roche Diagnostics GmbH, Mannheim, Germany). Blood samples (60 µl) were taken during the basal period (after 50 and 60 min) and during the clamp period (after 70, 80, and 90 min) to determine plasma concentrations of glucose, non-esterified fatty acids (NEFA), insulin, and plasma $^{14}$C-glucose specific activities. To estimate insulin-stimulated glucose uptake in individual tissues, 2-deoxy-D-[3H] glucose (2-[3H]DG; Amersham, Little Chalfont, UK) was
Oxymodulin and glucose metabolism

administered as a bolus (2 µCi) 40 minutes before the end of the clamp. At the end of the clamp, mice were sacrificed and skeletal muscle (hind limb) and white adipose tissue (abdomen) were isolated and immediately frozen in liquid nitrogen for subsequent analysis.

**Analytical procedures.** Plasma levels of glucose and NEFA were determined using commercially available kits (Instruchemie, Delfzijl, The Netherlands). Plasma insulin concentrations were measured by a mouse insulin ELISA (Mercodia AB, Uppsala, Sweden). Total plasma $^{14}$C-glucose was determined in 7.5 µl plasma and in supernatants after trichloroacetic acid (20%) precipitation and water evaporation to eliminate tritiated water as described earlier$^{26}$.

**Tissue analysis.** For determination of tissue 2-DG uptake, the homogenate of muscle and adipose tissue was boiled and the supernatant was subjected to an ion-exchange column to separate 2-DG-6-phosphate from 2-DG, as described previously$^{27}$.

**Calculations.** Areas under the curve (AUC) for glucose (AUC$_{glucose}$) and insulin (AUC$_{insulin}$) were calculated using the trapezoidal rule. The turnover rate of glucose (µmol/min/kg) was calculated during the basal period and under steady-state clamp conditions as the rate of tracer infusion (dpm/min) divided by the plasma-specific activity of $^{14}$C-glucose (dpm/µmol). The ratio was corrected for body weight. Endogenous glucose production (EGP) was calculated as the difference between the tracer-derived rate of glucose appearance and the glucose infusion rate. The whole body insulin sensitivity index was calculated as the ratio of glucose disposal to plasma insulin levels during hyperinsulinemic conditions. The hepatic insulin sensitivity index was calculated as the ratio of the relative suppression of EGP during the hyperinsulinemic condition to the change in plasma insulin levels from basal to hyperinsulinemic conditions. Tissue-specific glucose uptake was calculated from tissue 2-DG content, which was expressed as percentage of 2-DG of the dosage per gram of tissue, as previously described$^{27}$.

**Statistical analysis.** Differences between groups were determined with the Kruskal–Wallis non-parametric test for $k$ independent samples. When significant differences were found, the Mann–Whitney non-parametric test was used as a post hoc test to determine differences between two independent groups. A P-value of less than 0.05 was considered statistically significant. Data are presented as mean ± SD.
Chapter 2

Results

Glucose tolerance test

OXM significantly improved glucose tolerance in a dose-dependent way, as evidenced by a reduction of the area under the curve of glucose concentrations (AUC\textsubscript{glucose}: OXM, 7.5 nmol: 1564 ± 460, p<0.01; 2.5 nmol: 1828 ± 684, p<0.01; 0.75 nmol: 2322 ± 303, p<0.05; control: 2790 ± 222 nmol/l/120 min) (figure 1A and B). Insulin levels in response to glucose administration were significantly increased in animals receiving 7.5 nmol OXM compared to control animals (AUC\textsubscript{insulin}: 343 ± 113 vs. 231 ± 39 ng/ml/120 min, p<0.05) (figure 2A and B), but not in animals treated with 2.5 or 0.75 nmol of OXM.

![Figure 1.](image1)

![Figure 2.](image2)

Hyperinsulinemic euglycemic clamp

Plasma parameters and body weight

Body weight, plasma glucose, insulin, and NEFA concentrations in basal and hyperinsulinemic conditions are shown in table 1. Basal glucose and insulin levels were significantly higher and basal NEFA levels significantly lower in mice that received OXM.
compared to control mice. In hyperinsulinemic steady state clamp conditions, insulin levels remained significantly higher and plasma NEFA levels significantly lower in OXM treated mice compared to control mice.

Table 1. Body weight and plasma parameters under basal and hyperinsulinemic conditions in mice that received continuous intravenous infusion of vehicle (n=8) or OXM (n=7).

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Hyperinsulinemic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>OXM</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>37.1 ± 3.2</td>
<td>38.4 ± 1.8</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>5.9 ± 0.8</td>
<td>13.4 ± 2.8**</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>2.0 ± 1.4</td>
<td>14.1 ± 2.6***</td>
</tr>
<tr>
<td>NEFA, mmol/l</td>
<td>0.6 ± 0.2</td>
<td>0.2 ± 0.1**</td>
</tr>
</tbody>
</table>

Values are ± means SD. OXM, oxyntomodulin; NEFA, nonesterified fatty acids. *P<0.05 vs. control. **P<0.01 vs. control. ***P<0.001 vs. control.

Glucose turnover and tissue-specific glucose uptake
In basal conditions, EGP was significantly higher in the OXM-treated animals compared to control animals (82.2 ± 14.7 vs. 39.9 ± 5.7 µmol/min/kg, p<0.001). The rate of glucose infusion required to maintain euglycemia during insulin infusion was significantly higher in OXM-treated mice than in control mice (118.6 ± 30.8 vs. 38.8 ± 26.4 µl/h, respectively, p<0.001). This was because glucose disposal was significantly enhanced (88.1 ± 13.0 vs. 45.2 ± 6.9 µmol/min/kg in controls, p<0.001, figure 3A) and glucose production more suppressed (35.7 ± 15.5 vs. 15.8 ± 11.4% inhibition in controls, P<0.05, figure 4A) during insulin infusion in OXM-treated animals. Accordingly, insulin-mediated 2-deoxy-glucose uptake in adipose and muscle tissue were significantly higher in OXM-treated mice (adipose tissue: 1.3 ± 0.8 vs. 0.5 ± 0.2 µmol/g tissue in controls, p<0.01; muscle: 5.2 ± 2.9 vs. 2.2 ± 1.1 µmol/g tissue in controls, p<0.05). However, the plasma insulin concentrations attained by insulin infusion were significantly higher in OXM-treated mice compared to control (10.6 ± 2.8 vs. 4.4 ± 2.2 ng/ml, p<0.01). Therefore, we also present the data per unit of circulating insulin concentration. The whole body and hepatic insulin sensitivity indices were not different between groups (whole body insulin sensitivity index: 8.9 ± 2.6 vs. 12.2 ± 5.2 µmol/min/kg/ng insulin/ml, NS; hepatic insulin sensitivity index: 3.5 ± 1.5 vs. 3.3 ± 1.8% inhibition from basal per ng insulin/ml, NS, figure 3B and 4B respectively).
Figure 3. Hyperinsulinemic glucose disposal (A) and whole body insulin sensitivity index (B) in mice that received continuous intravenous infusion of vehicle (n=8) or OXM (n=7) during a hyperinsulinemic euglycemic clamp. Values represent mean ± SD. *P<0.001 vs. control.

Figure 4. Inhibition of endogenous glucose production (EGP) (A) and hepatic insulin sensitivity index (B) in mice that received continuous intravenous infusion of vehicle (n=8) or OXM (n=7) during a hyperinsulinemic euglycemic clamp. Values represent mean ± SD. *P<0.05 vs. control.

Discussion

This is the first study to show that systemic administration of OXM beneficially affects glucose metabolism in diet-induced insulin resistant C57Bl/6 mice. A single i.p. injection of OXM dose-dependently ameliorates glucose intolerance in these animals. The plasma insulin concentrations in response to glucose administration were higher in OXM treated animals, particularly after the highest dose. In apparent contrast, i.v. OXM administration clearly enhanced EGP in fasted animals. During the euglycemic clamp, i.v. OXM stimulated glucose uptake and curtailed EGP, suggesting that it improved insulin action. However, circulating insulin levels were more than double during insulin infusion in OXM treated mice, complicating the interpretation of the data. Indeed, per unit of ambient insulin concentration, glucose uptake and EGP were not different in OXM and placebo treated animals. In aggregate, our data indicate that systemic OXM administration improves glucose tolerance in insulin resistant C57Bl/6 mice, primarily because it elevates plasma
insulin levels. Minor effects on insulin action cannot be excluded. In fasting condition, systemic OXM injection stimulates EGP.

Oxyntomodulin dose-dependently improved glucose tolerance in mice maintained on a high fat diet. Post-load plasma glucose concentrations are the result of the concerted effects of insulin secretion, clearance and action on glucose metabolism. Insulin levels in response to glucose administration were significantly higher in OXM treated animals, suggesting that OXM stimulates insulin secretion and/or inhibits insulin clearance from the circulation. Indeed, OXM has been shown to stimulate glucose-induced insulin release in perfused rat pancreas, which may be mediated by binding of OXM to the GLP-1 and/or glucagon receptor in β-cells. A unique receptor for OXM has not been identified to date. However, OXM was shown to bind and activate GLP-1 and glucagon receptors, albeit with lower affinity than the cognate ligands. β-cells express GLP-1 as well as glucagon receptors and both ligands reinforce glucose-induced insulin secretion. Thus, OXM most likely triggered insulin secretion by simultaneous activation of GLP-1 and glucagon receptors on β-cells. However, interaction with (an)other receptor(s) cannot be excluded as OXM was shown to induce contraction of antral smooth muscle cells in an in vitro model in which GLP-1 and glucagon had no effect at all.

GLP-1 retards insulin clearance from the circulation in mice, which suggests that OXM may also slow insulin clearance to elevate glucose-induced plasma insulin levels. This inference is strongly supported by our finding that OXM administration more than doubled plasma insulin concentrations in the face of insulin infusion rates similar to those in placebo treated animals during the hyperinsulinemic clamp. In any case, our data clearly suggest that the beneficial effect of OXM on glucose tolerance is entirely due to elevation of post-load plasma insulin concentrations, because OXM did not significantly impact on insulin action as quantified by hyperinsulinemic euglycemic clamp.

OXM inhibits food intake in rats, mice and humans. Other gut peptides, that similarly decrease appetite, also modulate insulin action and glucose metabolism. In fact, many peptides involved in the control of feeding simultaneously regulate metabolism, illustrating the intuitively obvious notion that food intake and metabolism share common regulatory pathways. Therefore, we speculated that oxyntomodulin could also impact on insulin action. However, although OXM dose-dependently improved glucose tolerance, it did not appear to affect insulin action, as glucose disposal and EGP on a per-unit-of-circulating-insulin-concentration basis were not different in OXM versus placebo infused animals during the euglycemic clamp. Correction of glucose production and uptake rates for plasma insulin levels is rather complex, as insulin action does not linearly correlate with plasma insulin concentrations. Thus, glucose production and disposal expressed per unit of circulating insulin concentration may not be an optimal
measure of insulin's capacity to impact on these metabolic processes. However, we believe it's the best measure we have, given the fact that circulating insulin levels were almost twofold higher in OXM treated animals during the clamp. Therefore, we cannot exclude minor effects of OXM on insulin sensitivity, also in light of the data from the IPGTT, showing that the two lowest doses of OXM did improve glucose tolerance without increased insulin levels.

The failure of OXM to deeply impact on insulin action may have to do with its affinities for both the GLP-1 and the glucagon receptors. Although GLP-1 and glucagon similarly stimulate insulin secretion, these peptides generally relay opposing metabolic messages. GLP-1 inhibits food intake and simultaneously improves insulin action in diabetic mice, whereas glucagon antagonizes insulin action (it stimulates EGP and indirectly inhibits glucose disposal by stimulating lipolysis). Thus, simultaneous activation of both GLP-1 and glucagon receptors by systemic administration of OXM is unlikely to significantly impact on insulin action.

Our findings suggest that OXM may be the base of new pharmacological tools to ameliorate glucose intolerance in insulin resistant subjects. The mice examined in our studies were maintained on a high fat diet, which invariably induces obesity and insulin resistance in the C57Bl/6 strain. Thus, the fact that OXM can improve glucose tolerance in these animals indicates that this peptide (or any novel peptidomimetic) may be useful in the treatment of obesity and type 2 diabetes, like other proglucagon derived peptides. However, OXM enhanced glucose production in basal condition, probably by virtue of its capacity to activate the glucagon receptor in the liver. Obviously, stimulation of glucose production is an undesirable quality of drugs used to treat insulin resistance, which requires careful evaluation. At the end of the day, if it turns out to be a consistent metabolic effect of treatment with OXM (-mimetics), excessive glucose production clearly limits the clinical applicability of this peptide.

In conclusion, OXM beneficially affects glucose metabolism in diet-induced insulin resistant C57Bl/6 mice. It improves glucose tolerance, enhances glucose disposal by peripheral tissues, and reduces glucose production, primarily by elevating glucose induced plasma insulin levels.

This study was supported by The Dutch Scientific Research Council projects 907-00-002 (to E. P. M. Corssmit) and 980-10-017 (to H. Pijl).
References


