The role of hypothalamic pathways in the metabolic side effects of Olanzapine

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Chapter 2.1

Acute peripheral but not central administration of Olanzapine induces hyperglycemia associated with hepatic and extra-hepatic insulin resistance

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ABSTRACT

Atypical antipsychotic drugs such as Olanzapine (Ola) induce weight gain and metabolic changes associated with the development of type 2 diabetes. The mechanisms underlying the metabolic side effects of these centrally acting drugs are still unknown to a large extent. We compared the effects of peripheral (intragastric; 3 mg/kg/h) versus central (intracerebroventricular; 30 µg/kg/h) administration of Ola on glucose metabolism using the stable isotope dilution technique (Experiment 1) in combination with low and high hyperinsulinemic-euglycemic clamps (Experiments 2 and 3), in order to evaluate hepatic and extra-hepatic insulin sensitivity, in adult male Wistar rats. Blood glucose, plasma corticosterone and insulin levels were measured alongside endogenous glucose production and glucose disappearance. Livers were harvested to determine glycogen content.

Under basal conditions peripheral administration of Ola induced pronounced hyperglycemia without a significant increase in hepatic glucose production (Experiment 1). The clamp experiments revealed a clear insulin resistance both at hepatic (Experiment 2) and extra-hepatic levels (Experiment 3). The induction of insulin resistance in Experiments 2 and 3 was supported by decreased hepatic glycogen stores in Ola-treated rats. Central administration of Ola, however, did not result in any significant changes in blood glucose, plasma insulin or corticosterone concentrations nor in glucose production.

In conclusion, acute intragastric administration of Ola leads to hyperglycemia and insulin resistance in male rats. The metabolic side effects of Ola appear to be mediated primarily via a peripheral mechanism, and not to have a central origin.
INTRODUCTION

Atypical antipsychotic drugs (AAPDs) are increasingly replacing the use of typical antipsychotics due to their decreased risk for extrapyramidal side effects (Hoiberg and Nielsen 2006) and their higher efficacy in the treatment of negative symptoms of schizophrenia (Lee et al. 2002; Lublin et al. 2005; Sirota et al. 2006). However, some AAPDs are associated with unfavorable metabolic side effects such as weight gain and insulin resistance (Lublin et al. 2005; Wetterling 2001). Epidemiologic studies showed that Olanzapine (Ola) is one of the AAPDs that causes most pronounced weight gain (Nasrallah 2008). Also clinically, Ola represents one of the atypical antipsychotics with the greatest risk of inducing weight gain, metabolic disturbances or both (Allison et al. 1999). These metabolic side effects, especially weight gain, decrease patients compliance (Citrome and Volavka 2004) even though Ola is a very effective drug in terms of symptom reduction (Sirota et al. 2006; van Bruggen et al. 2003). Moreover, weight gain and insulin resistance are risk factors for type 2 diabetes and cardiovascular diseases (McIntyre et al. 2001).

The mechanisms underlying Ola-induced metabolic disturbances are still unclear. Ola is known to bind to a number of receptors, such as the histamine H1 receptor (Deng et al. 2010; Bymaster et al. 1996), the serotonin 5-HT2c receptor (Davoodi et al. 2008; Huang et al. 2006; Kirk et al. 2009), the adrenergic α2 and β3 receptors, the acetylcholine m3 receptor (high affinity) and the dopamine 2 (D2) receptor (low affinity) (Kapur et al. 2003; Nordstrom et al. 1993). In addition, it is not obvious whether the metabolic side effects are mediated by central or peripheral effects of the drug. The principal mechanism of action of AAPDs is clearly based on their actions in the central nervous system (CNS), but the receptors they bind to are also widely expressed in peripheral tissues such as the liver (Kawai et al. 1986; Nassar et al. 1986; Imoto et al. 1985; Ruddell et al. 2008; Vatamaniuk et al. 2003).

In rat models, it has been shown that an acute subcutaneous administration of Ola induces insulin resistance by increasing hepatic glucose production and decreasing glucose uptake (Chintoh et al. 2008). These findings indicate that the Ola-induced metabolic changes can occur rapidly, and even before the weight gain occurs, indicating that these effects are not secondary to the weight gain. Two studies investigated possible central effects of Ola using acute intracerebroventricular (ICV) infusions of the drug, however, whilst one study reported Ola to induce metabolic changes (Martins et al. 2010), the other study found no metabolic changes after the central administration (Ferno et al. 2011). Neither of these two studies published plasma levels of Ola post-infusion, therefore a possible peripheral effect due to leakage cannot be excluded.
In order to elucidate the metabolic side effects of Ola and the mechanism thereof, we compared the acute effects of a peripheral (intragastric, IG) versus a central (ICV) administration of Ola on glucose production and insulin sensitivity using a stable glucose isotope dilution technique in combination with hyperinsulinemic-euglycemic clamps.

**MATERIALS AND METHODS**

*Ethic statement*
All experiments were approved by the animal care committee of the Royal Netherlands Academy of Arts and Science.

*Animals*
Male Wistar rats (Harlan Nederland, Horst, The Netherlands) weighing 300–350 g were individually housed (cages 40x25x25 cm) and maintained on a 12h/12h light/dark cycle (lights on at 7:00am) at 21±1°C and 60±5% relative humidity. Food (standard rodent chow, Teklad) and water were available ad libitum. Experiments 1, 2 and 3 have been performed on separate sets of animals.

*Drugs*
The dose of Ola chosen in the present studies was selected to parallel the clinical setting based on 70% dopamine D₂ receptor occupancy, which represents a threshold in humans associated with optimal clinical response (Kapur et al. 2003). The route of administration was chosen such that a continuous infusion of freshly made solution was possible in freely moving, undisturbed animals. Using a surgically implanted IG catheter, animals were treated with a primed 36 mg/kg/h infusion during 5 minutes followed by a continuous 3 mg/kg/h infusion for 160 minutes (i.e., in total 3.66 mg/rat) of Ola (ChemPacific Corporation, Maryland) dissolved in acidified MilliQ water (pH=6). A second set of animals were treated with 360 µg/kg/h during 5 minutes and 30 µg/kg/h for 160 minutes (i.e., in total 36.6 µg per rat) administered via an ICV cannula, representing 1% of the peripheral dose. Solubility of the drug when applied in the ventricular compartment was tested separately and appeared to be maintained when added to artificial cerebrospinal fluid at physiological pH. Ola solution for IG infusion was prepared in MilliQ water acidified with HCl (1M) and then brought back to pH 6 using NaOH (1M).
Surgical procedures

After 7 days of habituation, animals were anesthetized by an intramuscular injection of 0.9 ml/kg Hypnorm (Janssen, High Wycombe, Buckinghamshire, UK) and a subcutaneous injection of 0.3 ml/kg Dormiculm (Roche, Almere, The Netherlands). Silicon catheters were placed into the right jugular vein and the left carotid artery for intravenous infusions and blood sampling. The vascular lines were closed using a mix of polyvinylpyruvidon (PVP; Sigma-Aldrich Corp., St. Louis, MO), heparin and amoxicillin. For the peripheral study, a silicon cannula was placed in the stomach during the same surgery. IG cannulas were placed through a 1 cm incision on the left side of the abdomen. For the central study, ICV probes were placed unilaterally into the lateral cerebral ventricle using a standard Kopf stereotaxic apparatus (Anteroposterior: -0.8mm, Lateral: 2.0mm, Ventral: -3.2mm, Angle: 0). Catheters and IG cannulas were fixed on the top of the head of the animal using dental cement. These techniques allowed us to perform all our experiments in freely moving animals. Experiments were performed only after recovery of the pre-surgical body weight and with animals in healthy shape, i.e. 7 – 10 days post-operative recovery.

Experimental procedures

During the experiments, animals were permanently connected to blood-sampling and infusion lines, which were attached to a metal collar and kept out of reach from the rats by means of a counter-balanced arm. This allowed all manipulations to be performed outside the cages without handling the animals. The metal collars were attached the day before the experiment. Before the day of the experiment, food was restricted to 20 g overnight. Two hours before the experiment, rats were handled to connect them to the blood sampling and infusion lines and all remaining food was removed.

1. Experiment 1: Basal endogenous glucose production

To assess endogenous glucose production (EGP), \([6,6^{2}H_{2}]\)glucose was used as a tracer. Blood samples were taken at t=-5 min for background enrichment (t=0 was at 11.00 a.m.), at t=90, t=95 and t=100 min to determine enrichment during the equilibrium state and every 20 min from t=120 till t=260 to determine enrichment during the experiment. Vehicle (MilliQ water at pH=6 to mimic the pH of the Ola solution for the IG infusion (1 ml/h) and Ringer for the ICV infusion (5 µl/h)) started together with a continuous [6,6-\(^2\)H\(_2\)]glucose infusion via the jugular vein in both groups at t=0. After the t=100 min blood sample (at 12.40 a.m.), vehicle infusion was changed to Ola or vehicle solution (36 mg/kg/h during 5 min and 300 µg/kg/h during 5 min and...
30 μg/kg/h till the end of the experiment for the ICV infusion). At the end of the experiment, animals were sacrificed by a lethal intravenous injection of pentobarbital. Trunk blood was collected for plasma Ola measurements at the end of the experiment. Samples of liver were snap frozen in liquid nitrogen and stored for glycogen measurements.

2. Experiments 2 and 3: Hyperinsulinemic-euglycemic clamps

Both hyperinsulinemic-euglycemic clamp experiments (Experiments 2 and 3) started with a continuous [6,6-$^2$H$_2$]glucose infusion and an IG vehicle infusion at t=0. Blood samples were collected at t=-5 min for background enrichment, and at t=90, t=95 and t=100 min to determine enrichment at the equilibrium state. Starting at t=100, insulin (Actrapid 100IU/ml; Novo Nordisk, Alphen aan de Rijn, The Netherlands) was administered in a primed (7.2 mU/kg/min for Experiment 2 and 21.6 mU/kg/min for Experiment 3 during 5 min) – continuous (3 mU/kg/min for Experiment 2 and 9 mU/kg/min for Experiment 3) intravenous infusion. A variable infusion of a 25% glucose solution containing 1% [6,6-$^2$H$_2$]glucose was used to maintain euglycemia, which was checked every 10 min by blood sampling. Thirty minutes after the start of the insulin infusion, the IG vehicle infusion was replaced by Ola or vehicle (36 mg/kg/h during 5 min and 3 mg/kg/h till the end of the experiment). At the end of the experiment, five final blood samples were collected with a 10 min interval from t=250 till t=290 to determine isotope enrichment during the hyperinsulinemic-euglycemic state. At the end of the experiment, animals were sacrificed by a lethal intravenous injection of pentobarbital. Trunk blood was collected for plasma Ola measurement at the end of the experiment. Samples of liver were snap frozen in liquid nitrogen and stored for glycogen measurements.

Laboratory methods/analysis

Glucose concentrations were determined using a blood glucometer (Abbott BV, Hoofdorp, Netherlands). Blood samples were collected in tubes containing heparin on ice and centrifuged at +4°C. Plasma was stored at -20°C until further analysis. Plasma insulin and corticosterone concentrations were measured using radioimmunoassay kits (Millipore, Billerica, USA and MP Biomedicals, Orangeburg, USA, respectively). Plasma [6,6-$^2$H$_2$]glucose enrichment was measured by gas chromatography-mass spectrometry (GCMS), and EGP was calculated by the methods of Steele (Steele 1959). Hepatic glycogen content was measured as described before with minor adaptations (Van der Vies J 1954). Briefly, 10–20 mg snap-frozen liver tissue was homogenized in 1 ml 5% trichloroacetic acid and incubated for 30 min at room temperature. After centrifugation for 10
min, glycogen was precipitated from the supernatant by adding 2 volumes of 95% ethanol and centrifuged for 30 min. The supernatant was discarded and the precipitate dissolved in a 1:60 dilution of Lugol’s reagent in 25% (wt/vol) potassium chloride containing 30 mM hydrochloric acid. The glycogen content was determined spectrophotometrically at 600 nm and normalized against the tissue weight.

Plasma Ola concentrations were measured by LC/MS/MS coupled to Quattro I Xe, using a 7 points calibration curve from 0.2 to 200 ng/ml and 3 quality standards (1 – 10 and 100 ng/ml) (Laboratoires Fournier, Solvay Pharmaceuticals now Abbott, Daix, France).

**Statistical analysis**

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS version 17.0. A p<0.05 was considered statistically significant.

For Experiment 1, an ANOVA with repeated measures was performed to compare glucose levels, EGP, corticosterone levels and insulin levels. Plasma Ola levels were compared using a one-way ANOVA. For non-detectable samples, 0.2 ng/ml (the detection level) was used for the statistical analysis. For Experiments 2 and 3, EGP and glucose uptake levels were compared using an ANOVA with repeated measures and a post-hoc analysis using one-way ANOVA. Plasma corticosterone and insulin levels were compared using ANOVA with repeated measures. Finally, liver glycogen contents were compared using a T-test.

**RESULTS**

**Experiment 1: Acute IG administration of Ola induces hyperglycemia, unlike ICV administration**

Plasma glucose concentrations were measured before and during the infusion of Ola (IG infusion in Figure 1a, ICV infusion in Figure 2a). Control and Ola groups showed similar basal blood glucose levels before the IG infusion started (i.e., 5.77 ± 0.37 mmol/L for the control group and 5.98 ± 0.17 mmol/L for the Ola-treated group). IG infusion of Ola in rats resulted in a significant increase (p<0.001) in glycemia 60 min after the start of the infusion, as compared to the control rats. Maximal glycemia levels were reached 2h after the start of Ola (8.68 ± 0.43 mmol/L), while the control rats did not show a significant difference (6.15 ± 0.26 mmol/L) (Figure 1a). ANOVA showed significant effects of Time (p<0.001), Group (p=0.001) and Group*Time (p<0.001). Rats receiving an ICV Ola infusion showed no significant difference in glycemia as compared to the control rats. Basal blood glucose levels were 5.51 ± 0.26 mmol/L and 5.66 ± 0.13 mmol/L and maximally increased to 6.13 ± 0.17
mmol/L and 6.23 ± 0.17 mmol/L during the infusion for the control and Ola groups, respectively (Group, p=0.635; Figure 2a).

EGP was measured by calculating the ratio of labeled and unlabeled glucose in the plasma (Figure 1b for IG infusion and Figure 2b for ICV infusion). At the start of the IG infusion of Ola, basal EGP was comparable for both groups (53.76 ± 2.14 μmol.kg⁻¹.min⁻¹ for control animals and 60.49 ± 5.8 μmol.kg⁻¹.min⁻¹ for Ola animals, p=0.687). Infusion of Ola did not show any effect on EGP (Group, p=0.356; Figure 1b). Also during the ICV infusion of Ola, no
significant changes in EGP were found (basal EGP 63.99 ± 1.17 μmol.kg⁻¹.min⁻¹ for controls and 65.83 ± 3.16 μmol.kg⁻¹.min⁻¹ for Ola animals and, at t=240, experimental EGP 69.9 ± 9.92 μmol.kg⁻¹.min⁻¹ for controls and 65.7 ± 3.42 μmol.kg⁻¹.min⁻¹ for Ola animals; Group, p=0.805; Figure 2b).

Corticosterone levels showed a steady increase over time in both intragastrically-treated groups, with a significant increase in the Ola-treated animals (Group, p=0.039; Figure 1c). In the ICV-treated groups, no significant effects of Time or Group were found for the plasma

Figure 2: Effects of ICV infusion of Ola. (Vehicle group n=6, Ola group n=9). 2a: Glucose evolution before (t=90 to t=100) and during (t=120 to t=260) ICV Ola infusion (30 μg/kg/h). No significant differences between the 2 groups were detected (ANOVA repeated measures; Time, p<0.001; Time×Group, p=0.59; Group, p=0.635). 2b: EGP before (t=90 to t=100) and during (t=120 to t=260) ICV Ola infusion. No significant changes were detected (ANOVA repeated measures; Time, p=0.731; Time×Group, p=0.709; Group, p=0.84). 2c: Corticosterone levels before (t=90 to t=100) and during (t=120 to t=260) ICV Ola infusion. No significant changes were detected (ANOVA repeated measures; Time, p=0.971; Time×Group, p=0.631; Group, p=0.546). 2d: Plasma insulin levels before (mean of time points t=90 and t=100) and at the end (mean of time points t=220 and t=260) of the ICV infusion of Ola. No significant changes were detected (ANOVA repeated measures; Time, p=0.722; Time×Group, p=0.638; Group, p=0.274). Vehicle-treated animals = white dots; Ola-treated animals = black dots.
corticosterone concentrations. Neither the IG nor ICV Ola infusion affected plasma insulin levels (Figures 1d and 2d) nor glucagon levels (data not shown).

**Plasma levels of Ola after IG and ICV administration of Ola**

Plasma Ola levels were assessed at the end of Experiment 1. Plasma Ola concentrations after IG infusion of Ola were $285.9 \pm 59.6$ ng/ml for treated group and non-detectable (i.e., limit of detection 0.2 ng/ml) for controls ($p<0.001$; Figure 3). After ICV administration, plasma levels of Ola were significantly lower than after the peripheral infusion (*Administration route*; $p<0.001$) and there was no significant difference between ICV controls (<0.2 ng/ml) and ICV Ola animals ($1.78 \pm 0.84$ ng/ml; $p=0.2$; Figure 3).

![Graph showing plasma Olanzapine levels after IG and ICV infusion of Ola.](image)

*Figure 3: Plasma Ola levels after IG and ICV infusion of Ola. (IG: Vehicle group n=5, Ola group n=6 and ICV Vehicle group n=8 and Ola group n=12) Plasma Ola levels are significantly higher in IG-Ola-treated than in IG-vehicle-infused animals (One-way ANOVA, $p<0.001$), or ICV-Ola animals (2-Way ANOVA, *Administration route*Treatment $p<0.001$). Plasma Ola levels of ICV-Ola animals are not significantly different from the ICV-Vehicle animals (One-way ANOVA, $p=0.2$). Vehicle-treated animals = white bars; Ola-treated animals = black bars; *$p<0.001$.*

**Experiment 2: Acute IG administration of Ola induces hepatic insulin resistance**

During the hyperinsulinemic-euglycemic clamp (Experiment 2), we assessed hepatic insulin sensitivity by raising the circulating plasma insulin concentration ~30% above the basal concentration of insulin (Figure 4d). Glucose levels were successfully clamped at an average of 6.21 ± 0.06 mmol/L, and no significant differences were noticed between the 2 groups (*Time*Group, $p=0.723$ and Group, $p=0.113$; Figure S1). The glucose infusion rate (GIR) needed to maintain euglycemia was significantly lower in the Ola-treated animals compared
to vehicle-treated animals (*Time*Group, p=0.001; Figure S2). Basal EGP was identical for both groups (53.56 ± 4.89 μmol.kg⁻¹.min⁻¹ for controls and 55.4 ± 2.87 μmol.kg⁻¹.min⁻¹ for Ola-treated rats). The physiological effect of a modest increase in circulating plasma insulin is evidenced by the 35% decrease of EGP in the control group (p=0.018). The inhibitory effect of insulin was clearly reduced by the Ola infusion resulting in a non-significant 14% decrease of EGP (p=0.111; Figure 4a). Vehicle-treated animals showed a lower EGP at the end of the insulin clamp compared to Ola-treated animals (p=0.06).

**Figure 4:** Effects of IG infusion of Ola on liver insulin sensitivity (Experiment 2) (Vehicle group n=8, Ola group n=7) 4a: EGP at basal (mean of 3 time points: t=90 to t=100; white bars) and during the hyperinsulinemic state (mean of 5 time points: t=250 to t=290; black bars). EGP significantly decreased during the hyperinsulinemic state for the vehicle group (p=0.018, One-way ANOVA) and remained unchanged in the Ola group (p=0.111, One-way ANOVA). 4b: Glucose uptake at basal (mean of 3 time points: t=90 to t=100; white bars) and during the hyperinsulinemic state (mean of 5 time points: t=250 to t=290; black bars). Glucose uptake is significantly increased in both vehicle (p=0.002, One-way ANOVA) and Ola group (p=0.046, One-way ANOVA). 4c: Plasma corticosterone levels were significantly elevated by the IG infusion of Ola (ANOVA repeated measures; *Time*, p<0.001; *Time*Group, p<0.001; Group, p=0.039). Vehicle-treated animals = white dots; Ola-treated animals = black dots. 4d: Plasma insulin levels were elevated 1.3-fold during the hyperinsulinemic state (mean of 3 time points; black bars) compared to the basal level (mean of 2 time points; white bars) (ANOVA repeated measures; *Time*, p=0.062; *Time*Group, p=0.956; Group, p=0.706). *p<0.05,**p<0.001.
Glucose disappearance showed a similar pattern (Figure 4b). Basal levels were identical for both groups (53.56 ± 4.89 μmol.kg⁻¹.min⁻¹ for controls and 55.4 ± 2.87 μmol.kg⁻¹.min⁻¹ for Ola-treated rats), but the insulin-induced increase of glucose uptake was much more pronounced in the control group (+26.57 μmol.kg⁻¹.min⁻¹) than in Ola-treated animals (+13.49 μmol.kg⁻¹.min⁻¹; Time*Group, p=0.048; Figure 4b).

![Figure 5: Effects of IG infusion of Ola (t=130 to t=290; 3 mg/kg/h) on glucose disappearance (Experiment 3). (Vehicle group n=8, Ola group n=7) 5a: EGP at basal (mean of 3 time points: t=90 to t=100; white bars) and during the hyperinsulinemic state (mean of 5 time points: t=250 to t=290; black bars). EGP is significantly decreased in both groups (p<0.001), but the decrease is smaller in the group treated with Ola (ANOVA repeated measures; Time, p<0.001; Time*Group, p=0.018; Group, p=0.604). 5b: Glucose disappearance at basal (mean of 3 time points: t=90 to t=100; white bars) and during the hyperinsulinemic state (mean of 5 time points: t=250 to t=290; black bars). Glucose disappearance is significantly increased in both groups (ANOVA repeated measures; Time, p<0.001; Time*Group, p=0.475; Group, p=0.005). This increase is significantly smaller for the group treated with Ola (p=0.014, One-Way ANOVA). 5c: Corticosterone levels are significantly increased by the Ola treatment (ANOVA repeated measures; Time, p<0.001; Time*Group, p=0.005; Group, p=0.035). Vehicle-treated animals = white dots; Ola-treated animals = black dots. 5d: Plasma insulin levels are elevated 4.4-fold in both groups during the hyperinsulinemic state (mean of 3 time points; black bars) compared to the basal level (mean of 2 time points; white bars) (ANOVA repeated measures; Time, p<0.001; Time*Group, p=0.787; Group, p=0.938). *p<0.05]
The results of the corticosterone data show that while in the control group, plasma corticosterone concentrations during the experiment were quite stable, the Ola-treated group showed a significant increase of their circulating corticosterone level (18.61 ± 5.70 ng/ml at baseline and 314.14 ± 53.46 ng/ml at the end of the experiment; *Time*×*Group*, *p*=0.028; Figure 4c).

**Experiment 3: Acute IG administration of Ola induces extra-hepatic insulin resistance**

In order to investigate the effect of Ola on glucose disappearance in more detail, we performed an additional clamp study, in which circulating insulin concentrations were increased >4-fold (Figure 5d). Glucose levels were successfully clamped at an average of 5.74 ± 0.051, and no significant differences were detected between the 2 groups (*Time*×*Group*, *p*=0.628 and *Group*, *p*=0.631; Figure S3). The GIR needed to maintain euglycemia was close to zero in the Ola group and significantly lower in the Ola-treated compared to vehicle-treated animals (*Time*×*Group*, *p*<0.001; Figure S4).

Basal EGP of the 2 groups did not differ (57.07 ± 9.01 μmol.kg⁻¹.min⁻¹ for the controls and 44.26 ± 2.40 μmol.kg⁻¹.min⁻¹ for the Ola-treated group, *p*=0.22). Control animals showed a strong decrease of EGP due to high plasma insulin levels, i.e. almost 75% inhibition. Ola-treated animals, however, showed a milder decrease, i.e., ~50% (*Time*×*Group*, *p*=0.018; Figure 5a). The glucose disappearance results showed a comparable pattern: similar basal levels, a large insulin-stimulated increase, but a smaller increase for the Ola group (*Group*, *p*=0.005; Figure 5b).

Corticosterone data were comparable to the “low” clamp group. The control group showed a slight increase of corticosterone during the experiment (21.29 ± 4.99 ng/ml at basal level and 87.42 ± 21.99 ng/ml at the end of the experiment), whereas the Ola-treated animals showed a significant increase of their circulating corticosterone levels (36.19 ± 12.35 ng/ml at baseline and 271.45 ± 51.22 ng/ml at the end of the experiment, *Group*, *p*=0.035; Figure 5c).

**Effect of acute Ola treatment on glycogen storage**

Relative hepatic glycogen content was assessed in all 3 experiments (Figure 6). Although mean hepatic glycogen levels appeared to be higher in Ola-treated animals in Experiment 1 (Figure 6a), this increase did not reach statistical significance (*p*=0.096). During the “low” hyperinsulinemic-euglycemic clamp (Experiment 2), the insulin infusion resulted in a physiologic increase in glycogen storage in the control group (as compared with the non-insulin treated animals in 6a). However, hepatic glycogen levels in Ola-treated animals were lower than in the control group (*p*=0.007) (Figure 6b). During the “high” hyperinsulinemic-
DISCUSSION

In this study, we compared the effects of peripheral and central administration of Ola on glucose metabolism. Acute IG administration of Ola clearly mimicked the adverse metabolic side effects known from clinical studies, as it induced both hyperglycemia and insulin...
resistance, both at hepatic and extra-hepatic levels. Acute ICV administration of Ola did not result in any of these changes, indicating that the initiation of the metabolic side effects of Ola is mainly based on a peripheral mechanism. Moreover, our results show that the unfavorable effects of Ola can occur independently of weight gain.

**Ola side effects: Central vs peripheral?**

Recently Martins et al. (2010) compared the effects of intravenous (IV) and ICV infusion of Ola on insulin sensitivity. While the results of their IV administration were comparable to our IG administration, the ICV results of that study are in clear contrast with the current study. Similar to the subcutaneous administration (Houseknecht et al. 2007) and IV administration (Martins et al. 2010) of Ola, our IG route of administration also resulted in hepatic and extra-hepatic insulin resistance. On the other hand, contrary to the lack of effect of ICV administration of Ola in our study, Martins et al. (2010) also reported an induction of hepatic insulin resistance with ICV administration. However, there are several differences in the experimental design that might explain these contrasting results. Most importantly, the dose of Ola used by Martins et al. (2010) was almost 10-fold higher than the dose used in this study (i.e., 330 µg/rat for Martins et al. (2010) and a total of 36 µg/rat in this study). Another study reporting the ICV administration of Ola (Ferno et al. 2011) showed a transient sedative effect after using a 50µg/rat dose but none at 20µg/rat. Our intermediate dose (36 µg/rat) indeed showed no sedative effects, but clearly part of the effects in Martins’ study might be linked to sedation (Martins et al. 2010). Next, we implanted our ICV cannulas into the lateral ventricle instead of the third ventricle, which was targeted in the study of Martins et al. (2010). Several hypothalamic nuclei, involved in the control of energy metabolism, are located close to the borders of the third ventricle. Therefore, in Martins’ study, these hypothalamic nuclei probably have been exposed to much higher concentrations of Ola than in our study. Finally, our study was performed in male Wistar rats and Martins et al. (2010) used male Sprague Dawley rats. The measurements of the plasma Ola concentrations that we performed in samples taken immediately at the end of the infusion showed a small, but detectable, amount of Ola (1.8 ± 0.8 ng/ml) after ICV administration, i.e. ~0.5% of those in the IG Ola group (~280 ng/ml). In IG and ICV vehicle groups, plasma Ola concentrations were below the detection level. Unfortunately, in the study of Martins et al. (2010) no plasma Ola measurements were provided. However, since even with our low dose, Ola passed from the ventricle to the plasma, higher doses of Ola most likely will increase the level of Ola in plasma even further and thus make it very difficult to distinguish between a central and a peripheral effect. In the light of the current data, it is plausible that a combination of the 3rd
ventricle injection site and a 10-fold higher dose would possibly result in a leakage of Ola to the peripheral circulation. Therefore, the conclusion that metabolic side effects of ICV Ola are mediated by the hypothalamus may be erroneous.

*Peripheral Ola induces hyperglycemia and insulin resistance*

IG Ola infusion rapidly influenced glucose metabolism, as in basal conditions blood glucose concentrations were already increased by 1 mmol/L only 60 minutes after the start of the infusion. The clamp experiments (Experiments 2 and 3) showed a clear hepatic and extra-hepatic insulin resistance that likely contributes to the increased glycemia following Ola infusion. Since in Experiment 1, hepatic glucose production was not significantly increased, this would indicate a non-hepatic reduction in glucose uptake as the primary mechanism of Ola to induce hyperglycemia. Corticosterone levels of Ola-treated animals were elevated, but it is unlikely that this hormone causes the observed hyperglycemia as the increase was not sufficient to induce changes in hepatic glucose production. On the other hand, glucocorticoids are also involved in the regulation of insulin secretion (Morgan et al. 2009; Giorgino et al. 1993), and, although in our study plasma insulin levels remained unchanged during Ola treatment, the lack of an increased insulin secretion could facilitate the hyperglycemic effect of Ola. Moreover, it has also been shown that glucocorticoids like corticosterone can impair insulin signaling, primarily by decreasing total IRS-1 protein expression and increasing Ser307 phosphorylation. IRS-1 serine phosphorylation at this site had been reported to decrease the affinity of IRS-1 for the insulin receptor and increase IRS-1 degradation (Aguirre et al. 2002), which seems sufficient to account for the glucocorticoid-induced decrease of insulin-stimulated glucose uptake in skeletal muscle (Morgan et al. 2009).

The clinical syndrome of glucocorticoid excess, Cushing’s syndrome, includes decreased insulin sensitivity, hyperglycemia and diabetes. Recently, it has been shown that selective glucocorticoid receptor (type 2) antagonists are able to prevent the weight gain in rats induced by chronic Ola treatment (Belanoff et al. 2011). Belanoff et al. (2011) suggest that, since the weight gain reduction is not mediated by a decrease in food intake, the selective glucocorticoid receptor (type 2) antagonist might act on metabolic processes rather than controlling appetite. However, the mechanisms underlying glucocorticoid antagonist mediated inhibition of weight gain have not been fully elucidated yet. Nevertheless, these data indicate the glucocorticoid receptor as an interesting target for preventing the metabolic side effects of Ola.
In conclusion, we show that the primary side of action for the metabolic side effects of Ola are based on a peripheral mechanism, since ICV treatment did not result in any acute glucoregulatory changes. Nevertheless, it is still possible that subsequent to these primary events in the periphery an afferent signal is transmitted to the central nervous system (Stefanidis et al. 2009) and central mechanisms are thus implicated in subsequent steps of the metabolic side effects of Ola.

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Supplementary figures:

Figure S1: Plasma glucose levels during Experiment 2.
Plasma glucose levels of the control (open dots) and Ola-treated animals (closed dots) did not significantly differ during the clamp experiment (ANOVA repeated measures; Time*Group, p=0.723; Group, p=0.113).

Figure S2: Glucose infusion rate during Experiment 2.
The glucose infusion rate in the Ola-treated animals (closed dots) is significantly lower than that in the control group (open dots) (ANOVA repeated measures; Time, p<0.001; Time*Group, p=0.001; Group, p<0.001).

Figure S3: Plasma glucose levels during Experiment 3.
Plasma glucose levels of the control (open dots) and Ola-treated animals (closed dots) did not significantly differ during the clamp experiment (ANOVA repeated measures; Time*Group, p=0.028; Group, p=0.031).

Figure S4: Glucose infusion rate during Experiment 3.
The glucose infusion rate in the Ola-treated animals (closed dots) is significantly lower than that in the control group (open dots) (ANOVA repeated measures; Time, p<0.001; Time*Group, p<0.001; Group, p<0.245).
Reference List


