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Title page

CNTO736, a Novel GLP-1 Receptor Agonist, Ameliorates Insulin Resistance and Inhibits Very Low Density Lipoprotein Production in High-Fat-Fed Mice

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Running title page

CNTO736 improves glucose and VLDL metabolism in mice

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Abbreviations: ApoB, apolipoprotein B; DPP-4, dipeptidyl-peptidase-4; EGP, endogenous glucose production; Ex-4, exendin-4; GIR, glucose infusion rate; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor; i.c.v., intracerebroventricular; NPY, neuropeptide Y; PBS, phosphate buffered saline; T2DM, type 2 diabetes mellitus; TG, triglycerides.

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Abstract

CNTO736 is a glucagon-like peptide-1 (GLP-1) receptor agonist that incorporates a GLP-1 peptide analogue linked to the Mimetibody™ platform. We evaluate the potential of acute and chronic CNTO736 treatment on insulin sensitivity and very low density lipoprotein (VLDL) metabolism. For acute studies, diet-induced insulin resistant C57Bl/6 mice received a single i.p. injection of CNTO736 or vehicle. Chronic effects were studied following 4 weeks daily i.p. administration. A hyperinsulinemic-euglycemic clamp monitored insulin sensitivity. A single dose of CNTO736 reduced fasting plasma glucose levels (CNTO736: 4.4 ± 1.0; control: 6.3 ± 2.4 mM) and endogenous glucose production (EGP) (CNTO736: 39 ± 11; control: 53 ± 13 µmol/min/kg) and increased insulin mediated glucose uptake (CNTO736: 76 ± 25; control: 54 ± 13 µmol/min/kg). Chronic administration of CNTO736 reduced fasting glucose levels (CNTO736: 4.1 ± 0.8; control 6.0 ± 1.0 mM), improved insulin dependent glucose uptake (CNTO736: 84 ± 19; control: 61 ± 15 µmol/min/kg), and enhanced inhibition of EGP (CNTO736: 91 ± 18; control: 80 ± 10 % inhibition). In addition, chronic dosing with CNTO736 reduced fasting EGP (CNTO736: 39 ± 9; control: 50 ± 8 µmol/min/kg) and VLDL production (CNTO736: 157 ± 23; control: 216 ± 36 µmol/h/kg). These results indicate that CNTO736 reinforces insulin's action on glucose disposal and production in diet-induced insulin resistant mice. In addition, CNTO736 reduces basal hepatic glucose and VLDL output in these animals. The data suggest that CNTO736 may be a useful tool in the treatment of type 2 diabetes.

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Introduction

Glucagon-like peptide 1 (GLP-1) is an incretin hormone synthesized in enteroendocrine L-cells and brain tissue (Kreymann, et al., 1987;Larsen, et al., 1997). It is released by the gut in response to food intake to stimulate glucose-induced insulin production (Holst, et al., 1987;Kreymann, et al., 1987). In addition, GLP-1 exerts multiple other effects, including inhibition of food intake, slowing of gastric emptying and inhibition of glucagon secretion (Turton, et al., 1996;Willms, et al., 1996). Some, but certainly not all, studies suggest that GLP-1 secretion in response to meals is reduced in patients with type 2 diabetes mellitus (T2DM) (Lugari, et al., 2002;Toft-Nielsen, et al., 2001;Vilsboll, et al., 2001), which attenuates postprandial insulin release and potentially blunts satiety. Continuous infusion of GLP-1 lowers circulating glucose levels after ingestion of a meal and normalizes fasting hyperglycemia in these patients (Nauck, et al., 1998;Willms, et al., 1996). Native GLP-1 is not an appropriate candidate molecule for therapeutic intervention, because of its rapid degradation by dipeptidyl-peptidase-4 (DPP-4) (Deacon, et al., 1995). However, GLP-1 analogues with extended *in vivo* stability are currently under development by many pharmaceutical companies. Byetta™ (exendin-4 (Ex-4)) was approved in 2005 to treat T2DM (Bond, 2006).

We have developed the Mimetibody™ platform for display and delivery of bioactive peptides (O'Neil and Picha, 2005). Mimetibodies™ have longer terminal half-lives relative to peptides due to their increased size and antibody Fc properties. CNTO736 incorporates a DPP-4 resistant GLP-1 peptide analogue into the Mimetibody™ platform. Despite these physical adaptations, it retains GLP-1-like biological activity: it stimulates glucose-dependent insulin secretion, inhibits food intake and gastric emptying, and improves glucose tolerance in diet-induced obese mice in a GLP-1 receptor dependent manner (Picha, et al., 2008).

Here, we further explore the pharmacological characteristics of CNTO736 in terms of its metabolic effects. In particular we evaluate the acute and chronic impact of the compound on insulin sensitivity of glucose and lipid metabolism. Overproduction of very low density lipoprotein (VLDL), which is a prominent feature of diabetic dyslipidemia (as the proximate cause of hypertriglyceridemia), contributes significantly to cardiovascular risk in T2DM

patients (Adiels, et al., 2005). A few studies show that GLP-1 treatment and DPP-4 inhibition reduce circulating triglyceride (TG) levels in healthy humans and T2DM patients, which suggest that these drugs can inhibit VLDL production and/or clearance (Juntti-Berggren, et al., 1996;Matikainen, et al., 2006;Meier, et al., 2006).

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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 10 weeks to induce insulin resistance (Surwit, et al., 1988). All animal experiments were approved by the Animal Ethics Committee from the Leiden University Medical Center, Leiden, The Netherlands.

Expression and Purification of CNTO736. CNTO736 was constructed by fusing a GLP-1 peptide analogue to a flexible Gly/Ser linker and a fragment of a VH domain linked directly to the CH2 and CH3 domains of an Fc (Picha, et al., 2008). A gene encoding CNTO736 was cloned into a vector for mammalian expression under control of the CMV promoter. For transient expression, HEK 293E cells were expanded (DMEM (Invitrogen, Carlsbad, CA, USA) + 10% FBS) and used to seed a 10-tier cell factory (5×10^7 cells in growth medium (1200 ml)). 24 h after seeding, the cells were transfected. 24 h later, the growth medium/transfection mix was removed and replaced with 293-SFMII medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5 mmol/l sodium butyrate (1200 ml). Four days later, the conditioned medium was harvested, filtered, and stored at 4^oC until purification. CNTO736 was purified using a Protein A MabSelect column (GE Healthcare, Piscataway, NJ, USA) and Immunopure Gentle Ag/Ab binding and elution buffers (Pierce, Rockland, IL, USA). The purified product was dialyzed into 20 mmol/l Tris, pH 7.4 prior to concentration. The final column was a Superdex 200 column (GE Healthcare, Piscataway, NJ, USA) in phosphate buffered saline (PBS). Selected fractions were pooled and concentrated.

Treatments. Mice enrolled in the *acute* study of CNTO736 and Ex-4 (generic name: exenatide) were divided into 4 groups. Each group received a single i.p. injection of CNTO736 (0.1 or 1.0 mg/kg, dissolved in PBS), Ex-4 (7.1 µg/kg, dissolved in PBS. This dose of Ex-4 is on a molar base equivalent to 0.1 mg/kg CNTO736) (Sigma-Aldrich, Zwijndrecht, The Netherlands), or PBS in a volume of 100 µl at 08.00 a.m. at the end of the 10 week high-

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fat diet period. The clamp studies began exactly 1 h later. Mice enrolled in the *chronic* study of CNTO736 and Ex-4 were matched for body weight and fasting plasma glucose concentration after 6 weeks of high-fat-feeding, where after they were divided into 4 groups. Each group received daily i.p. doses of CNTO736 (0.1 or 1.0 mg/kg), Ex-4 (7.1 µg/kg), or PBS in a volume of 100 µl at 08.00 a.m. during the remaining 4 weeks on diet. At 08.00 a.m. on the last day of the 10 week high-fat diet period, animals were given a last i.p. dose and the clamp studies were initiated 1 h later.

Hyperinsulinemic-euglycemic clamp study. Mice were fasted for 10 h with food withdrawn at 23:00 p.m. the day prior to the start of the study. Hyperinsulinemic-euglycemic clamps started 1 h after the last dose. 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). First, basal rates of glucose and glycerol turnover were determined by giving a primed (p) continuous (c) i.v. infusion of D-[U-^{14}C]Glucose (p: 0.2 µCi; c: 0.3 µCi/h, GE Healthcare, Little Chalfont, U.K.) and [1-(3)-^{3}H]Glycerol (p: 0.6 µCi; c: 0.9 µCi/h, GE Healthcare) for 60 min. Subsequently, insulin was administered in a primed (4.5 mU) continuous (6.8 mU/h) i.v. infusion for 90 min 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 via tail bleeding 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, NEFA, insulin, glycerol, and plasma D-[U-^{14}C]Glucose and [1-(3)-^{3}H]Glycerol specific activities. At the end of the clamp, mice were used to determine VLDL-production.

VLDL production. VLDL production was determined in basal conditions (1h after the last treatment) and in hyperinsulinemic conditions (after the clamp experiment) in parallel experiments. Mice were fasted for 10h with food withdrawn at 23:00 p.m. the day prior to the start of the study. During the experiment, mice were sedated with 6.25 mg/kg acepromazine

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(Alfasan), 6.25 mg/kg midazolam (Roche), and 0.3125 mg/kg fentanyl (Janssen-Cilag). At t=0 min blood was taken via tail bleeding and mice were i.v. injected with 500 mg of tyloxapol (Triton WR-1339, Sigma-Aldrich) per kg body weight as a 10% (w/w) solution in sterile saline, which completely blocks VLDL clearance from serum (Aalto-Setala, et al., 1992). Additional blood samples were taken at t=10, 20, 40, and 60 min after tyloxapol injection and used for determination of plasma TG concentration. Plasma TG concentrations were related to body weight and hepatic VLDL-TG production rates were calculated from the linear increase in TG in time and expressed as µmol/h/kg. After the last sampling mice were sacrificed by cervical dislocation and livers were immediately removed from the mice and snap-frozen in liquid nitrogen for determination of hepatic TG content.

Analytical procedures. Plasma levels of glucose, NEFA, TG, and glycerol were determined using commercially available kits (Instruchemie, Delfzijl, The Netherlands). Plasma insulin (Mercodia AB, Uppsala, Sweden) and glucagon (Alpco, Salem, NH, USA) concentrations were measured by ELISA. Due to limited plasma, samples were pooled for glucagon measurements. Total plasma ^{14}C-glucose and ^{3}H-glycerol were determined in 7.5 µl plasma and in supernatants after trichloroacetic acid (20%) precipitation and water evaporation to eliminate tritiated water.

Hepatic TG content. A small piece of liver was homogenated in 400 µl PBS and 1.5 ml CH_3OH:$CHCl_3$ (2:1, v/v) was added. After centrifugation, TG was extracted from the supernatant with $CHCl_3$ and H_2O (1:1, v/v) and the $CHCl_3$ phase was dried. TG was dissolved in H_2O with 2% Triton-X100 (Sigma). TG levels were assayed as described above.

Calculations. The turnover rates of glucose and glycerol (µmol/min/kg) were 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 D-[U-^{14}C]Glucose or [1-(3)-^{3}H]Glycerol (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. All metabolic parameters were expressed per kg of body weight.

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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 follow-up 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.

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Results

Plasma metabolites and body weight. Body weight, plasma glucose, NEFA, insulin, glucagon, and glycerol before chronic treatment and in basal and hyperinsulinemic conditions after acute and chronic treatment are shown in table 1.

Acute effects of a single injection

The fasting plasma glucose concentration was significantly reduced by a single dose of CNTO736 (1.0 mg/kg). Similarly, Ex-4 and a lower dose of CNTO736 (0.1 mg/kg) decreased fasting blood glucose, although the difference did not reach statistical significance. Other fasting metabolite concentrations (NEFA, insulin, glucagon, and glycerol) were not affected by any single injection.

In the hyperinsulinemic state, insulin levels were slightly, but significantly elevated after a single dose of Ex-4 compared to control mice, although insulin infusion rates were identical and glucose concentrations were clamped at a similar level in all groups. Perhaps as a result, plasma NEFA concentrations were slightly more suppressed in response to insulin infusion in mice treated with Ex-4 compared to control, whereas CNTO736 did not affect circulating NEFA levels during hyperinsulinemia.

Effects of 4 weeks of daily injections

Chronic administration of both drugs significantly reduced the fasting plasma glucose concentration compared to baseline values, while glucose levels remained high in the control group. The other plasma parameters did not differ between groups.

In the steady state clamp condition, insulin and glucose concentrations were similar in all groups and hyperinsulinemia suppressed NEFA levels to a similar extent in all groups. Body weight was significantly reduced by chronic administration of the highest dose of CNTO736 only.

Glucose turnover.

Acute effects of a single injection

A single dose of CNTO736 (1.0 mg/kg) inhibited endogenous glucose production (EGP) in the basal condition. Ex-4 reduced EGP to a similar extent, but the difference with control did not reach statistical significance. The lowest dose of CNTO736 (0.1 mg/kg) did not affect glucose metabolism in basal conditions (control: 53 ± 13; Ex-4: 40 ± 11; 0.1 mg/kg CNTO736: 47 ± 10; 1.0 mg/kg CNTO736: 39 ± 11 µmol/min/kg, $p<0.05$; figure 1).

The high dose of CNTO736 (1.0 mg/kg) and Ex-4 significantly increased the rate of glucose infusion necessary to maintain euglycemia during the insulin infusion relative to the control (control: 52 ± 14; Ex-4: 75 ± 13, $p<0.05$; 0.1 mg/kg CNTO736: 63 ± 24; 1.0 mg/kg CNTO736: 75 ± 16 µmol/min/kg, $p<0.05$; figure 2). This was largely because both drugs enhanced the ability of insulin to stimulate glucose uptake (control: 54 ± 13; Ex-4: 72 ± 17, $p<0.05$; 0.1 mg/kg CNTO736: 69 ± 20; 1.0 mg/kg CNTO736: 76 ± 25 µmol/min/kg, $p<0.05$). In contrast, neither drug acutely affected insulin's inhibitory action on EGP (control: 89 ± 12; Ex-4: 90 ± 14; 0.1 mg/kg CNTO736: 84 ± 14; 1.0 mg/kg CNTO736: 79 ± 19 % from basal; figure 4). The lowest dose of CNTO736 did not affect insulin action in this experimental context.

Effects of 4 weeks of daily injections

Chronic dosing of CNTO736 (1.0 and 0.1 mg/kg) significantly inhibited EGP in basal condition. In contrast, Ex-4 did not affect EGP in the fasting condition (control: 50 ± 8; Ex-4: 51 ± 18; 0.1 mg/kg CNTO736: 35 ± 9, $p<0.05$; 1.0 mg/kg CNTO736: 39 ± 9 µmol/min/kg, $p<0.05$; figure 1).

Chronic administration of CNTO736 and Ex-4 increased the rate of glucose infusion necessary to maintain euglycemia during insulin infusion ((control: 47 ± 18; Ex-4: 88 ± 16, $p<0.05$; 0.1 mg/kg CNTO736: 80 ± 12, $p<0.05$; 1.0 mg/kg CNTO736: 89 ± 16 µmol/min/kg, $p<0.05$; figure 2). Chronic administration of both 1.0 mg/kg CNTO736 and Ex-4 enhanced insulin stimulated glucose disposal relative to control (CNTO736: 84 ± 19; Ex-4: 83 ± 19; control: 61 ± 15 µmol/min/kg). The lowest dose of CNTO736 (0.1 mg/kg) did not significantly affect glucose uptake during hyperinsulinemia relative to the control (CNTO736: 74 ± 15; control: 61 ± 15 µmol/min/kg). However, if expressed as percentage increase of baseline values, 0.1 mg/kg CNTO736 significantly increased glucose disposal during hyperinsulinemia (control: 30 ± 36; Ex-4: 76 ± 49, $p<0.05$; 0.1 mg/kg CNTO736: 125 ± 69, $p<0.01$; 1.0 mg/kg

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CNTO736: 132 ± 77 % from basal, p<0.01; figure 3). All chronic interventions significantly reinforced insulin's capacity to inhibit EGP (control: 80 ± 10; Ex-4: 98 ± 4, p<0.05; 0.1 mg/kg CNTO736: 95 ± 10, p<0.05; 1.0 mg/kg CNTO736: 91 ± 18 % from basal, p<0.05; figure 4).

Glycerol turnover.

Acute effects of a single injection

Basal rates of glycerol turnover were not different after a single dose of CNTO736, Ex-4, or vehicle. None of the treatments affected insulin's ability to inhibit glycerol turnover (figure 5).

Effects of 4 weeks of daily injections

Chronic administration of CNTO736 and Ex-4 did not affect glycerol turnover in basal conditions. Hyperinsulinemia suppressed glycerol turnover to a similar extent in all groups (figure 5).

VLDL production.

Acute effects of a single injection

VLDL production after one single injection was similar in all groups both in basal and hyperinsulinemic conditions (table 2).

Effects of 4 weeks of daily injections

Chronic treatment decreased plasma TG levels to a similar extent in all groups compared to baseline values (table 2). Chronic administration of CNTO736 (0.1 and 1.0 mg/kg) led to a significant decrease in VLDL production both in basal and in hyperinsulinemic conditions, whereas chronic Ex-4 treatment did not affect VLDL production (table 2 and figure 5).

Hepatic TG content.

Hepatic TG content did not differ between groups (control: 118 ± 10; Ex-4: 112 ± 14; 0.1 mg/kg CNTO736: 113 ± 27; 1.0 mg/kg CNTO736: 120 ± 29 µg TG/ mg protein).

Discussion

Here we show that CNTO736, a novel GLP-1 receptor agonist, beneficially affects glucose and VLDL metabolism in diet-induced insulin resistant C57Bl/6 mice. In particular, a single injection of the highest dose of CNTO736 (1.0 mg/kg) acutely reduced fasting plasma glucose concentration and inhibited basal endogenous glucose production (EGP). Moreover, the capacity of insulin to stimulate glucose disposal was acutely reinforced by CNTO736. Chronic administration also clearly reduced plasma glucose levels and EGP during the fasting state and reinforced insulin's action on glucose disposal and production. Moreover, chronic treatment with CNTO736 significantly inhibited VLDL production. Ex-4 had a similar impact on glucose disposal and production in the hyperinsulinemic state, indicating that these effects are likely attributable to common GLP-1-like actions of the compounds. However, chronic Ex-4 treatment did not affect basal EGP or VLDL production, suggesting that CNTO736 and Ex-4 differentially act to modulate metabolism. The half-life of CNTO736 (15-20 h) in mice is considerably longer than that of Ex-4 (~2 h) (Picha, et al., 2008). At this time, we do not know whether the difference between Ex-4 and CNTO736 on fasting EGP and VLDL production is due to sustained exposure of CNTO736 because of its longer plasma half-life or due to some other unique characteristic that is specific to CNTO736. However, Ex-4 and CNTO736 stimulated glucose disposal and inhibited glucose production in hyperinsulinemic conditions to a similar degree, which suggests that compound kinetics cannot fully explain the CNTO736-specific effect on fasting EGP and VLDL production.

The data corroborate earlier reports indicating that GLP-1 and its analogues in the long run ameliorate whole body insulin resistance in obese animal models (Gedulin, et al., 2005;Green, et al., 2006;Young, et al., 1999) and in T2DM patients (Zander, et al., 2002). They further extend our knowledge of the precise actions of GLP-1 analogues on distinct components of glucose flux in insulin resistant animals, inasmuch as they show that these compounds acutely reinforce insulin's ability to promote glucose disposal and boost insulin action on both glucose uptake and production in the long term.

Interestingly, the data also clearly show that CNTO736 inhibits VLDL production both in the fasting state and during hyperinsulinemia, whereas Ex-4 does not. To our knowledge, this is the first report of a GLP-1 analogue affecting VLDL synthesis. Native GLP-1 reduces

postprandial plasma triglyceride levels in healthy, normal-weight humans and T2DM patients, and vildagliptin, a DPP-4 inhibitor that increases plasma GLP-1 levels, lowers the postprandial concentration of triglyceride-rich chylomicron particles in type 2 diabetic patients (Juntti-Berggren, et al., 1996;Matikainen, et al., 2006;Meier, et al., 2006). T2DM is a complex metabolic disorder, frequently marked by more anomalies than hyperglycemia alone. Overproduction of VLDL by the liver is the proximate cause of hypertriglyceridemia in patients with T2DM (Adiels, et al., 2005), and hypertriglyceridemia contributes significantly to cardiovascular risk (Costa, et al., 2006). Thus, the capacity to inhibit VLDL production is a favorable quality of drugs designed to treat T2DM.

The mechanisms underlying the beneficial impact of GLP-1 analogues on glucose metabolism remain to be established. They facilitate glucose induced insulin release by beta cells of course, which dampens postprandial hyperglycemia and thereby ameliorates glucose toxicity. The effects of chronic administration of both CNTO736 and Ex-4 on glucose disposal and production are conceivably at least in part attributable to this feature of the drugs. However, the impact of a single drug dose on glucose metabolism clearly indicates that other, direct mechanisms are involved. CNTO736 and Ex-4 may act on peripheral tissues directly via the GLP-1 receptor (GLP-1R). However, it is unclear if the liver expresses GLP-1R, since there is conflicting data (Bullock, et al., 1996;Yamato, et al., 1997). CNTO736 and Ex-4 may reinforce insulin's action via neural routes that have recently emerged as key players in the control of glucose and lipid metabolism (Prodi and Obici, 2006). GLP-1 receptors that mediate its anorexigenic action are expressed in multiple hypothalamic nuclei and peripheral dosing of CNTO736 could have allowed the molecule to reach the hypothalamus (Picha, et al., 2008). One possibility is that CNTO736 and Ex-4 in the hypothalamus modulate the NPY pathway. It has been shown previously that intracerebroventricular (i.c.v.) administration of GLP-1 completely prevents the orexigenic effects of neuropeptide Y (NPY), which suggests that GLP-1 acts by blocking NPY transmission to inhibit food consumption (Furuse, et al., 1997). I.c.v. administration of NPY acutely impairs insulin's ability to suppress EGP (van den Hoek, et al., 2004b). Also, animal models of obesity and type 2 diabetes (including diet-induced obesity) are marked by elevated NPY expression in hypothalamic nuclei (Huang, et al., 2003;Wilding, et al., 1993). For these reasons, it is conceivable that these exceedingly

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active NPY neurons are involved in the pathophysiology of enhanced EGP in the face of hyperinsulinemia in these models (and obese humans). Peptide YY_{3-36}, which inhibits NPY release in the arcuate nucleus, enhances insulin sensitivity in the same experimental context (van den Hoek, et al., 2004a). Therefore, chronic administration of CNTO736 may have antagonized NPY-induced insulin resistance to explain the findings presented here.

Alternatively, CNTO736 and Ex-4 may activate neurons in the nucleus of the solitary tract (NTS) in the brain stem via afferent vagal inputs to modulate glucose metabolism. GLP-1 evokes vagal afferent nerve activity to initiate a hepato-pancreatic reflex that is critically involved in the control of insulin release (Nakabayashi, et al., 1996). It is conceivable, that vagal afferent output in this context is not limited to the pancreas, but affects liver and other visceral organs as well to modulate glucose production. Moreover, in addition to its direct effects on hypothalamic neurons, GLP-1 conveys its message to the hypothalamus via the vagus nerve and the NTS (Abbott, et al., 2005). Indeed, vagal ablation attenuates the anorexigenic effects of peripheral GLP-1 administration (Abbott, et al., 2005). In analogy, activation of vagal afferents by GLP-1 may impact on glucose metabolism via the hypothalamus. Finally, it remains possible that GLP-1 acts on the liver and other peripheral tissues via a structurally and functionally distinct GLP-1R. Indeed, GLP-1 was reported to have insulin-like actions in liver and skeletal muscle which are not mediated by the classical GLP-1R (Ikezawa, et al., 2003;Marquez, et al., 1998).

Chronic administration of CNTO736 clearly inhibited VLDL production in basal and hyperinsulinemic conditions. The assembly of VLDL particles in the endoplasmatic reticulum of hepatocytes is dependent on the intracellular presence of triglycerides, other lipids, and apolipoprotein B (apoB) as its major components. Correct apoB lipidation and translocation probably limit the rate of VLDL secretion (Hussain, et al., 2003). Thus, the supply of NEFA, released by lipolysis of circulating and stored triglycerides, to the liver is a major determinant of VLDL production (Julius, 2003). However, CNTO736 did not affect lipolysis or insulin's capacity to suppress this process and, accordingly, it did not change plasma NEFA concentrations. Moreover, chronic CNTO736 treatment did not impact on liver TG content. Therefore, CNTO736 does not appear to inhibit VLDL production via reduction of triglyceride lipolysis and diminution of NEFA supply to the liver.

CNTO736 may affect liver function via one of the mechanisms discussed above. ICV administration of NPY acutely impairs the capacity of insulin to inhibit VLDL production (van den Hoek, et al., 2004b). This observation suggests that neural mechanisms are involved in the control of VLDL metabolism. Thus, in analogy with hypotheses pertaining to the effect of CNTO736 on glucose metabolism discussed above, CNTO736 may modulate VLDL release via neural routes that involve the hypothalamus, brain stem, and vagal nerve. It is unclear why chronic treatment with Ex-4 did not result in similar effects on basal EGP and VLDL production. In this context, it is interesting to note that Ex-4 may have less affinity for the vagal GLP-1R than GLP-1 itself (Nishizawa, et al., 2000). It is tempting to speculate, that this explains the difference in effect of chronic treatment with CNTO736 and Ex-4 on EGP and VLDL production.

The pathophysiological consequence of the capacity of CNTO736 to inhibit VLDL secretion is uncertain, since circulating TG levels were not affected. It is also important to note, that the beneficial impact of CNTO736 on VLDL production is not simply due to a reduction of body weight, since a robust effect was also evident after chronic treatment with the lowest dose of the drug, which did not impact on body weight.

The current study suggests that CNTO736 has properties to provide better metabolic control in patients with T2DM. Chronic administration of the drug clearly reinforces insulin action on glucose disposal and production in insulin resistant mice. Moreover, chronic CNTO736 treatment inhibits fasting EGP and VLDL synthesis in the same experimental context. Administration of Ex-4 has similar effects on insulin mediated glucose metabolism, suggesting that common, GLP-1-like effects of these compounds underlie their glucoregulatory action. In contrast, Ex-4 treatment did not impact fasting EGP and VLDL production. This could imply that CNTO736 has a unique property that is responsible for inhibiting glucose production and VLDL release. We speculate that CNTO736 acts via GLP-1 receptors on vagal afferents and hypothalamic neurons to modulate glucose and VLDL metabolism.

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Footnotes

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Legends for figures

Figure 1. Basal endogenous glucose production (EGP) in mice that received acute or chronic i.p. injection of CNTO736, exendin-4, or vehicle during a hyperinsulinemic euglycemic clamp. Values represent mean ± SD for at least 8 mice per group. *p <0.05 vs control. **p < 0.01 vs control

Figure 2. Glucose infusion rate (GIR) in mice that received acute or chronic i.p. injection of CNTO736, exendin-4, or vehicle during a hyperinsulinemic euglycemic clamp. Values represent mean ± SD for at least 8 mice per group. *p <0.05 vs control.

Figure 3. Stimulation of glucose disposal by insulin in mice that received acute or chronic i.p. injection of CNTO736, exendin-4, or vehicle during a hyperinsulinemic euglycemic clamp. Values represent mean ± SD for at least 8 mice per group. *p <0.05 vs control. **p < 0.01 vs control

Figure 4. Inhibition of endogenous glucose production (EGP) by insulin in mice that received acute or chronic i.p. injection of CNTO736, exendin-4, or vehicle during a hyperinsulinemic euglycemic clamp. Values represent mean ± SD for at least 8 mice per group. **p <0.01 vs control.

Figure 5. Glycerol turnover in mice that received acute or chronic i.p. injection of CNTO736, exendin-4, or vehicle before (basal) and after (hyperinsulinemic) the initiation of a hyperinsulinemic euglycemic clamp. Values represent mean ± SD for at least 8 mice per group. *p <0.05 vs basal.

Figure 6. VLDL production rate in mice that received chronic i.p. administration of CNTO736, exendin-4, or vehicle in basal (a) or hyperinsulinemic (b) conditions. white squares = control; black squares = exendin-4; white circles = CNTO736 (0.1 mg/kg); black circles = CNTO736

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(1.0 mg/kg). Values represent mean ± SD for at least 9 mice per group. $^{\dagger}p$ <0.05 vs control and vs exendin-4. **p <0.05 vs control. *$^{\dagger}p$ <0.05 vs exendin-4.

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Tables

Table 1. Plasma parameters in mice before chronic treatment and in basal or hyperinsulinemic conditions after a single or chronic i.p. injection of CNTO736, exendin-4 (Ex-4), or vehicle. Values represent mean ± SD for at least 8 mice per group. *p<0.05 vs control. $^{\dagger\dagger}p$ <0.01 vs start treatment.

		Acute study				Chronic study			
				CNTO736				CNTO736	
		Control	Ex-4	0.1 mg/kg	1.0 mg/kg	Control	Ex-4	0.1 mg/kg	1.0 mg/kg
Body weight (g)	Start treatment	-	-	-	-	26.7 ± 1.2	27.0 ± 1.9	26.6 ± 1.7	26.8 ± 1.4
	12 wks diet/ after treatment	30.9 ± 2.4	31.4 ± 0.9	31.3 ± 1.6	31.2 ± 2.2	27.3 ± 1.8	26.3 ± 2.0	26.1 ± 1.9	25.1 ± 1.3††
Glucose (mM)	Start treatment	-	-	-	-	6.5 ± 0.8	6.8 ± 0.8	6.7 ± 1.1	6.6 ± 1.4
	Basal	6.3 ± 2.4	4.6 ± 1.1	5.5 ± 1.2	4.4 ± 1.0*	6.0 ± 1.0	4.9 ± 1.1 ††	4.3 ± 1.0 ††	4.1 ± 0.8 ††
	Hyperinsulinemic	7.3 ± 1.0	7.7 ± 0.9	7.3 ± 0.5	8.0 ± 0.8	6.9 ± 0.9	7.3 ± 0.9	7.6 ± 1.1	7.6 ± 0.9
FFA (mM)	Start treatment	-	-	-	-	0.9 ± 0.2	0.9 ± 0.2	1.0 ± 0.1	1.0 ± 0.2
	Basal	1.3 ± 0.6	1.0 ± 0.2	1.1 ± 0.5	1.1 ± 0.5	1.1 ± 0.3	1.0 ± 0.3	1.1 ± 0.3	1.1 ± 0.2
	Hyperinsulinemic	0.7 ± 0.3	0.5 ± 0.2*	0.7 ± 0.3	0.6 ± 0.4	0.5 ± 0.2	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
Insulin (ng/ml)	Start treatment	-	-	-	-	1.1 ± 0.4	1.0 ± 0.5	1.0 ± 0.5	1.1 ± 0.4
	Basal	1.1 ± 0.8	0.7 ± 0.5	1.1 ± 0.8	0.9 ± 0.6	1.0 ± 0.7	1.4 ± 0.7	1.1 ± 0.6	1.4 ± 1.3
	Hyperinsulinemic	3.1 ± 1.1	4.4 ± 0.8*	3.5 ± 1.3	4.5 ± 1.7	5.4 ± 2.4	4.4 ± 0.9	4.6 ± 1.0	5.0 ± 1.9
Glucagon (pg/ml)	Start treatment	-	-	-	-	491	502	551	580
	Basal	556	513	455	592	565	787	699	675
	Hyperinsulinemic	569	509	476	670	575	631	580	632
Glycerol (mM)	Start treatment	-	-	-	-	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0
	Basal	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1
	Hyperinsulinemic	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1

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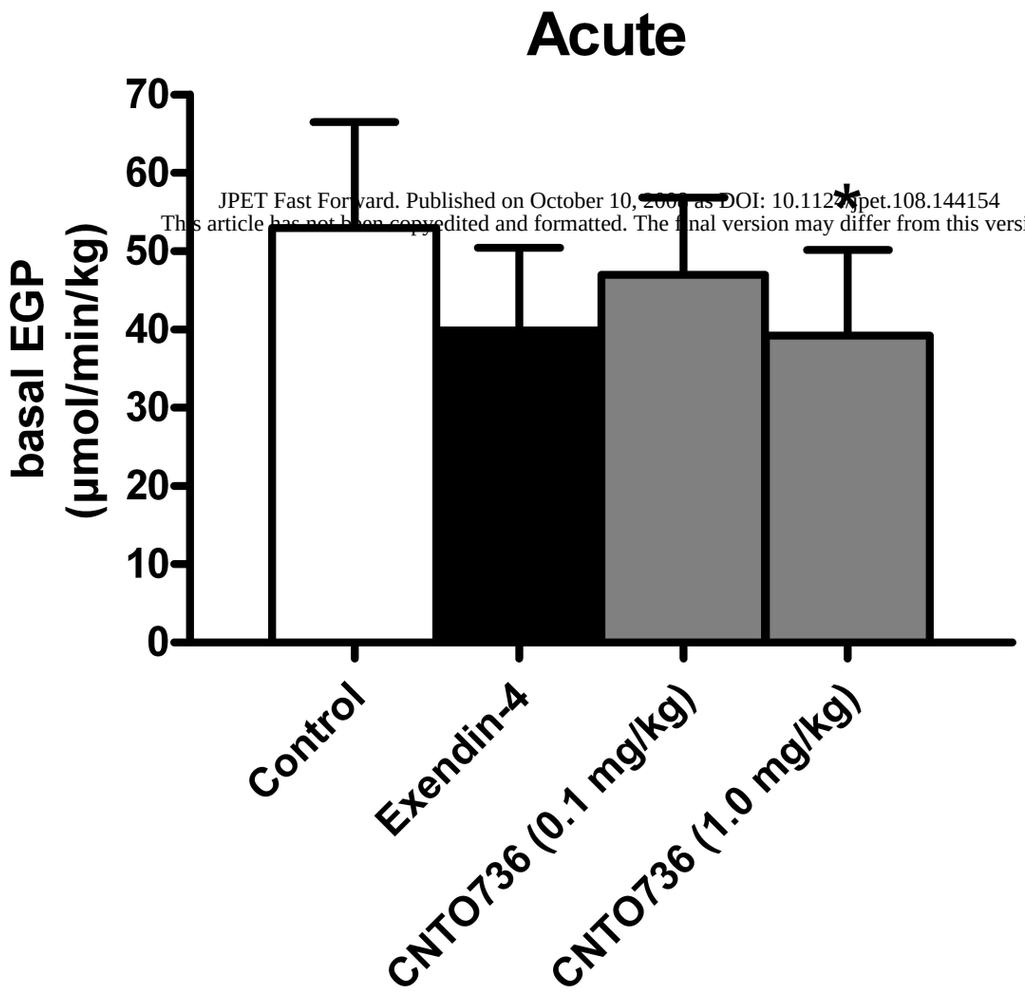
Table 2. Body weight, plasma TG levels and VLDL production rate under basal or hyperinsulinemic conditions in mice that received a single or chronic i.p. injection of CNTO736 (0.1 or 1.0 mg/kg), exendin-4 (Ex-4), or vehicle. Values represent mean ± SD for at least 8 mice per group. *p <0.05 vs control and exendin-4. $^{\dagger\dagger}p$ <0.01 vs start treatment.

		Acute study				Chronic study			
				CNTO736				CNTO736	
		Control	Ex-4	0.1 mg/kg	1.0 mg/kg	Control	Ex-4	0.1 mg/kg	1.0 mg/kg
Body weight (g)	Start treatment	-	-	-	-	27.0 ± 1.1	27.2 ± 1.7	27.3 ± 1.8	26.8 ± 1.3
	12 wks diet/ end treatment	31.6 ± 2.5	31.4 ± 1.9	31.5 ± 1.8	31.5 ± 2.5	27.5 ± 1.6	26.6 ± 1.8	26.6 ± 1.8	25.2 ± 1.2††
TG (mM)	Start treatment	-	-	-	-	0.79 ± 0.3	0.77 ± 0.2	0.78 ± 0.2	0.81 ± 0.3
	12 wks diet/ end treatment	0.39 ± 0.1	0.35 ± 0.1	0.34 ± 0.1	0.39 ± 0.1	0.56 ± 0.2††	0.52 ± 0.1††	0.53 ± 0.1††	0.52 ± 0.1††
VLDL production rate (µmol/h/kg)	Basal	229 ± 61	237 ± 16	205 ± 34	220 ± 48	216 ± 36	212 ± 55	160 ± 30*	157 ± 23*
	Hyper insulinemic	190 ± 45	202 ± 47	196 ± 45	186 ± 32	176 ± 51	179 ± 40	117 ± 37*	111 ± 32*

Figure 1



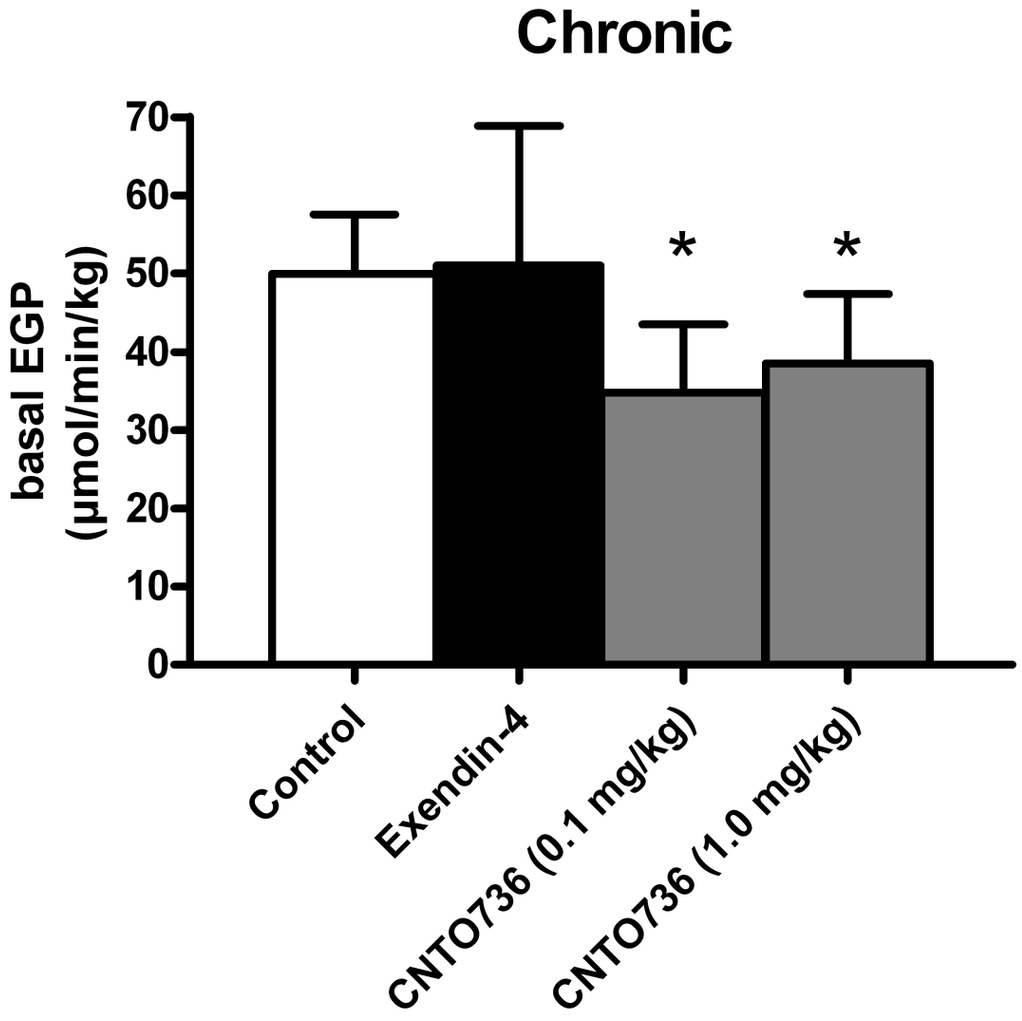
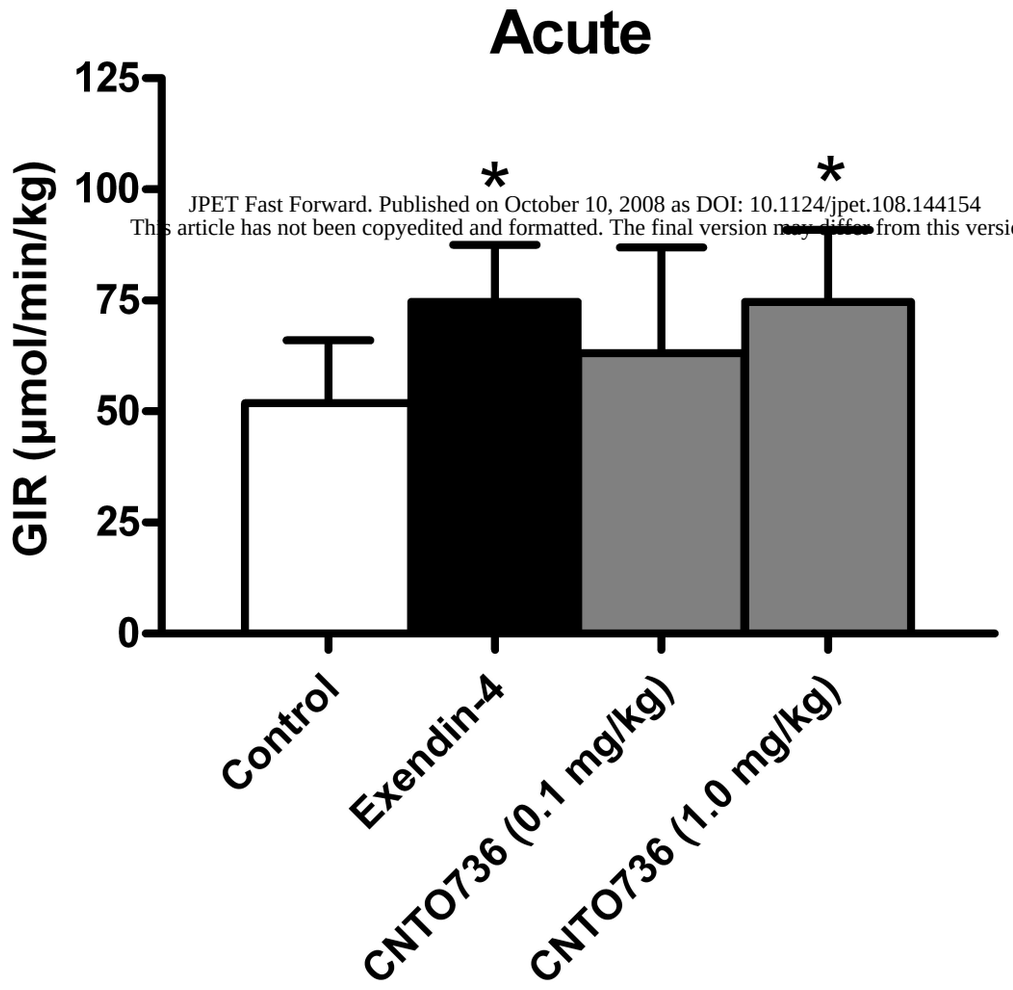


Figure 2



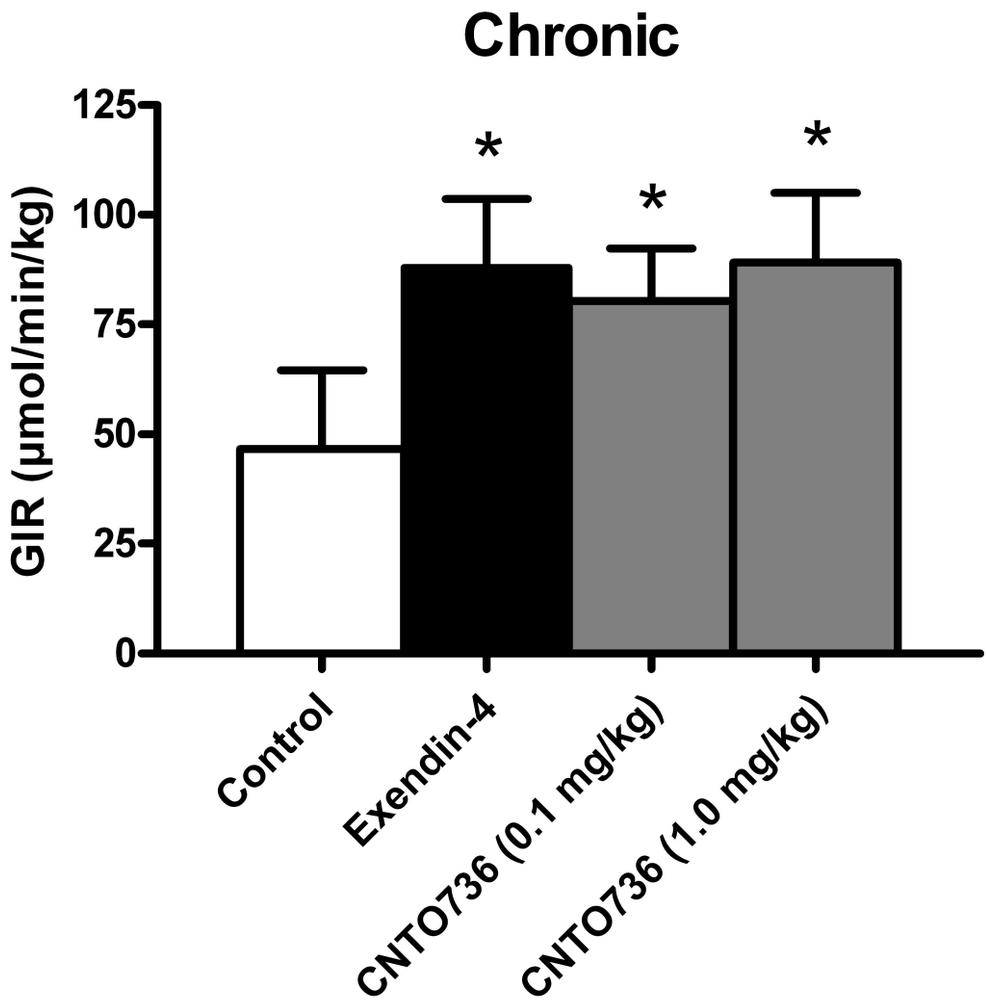


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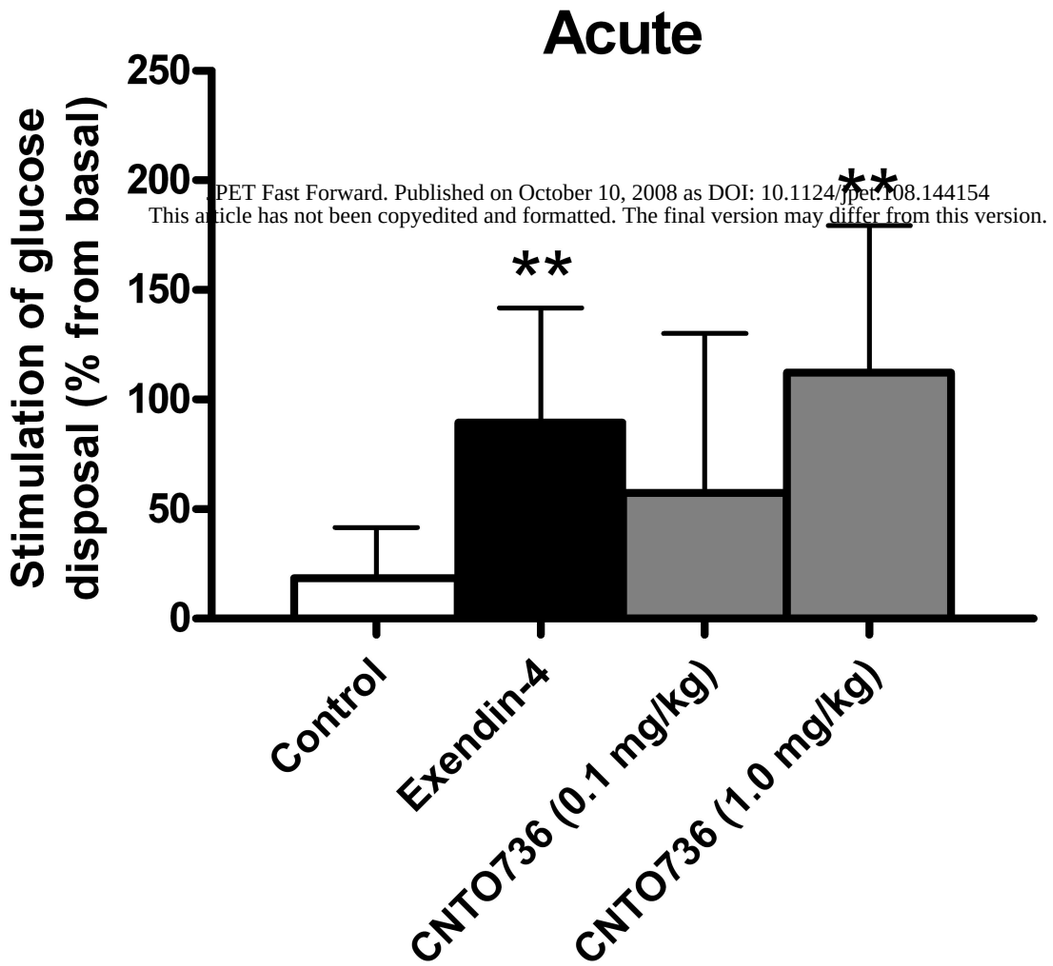
Figure 3



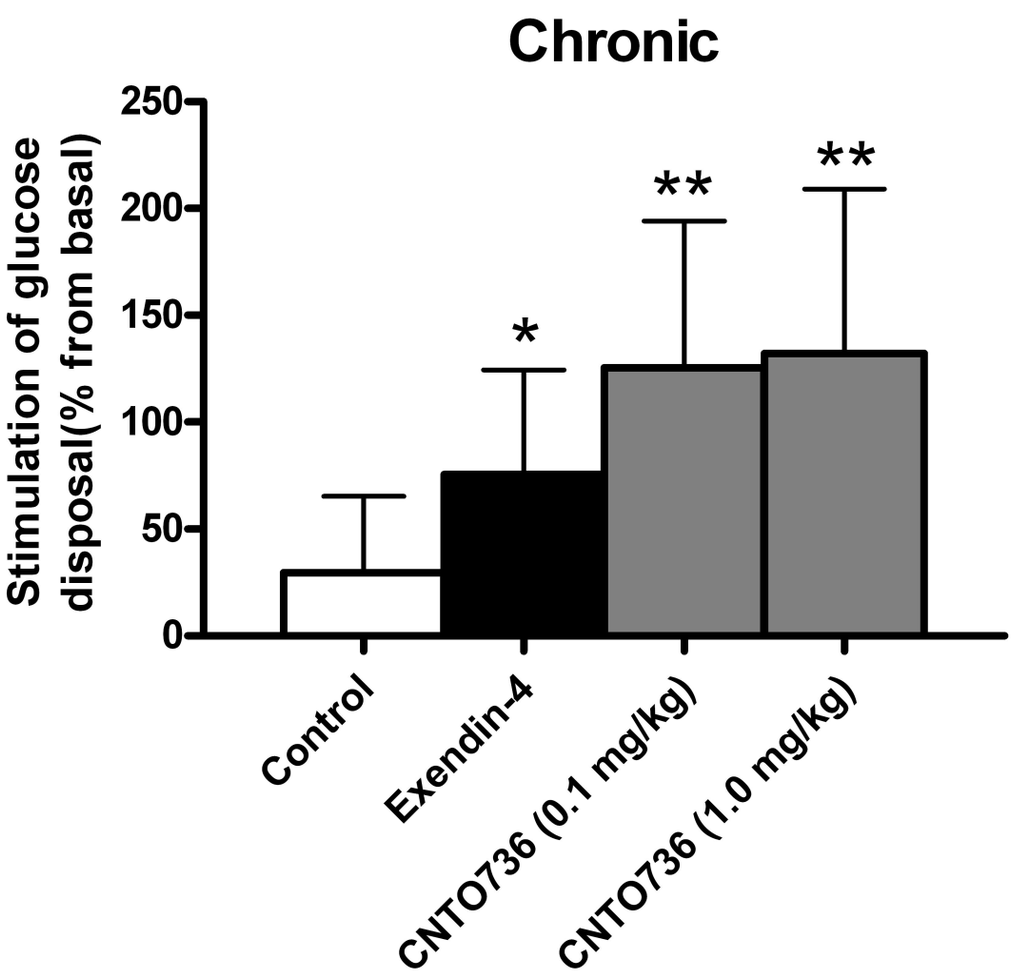
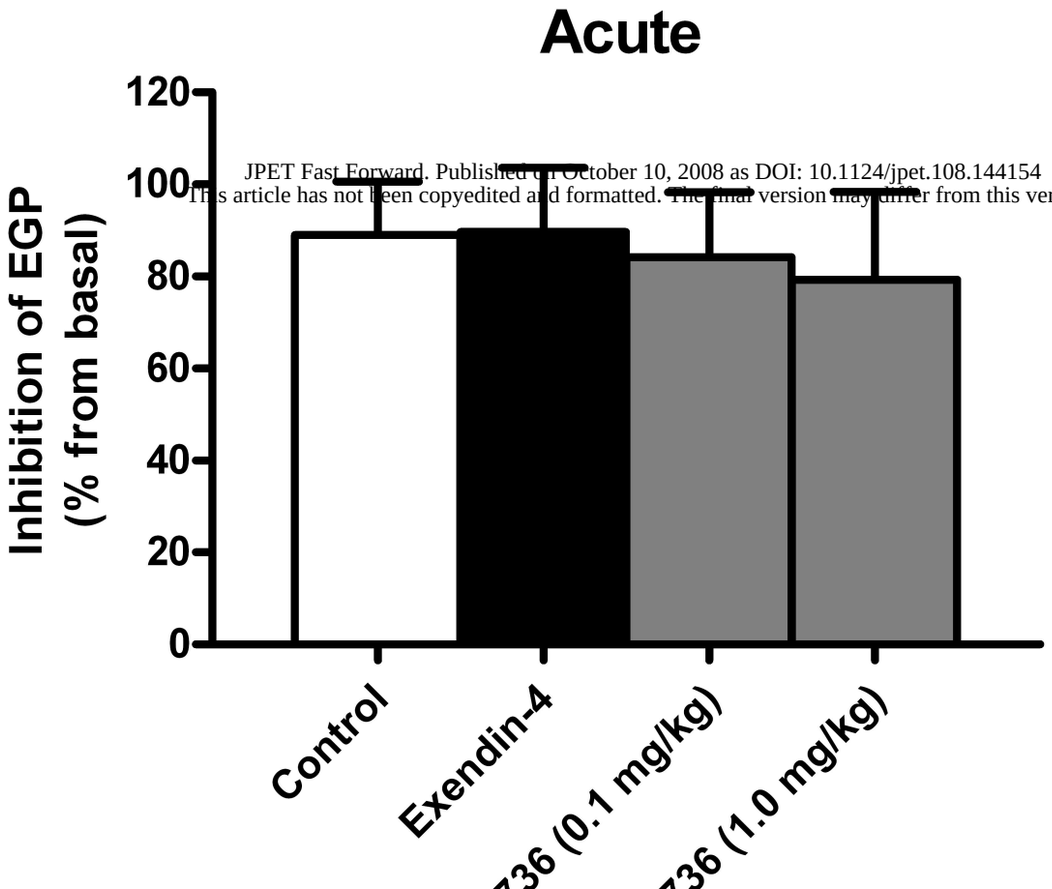


Figure 4



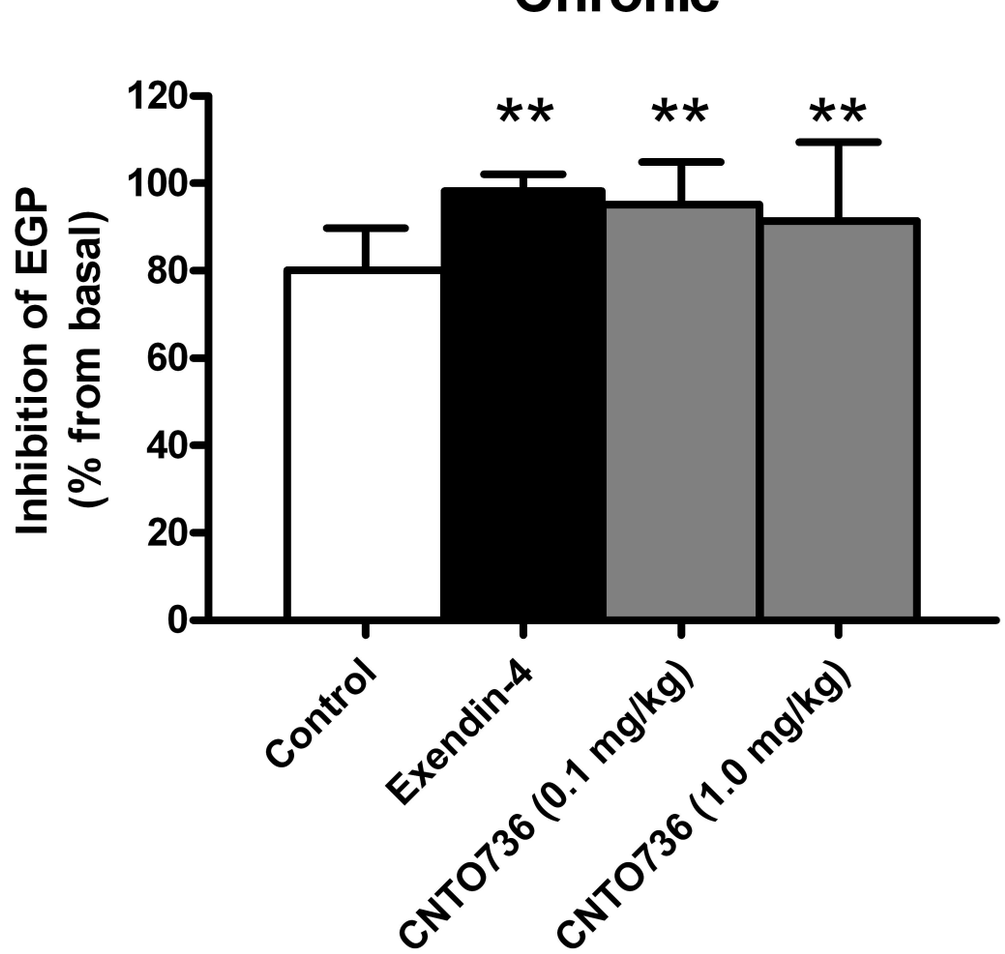


Figure 5

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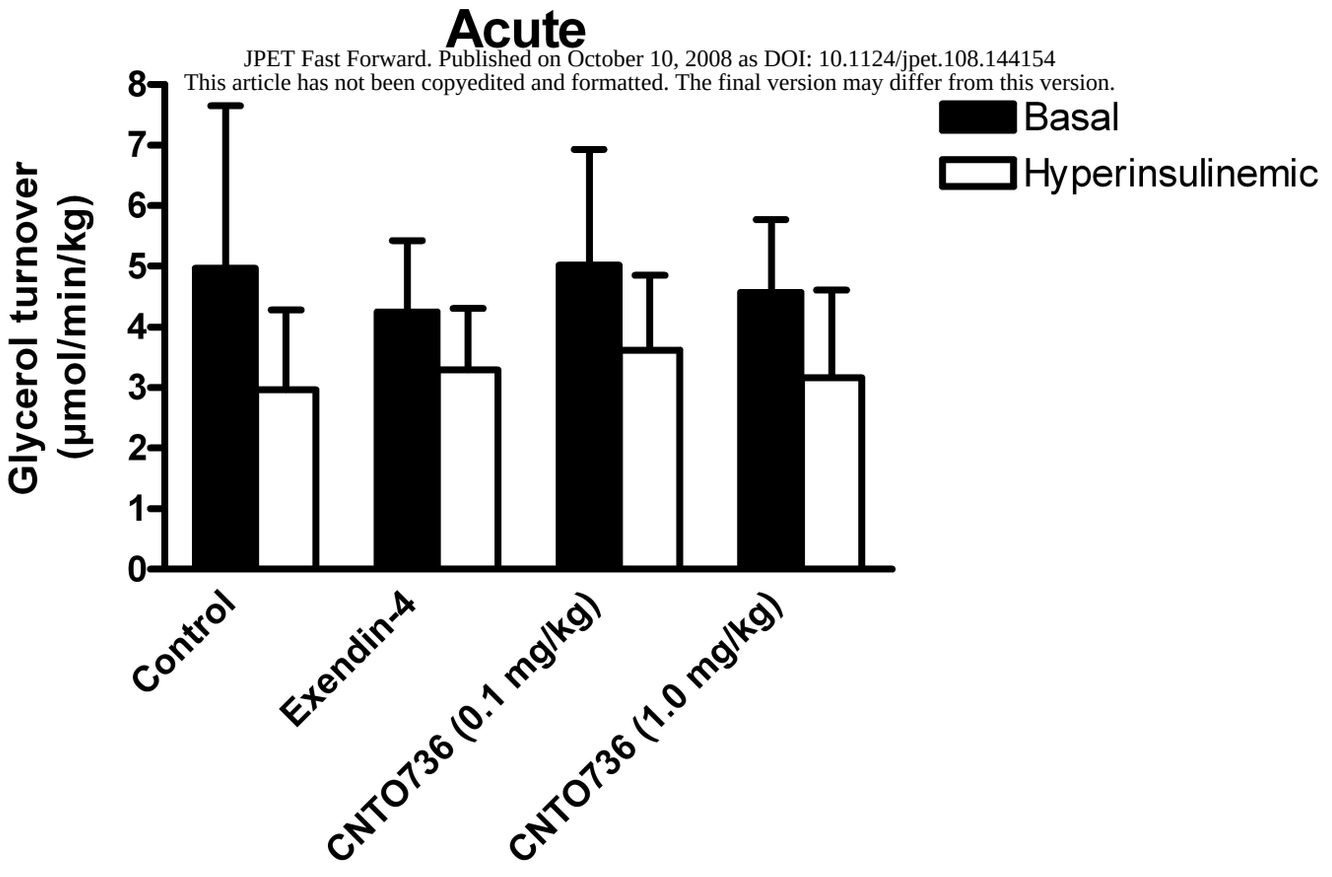


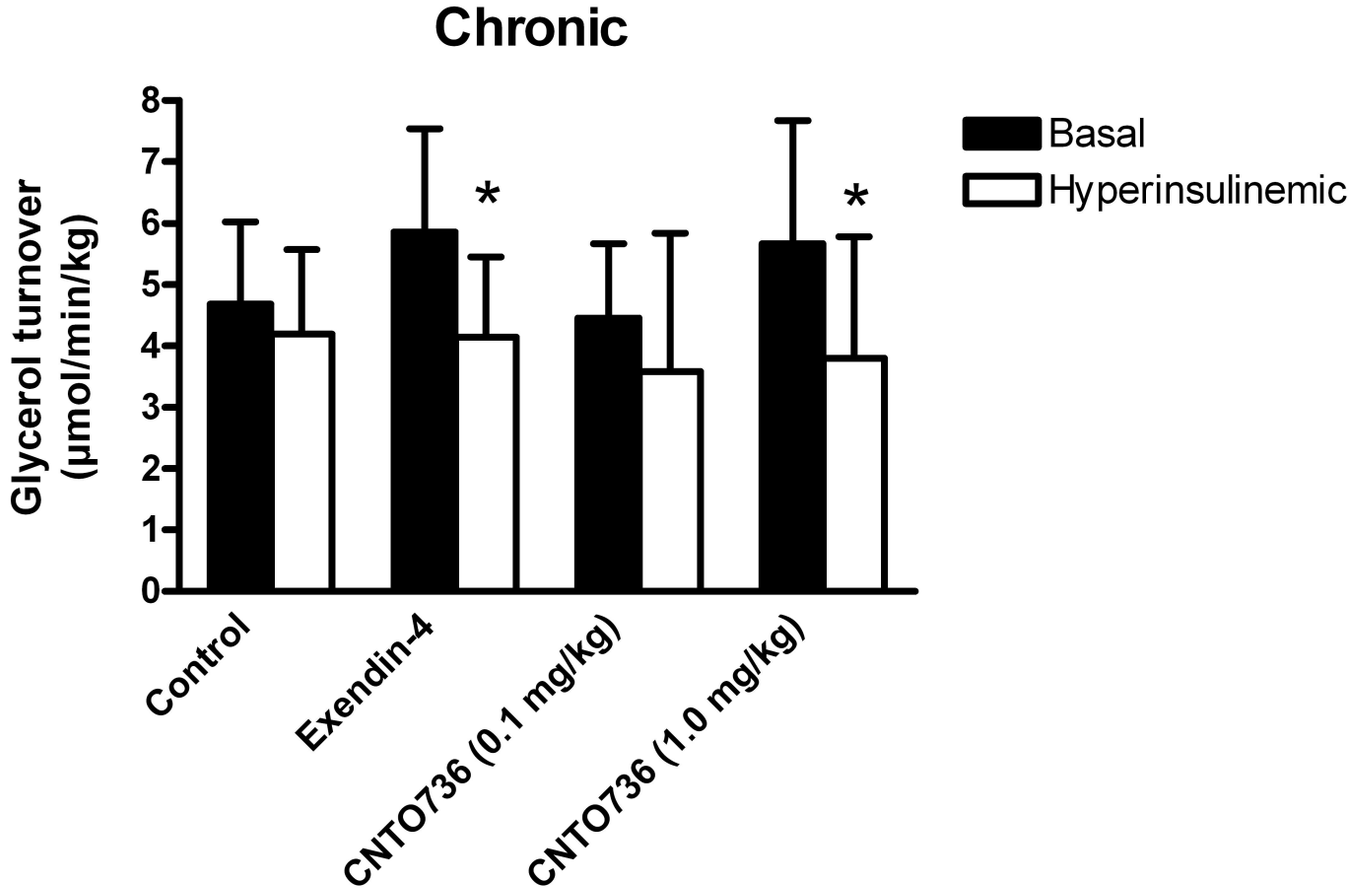
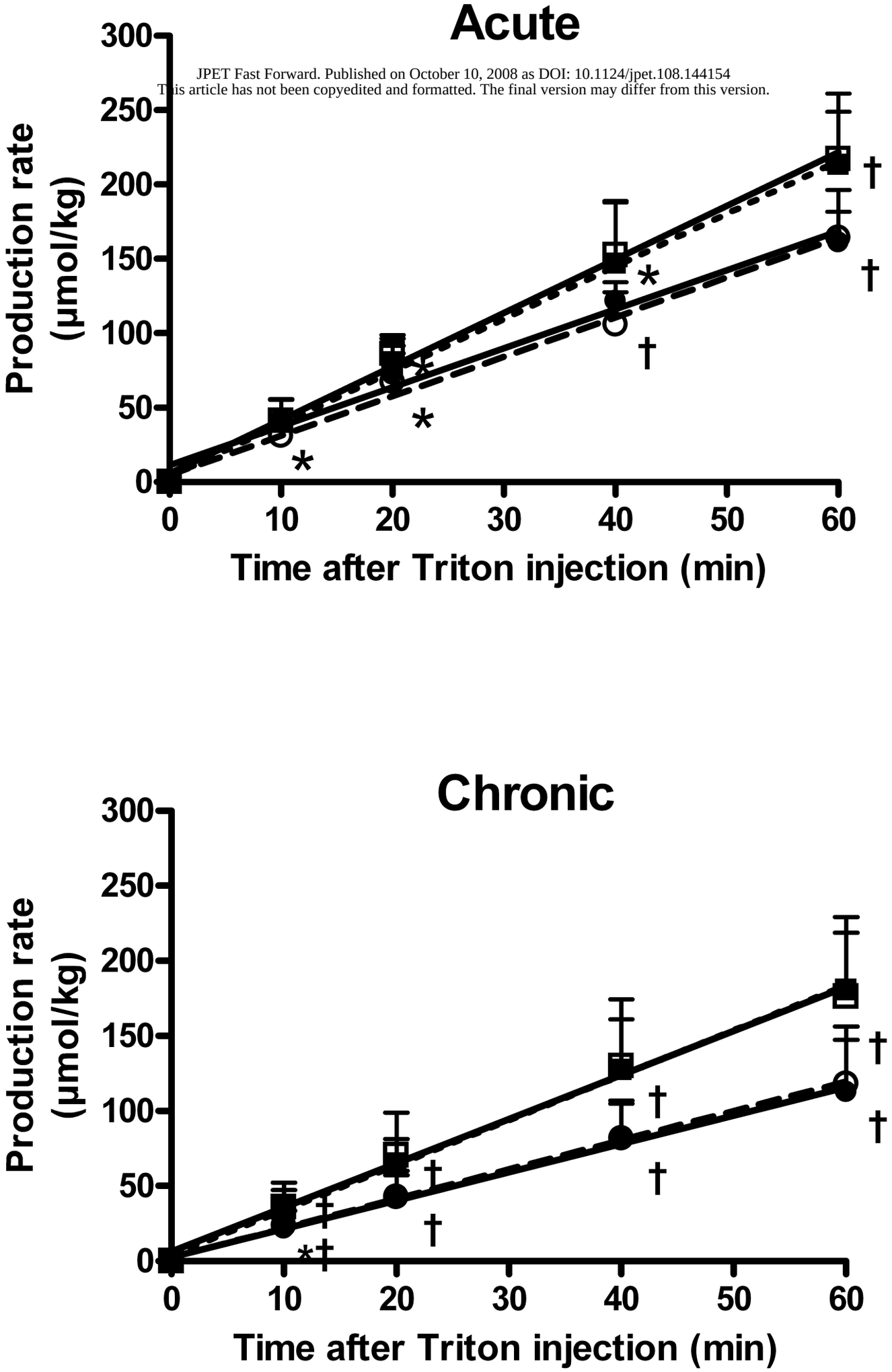


Figure 6



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