DOSING PROFILE PROFOUNDLY INFLUENCES NICOTINIC ACID’S ABILITY TO IMPROVE METABOLIC CONTROL IN RATS

Tobias Kroon1,2, Ann Kjellstedt1, Pia Thalén1, Johan Gabrielsson2, Nicholas D. Oakes1

1AstraZeneca R&D, Mölndal, Sweden

2Division of Pharmacology and Toxicology, Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden

Running title: Insulin sensitization in obese Zucker rats by nicotinic acid

Address Correspondence to:

Tobias Kroon
AstraZeneca R&D Mölndal
S-431 83
Mölndal
Sweden
Telephone: +46 31 7762693
Fax: +46 31 7763704
E-mail: Tobias.Kroon@astrazeneca.com
ABSTRACT

Acute nicotinic acid (NiAc) administration results in rapid reduction of plasma free fatty acid (FFA) concentrations. However, sustained NiAc exposure is associated with tolerance development resulting in return of FFA to pre-treatment levels. The aim of this study was to determine whether a 12h rectangular exposure profile (intermittent dose group) could avoid tolerance development and thereby reverse insulin resistance induced by lipid-overload. FFA lowering was assessed in male Sprague Dawley (Lean) and obese Zucker rats (Obese) in response to a 5h NiAc infusion, in either NiAc-naïve animals, or after 5 days of continuous (24h/day) or intermittent (12h/day) NiAc dosing (via implantable, programmable mini-pump). We found that intermittent dosing over 5 days preserved NiAc-induced FFA lowering, comparable to dosing in NiAc-naïve animals. By contrast, following 5 days continuous administration, NiAc-induced FFA lowering was lost. The effect of intermittent NiAc infusion on insulin sensitivity was assessed in obese Zucker rats using hyperinsulinemic-isoglycemic clamps. The acute effect of NiAc to elevate GIR (vs. saline control) was indeed preserved with intermittent dosing, while being lost upon continuous infusion. In conclusion, an intermittent but not continuous NiAc dosing strategy, succeeded in retaining NiAc’s ability to lower FFA and improve insulin sensitivity in obese Zucker rats.

Keywords: Lipolysis and Fatty Acid Metabolism, Diabetes, Insulin, Adipose Tissue, Drug Therapy/Hypolipidemic Drugs, Niacin, GPR109A, Tachyphylaxis
INTRODUCTION

Lipid overload in non-adipose tissues has been linked to the pathogenesis of insulin resistance and atherogenesis (1-4). A potential means for reversing peripheral lipid overload is to restrict the release of free fatty acids (FFA) from adipose tissues. A number of independent mechanisms have been explored and provide evidence supporting this concept. This includes inactivation of hormone sensitive lipase (HSL) (5, 6) and A1-adenosine receptor agonists (7). In addition, several other G protein-coupled receptors (GPCRs) are involved in controlling adipocyte FFA release, including GPR43, GPR81 and GPR109A (8-10).

The GPR109A agonist nicotinic acid (NiAc) has been used clinically ever since its anti-dyslipidemic effects (HDL elevation and reductions of total cholesterol, LDL-cholesterol and TG) were discovered over 50 years ago (11-15). Although NiAc potently lowers FFA acutely, large-scale clinical studies, with repeated oral NiAc administration, often report increased levels of fasting glycemia (16-19). NiAc has not been optimized to achieve durable and therapeutically meaningful FFA lowering. By this we specifically mean reducing around-the-clock FFA area under the curve. In theory this might be achieved by sustained NiAc exposure however the FFA lowering effect seen initially appears to be lost over time despite maintained NiAc exposure (tolerance development) (20). Time dependent loss of both FFA lowering and glucose control improvement also occur in patients with type 2 diabetes, treated with the NiAc analog acipimox (21, 22). To avoid tolerance development, drug holidays are needed. However, at the end of each NiAc exposure period there is the risk of FFA rebound (here referring to the situation where FFA overshoots pre-treatment levels in connection with NiAc decline) due to the short NiAc plasma half-life (23). FFA rebound is associated with impaired glucose
control (24, 25). The question of whether there might be an optimal balance between periods of continuous exposure (which would minimize rebound) and drug holidays (which would minimize tolerance) in order to achieve maximal FFA lowering, has not been addressed.

In this study effects of daily NiAc dosing profile on NiAc’s acute ability to lower FFA levels and improve insulin sensitivity were examined. More specifically, NiAc-induced FFA lowering following pre-treatment with two well defined NiAc dosing regimens were compared to the response in NiAc naïve animals. The pre-treatment regimens were: a 12h rectangular exposure profile (intermittent dose group) and a sustained exposure profile (continuous dose group). These profiles were produced using implantable, programmable mini-pumps in lean healthy and obese insulin resistant Zucker rats. Insulin sensitivity in obese Zucker rats, was assessed using hyperinsulinemic-isoglycemic clamps. Additionally, we examined the impact of the alternative dosing regimens on expression of selected adipose tissue genes involved in FFA mobilization.
MATERIALS AND METHODS

Animals: Experimental procedures were approved by the local Ethics Committee for Animal Experimentation (Gothenburg region, Sweden). Male Sprague Dawley (Lean) and obese Zucker rats (fa/fa, Obese) were purchased from Harlan Laboratories B.V. (The Netherlands). Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care accredited animal facility with climate-control: room temperature 20-22°C, relative humidity 40-60%, and with a 12h light-dark cycle (lights on at 06:00h). The animals were housed in groups of 5, given free access to standard rodent chow (R70, Laktamin AB, Stockholm, Sweden) and regular tap water. Peri-operative body weight was well matched within each group (Lean: 471±4 g, Obese: 649±7 g) and stable prior to the acute study; with insignificant changes following surgery and 5 days of saline/NiAc pre-treatment (Lean: ↑3.7±0.5%, Obese: ↑0.1±0.2%).

Studies overview: We wanted to compare the ability of continuous vs. intermittent NiAc administration to suppress FFA levels, in metabolically healthy and insulin resistant rats. To this end, Study I was conducted in conscious Lean and Obese animals to compare acute FFA lowering with the situation following 5 days of either continuous or intermittent NiAc administration. In Obese only, an additional group was studied following 11 days of intermittent NiAc dosing. In Study II, the effects of acute, continuous and intermittent NiAc infusion on whole body insulin sensitivity were assessed in Obese animals under hyperinsulinemic-isoglycemic clamp conditions. Study III was performed to examine the impact of the alternative dosing regimens on expression of selected adipose tissue genes involved in FFA mobilization in Lean and Obese groups using quantitative RT-PCR (Taqman).
Nicotinic acid dose selection and formulation: A key aspect of the study design was to achieve steady state plasma NiAc concentrations corresponding to therapeutically relevant levels in the rat (~1 µM). The dose selection was based upon the previously obtained relationship between FFA lowering and NiAc infusion rate in NiAc naïve Lean and Obese rats (26). NiAc (pyridine-3-carboxylic acid, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterile water and adjusted to physiological pH using sodium hydroxide. The final concentrations of the NiAc dosing solutions were ~1 M. Vehicle, for control animals, consisted of sodium chloride solutions at equimolar concentrations. Freshly prepared formulations were loaded into the infusion pump (see below), via a 0.2 µm sterile filter (Acrodisc®, Pall Corporation, Ann Arbor, MI, USA), just before pump implantation.

Surgical preparation: In order to acclimate to individual housing, animals were moved to separate cages 3 days prior to surgery. To prevent potential infections in conjunction with surgery, oral antibiotic treatment was initiated 1 day prior to surgery and then once daily for 3 days (sulfamethoxazole and trimethoprim 40 mg mL⁻¹ + 8 mg mL⁻¹; Bactrim®, 0.2 mL/animal, Roche Ltd, Basel, Switzerland). Surgery was performed under isoflurane (Forene®, Abbott Scandinavia AB, Solna, Sweden) anesthesia, with body temperature maintained at 37°C. For NiAc/saline administration, a programmable mini pump (iPrecio® SMP200 Micro Infusion Pump, Primetech Corporation, Tokyo, Japan) was implanted subcutaneously, via a dorsal skin incision. To allow blood sampling from animals in Study I, a polyurethane catheter (Instech Laboratories Inc, Plymouth Meeting, PA USA) was placed in the right jugular vein via an incision in the neck. In order to maintain its patency up to the acute experiment, the jugular catheter was filled with sterile 45.5% (wt/wt) PVP (polyvinylpyrrolidone, K30, MW ~40,000
Fluka, Sigma-Aldrich, Sweden) dissolved in a Na-citrate solution (20.6 mM, Pharmaceutical and Analytical R&D, AstraZeneca, Mölndal, Sweden), sealed and exteriorized at the nape of the neck. Each animal received a post-operative, subcutaneous analgesic injection (buprenorphine, Temgesic®, 1.85 µg kg⁻¹, RB Pharmaceuticals Ltd, Berkshire, GB). Animals were then housed individually and allowed three days of recovery before start of the pre-programmed pump infusion. Throughout the study, body weight and general health status was monitored and recorded daily.

**Treatment:** Both Lean and Obese animals were divided into 3 dose groups and NiAc was given acutely (NiAc naïve) or following 5 days with either continuous (Cont. NiAc) or intermittent (Inter. NiAc) administration. Each dose group was matched with corresponding saline infused controls. Infusions were given subcutaneously at 0.17 µmol min⁻¹ kg⁻¹, corresponding to 10.2 µL min⁻¹ kg⁻¹. The intermittent infusion protocol was programmed as a 12h on-off cycle (infusion on at 13:00h). During the last day of the treatment period an overnight fast was initiated (food removed at 24:00h with water freely available) and animals entered into one out of three terminal acute experiments: Study I, Study II or Study III. An overview of study protocols is summarized schematically in Fig. 1.

**Study I (NiAc induced FFA lowering).** In the morning of the acute experimental day, the jugular catheter was connected to a swivel system to enable blood sampling in unrestrained animals. Jugular catheter patency was maintained by continuous infusion (5 µl min⁻¹) of Na-citrate solution (20.6 mM). After a 3-4h adaptation period, at ~12:00h, the basal phase of the acute experiment (Basal Period) commenced with 2-3 blood samples drawn between -60 and -5
min relative to commencement of NiAc/saline infusion (note that, in the Cont. NiAc groups, infusion pumps were on throughout this sampling period, Fig. 1). Samples were then drawn during the infusion phase (Infusion Period) at 30, 60 and 90 min, as well as, at 2.0, 3.0, 4.0 and 4.5h with infusion stop at 5.0h. During the post infusion phase (Post Infusion Period), blood samples were drawn at 5.2, 5.3, 5.5, 6.0, 7.0 and 8.0h. Blood samples were used to determine plasma concentrations of NiAc, FFA, glucose and insulin. Blood sample volume ranged between 30-150 µL with a combined loss of less than 5% of the total blood volume. Blood was collected in potassium-EDTA coated tubes, and briefly kept on ice until centrifugation and storage at -20 °C pending analysis.

**Study II (hyperinsulinemic-isoglycemic clamps).** Whole body insulin sensitivity was assessed in anesthetized Obese animals using hyperinsulinemic-isoglycemic clamps. Animals were anesthetized at ~08:00h (Na-thiobutabarbitol, Inactin®, 180 mg kg⁻¹, i.p., RBI, Natick, MA, USA), tracheotomized with PE 240 tubing and breathed spontaneously. One catheter (PE 50 tubing) was placed in the left carotid artery for blood sampling, as well as recording of arterial blood pressure and heart rate. Four catheters (PE 10 tubing) were placed together in the right external jugular vein for infusions of NiAc, insulin and glucose and for administering top-up doses of anesthetic, if needed. The catheters were filled with Na-citrate solution (20.6 mM) in normal saline to prevent clotting. The arterial catheter patency was maintained by continuous infusion of Na-citrate (20.6 mM in saline, 5 µl min⁻¹) from shortly after carotid catheterization until the conclusion of the experiment. Body temperature was monitored using a rectal thermocouple and maintained at 37.5 °C by means of servo controlled external heating.
Animals were allowed a stabilization period of ~150 min from surgical completion. Following this, a 30 min Basal Period (with no NiAc/saline infusions in any group except Cont. NiAc) preceded commencement of iv-infused NiAc/saline, performed by external syringe pumps (CMA 1100, Carnegie Medicin, Solna, Sweden). An initial 60 min Pre-Clamp period preceded the start of insulin infusion for the clamps. Human insulin (Actrapid®, Novo Nordisk, Bagsvaerd, Denmark) was infused at a constant rate based on estimated lean body mass (lbm, (27)) at 60 pmol kg\textsubscript{lbm}^{-1} min\textsuperscript{-1} via one dedicated jugular vein catheter using a syringe pump. The target plasma glucose level for the clamp was determined for each animal to be equal to its own basal level (isoglycemia). This was obtained from the average of at least 3 stable consecutive samples during the Pre-Clamp period. Plasma glucose was clamped with a variable rate infusion of 20% (w:v) glucose using a syringe pump (Model 22 I/W, Harvard Apparatus Inc., South Natick, MA, USA) via a dedicated jugular vein catheter. During the first hour of the clamp, arterial plasma glucose was measured every 5 min and during the second hour every 10 min, using a glucose analyzer (ACCU-CHEK® Compact Plus, Roche Diagnostics Indianapolis, Indiana, USA; <10 µL blood per sample). Steady state, in both plasma glucose level (within ± 10% of the target level) and glucose infusion rate (GIR), was generally achieved within 90 min of clamp start. Additional blood samples (100 µL) were collected into potassium-EDTA coated tubes during: the Basal Period (at -90 and -75 min relative to insulin infusion start), the Pre-Clamp period (at -30, -15 min) and the Clamp (60 and 120 min). Blood samples were centrifuged immediately and stored at -20 °C pending analysis of plasma FFA and insulin concentrations.
Study III (NiAc induced changes in adipose tissue gene expression). Following 5 days of continuous or intermittent saline/NiAc treatment (Fig. 1A) and 5h of continuous infusion of NiAc/saline (Fig. 1B), animals were anesthetized with isoflurane and tissues (liver and epididymal adipose tissue) were dissected, snap frozen in liquid nitrogen and stored at -80°C pending analysis.

Analytical methods: Plasma FFA was analyzed using an enzymatic colorimetric method (Wako Chemicals GmbH, Neuss, Germany). Plasma glucose was measured using a portable blood glucose monitoring device (ACCU-CHEK® Compact Plus, Roche Diagnostics Indianapolis, Indiana, USA). Obese plasma insulin was analyzed with a radioimmunoassay kit (rat insulin RIA kit, Millipore Corporation, St. Charles, Missouri, USA), while Lean plasma insulin concentrations were determined using a colorimetric ELISA kit (Ultra Sensitive Rat Insulin ELISA Kit, Crystal Chem INC, Downers Grove, IL, USA). The ELISA was used for Lean rats to minimize blood sample volume (only 5μl plasma required vs. ~50 μl plasma for RIA). The RIA was used for the Obese rats because high lipid levels in the plasma of these animals interfere with the ELISA but not the RIA measurement. Because of the hyperinsulinemia in the obese animals only 5 μl of plasma was required. For Lean rat plasma (with low lipid levels) the absolute insulin measurement are equivalent for the RIA and ELISA assays based on an in-house comparison. For plasma samples collected during the glucose clamp study (Study II), total (rat + human) insulin was determined using the rat RIA and human insulin was determined by a species specific RIA (human insulin specific RIA kit, Millipore). Plasma NiAc concentrations were analyzed using LC-MS/MS with a hydrophilic interaction liquid chromatography (HILIC) approach, separated on a 50 x 2.1 mm Biobasic AX column, with 5 μm particles (Thermo Hypersil-Keystone,
Triglyceride content of liver was measured using an enzymatic colorimetric method (Horiba ABX, France). Area under the concentration-time curves (AUC) for FFA, insulin and glucose were calculated by trapezoidal approximation, using GraphPad Prism 6.01 (GraphPad Software Inc., La Jolla, CA, USA). HOMA-IR was calculated for Study II based on the product of Basal Period plasma insulin and glucose.

RNA was extracted and isolated according to the manufacturer’s instructions using RNeasy® Mini Kit (Qiagen AB, Solna, Sweden). RNA concentration was measured and purity assessed using a Nanodrop (ThermoFisher, Wilmington, USA). cDNA was reverse transcribed from up to 2.5μg RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA). Differences in gene expression (relative to 36B4) was determined by quantitative RT-PCR (Taqman) using a 7900HT system with SDS2.3 software.

Statistics: Statistical significance of post-hoc comparisons were evaluated based on 1-way ANOVA with Sidak’s multiple comparisons test, performed using GraphPad Prism 6.01 (GraphPad Software Inc., La Jolla, CA, USA). For Study I, statistical comparisons were made on area under concentration-time curves (AUC) estimates, for FFA, insulin and glucose. Throughout, results are reported as mean ± SE. P<0.05 was considered statistically significant.
RESULTS

Study I: Metabolic effects of continuous vs. intermittent NiAc dosing in lean and obese rats

Nicotinic acid exposure

The target steady state plasma NiAc concentration of ~1 µM was successfully achieved by subcutaneously infusing 0.17 µmol min⁻¹ kg⁻¹ in all Lean and Obese groups (Fig. 2). This exposure level was selected based on previous data showing near maximal FFA suppression in NiAc naïve Sprague Dawley rats (26). Plasma NiAc concentrations declined rapidly across all dosed groups during the Post Infusion Period. In control (saline infused) animals, endogenous NiAc levels were below the detection limit (6 nM).

Free fatty acids

Plasma FFA concentration-time profiles are shown in Fig. 3. Comparisons between groups are based on analysis of area under the concentration-time curves (AUCs) for the 5h Infusion Period, 3h Post Infusion Period and the combined 8h observation period (Fig. 4).

Lean rats: Previous exposure to NiAc, either intermittent or continuous, had no effect on average Basal Period FFA (P>0.05, vs. saline control). During the Infusion Period, FFA AUC was lower in the NiAc naïve group compared with saline control (P<0.05, Fig. 4A). In the Cont. NiAc group, FFA AUC was not reduced compared to saline controls (P>0.05, Fig. 4A) despite ongoing NiAc infusion/exposure (Fig. 2) indicating complete tolerance after 5 days of NiAc exposure. In contrast, the FFA lowering response was fully preserved following intermittent
infusion for 5 days compared to NiAc naïve animals, with similar AUC values in the two groups (P>0.05, Fig. 4A). Thus, intermittent dosing was successful in avoiding FFA lowering tolerance.

In the Lean NiAc naïve group, during the Post Infusion Period, there was clear evidence of a marked rebound (Fig. 3) with FFA AUC greater than saline control for the same period (P<0.05, Fig 4B). Interestingly, the Cont. NiAc group also exhibited a rebound (Fig. 3) with AUC greater than saline control (P<0.001, Fig 4B), consistent with a previous study (20).

Remarkably, in the Lean animals over the whole 8h observation period none of the NiAc protocols reduced total FFA AUC (Fig. 4C). Thus in the NiAc naïve and Inter. NiAc groups, the FFA rebound during the Post Infusion Period tended to cancel the FFA lowering achieved during the Infusion Period. In the Cont. NiAc group, the total FFA AUC was actually elevated compared to both NiAc naïve and Inter. NiAc groups (P<0.05, Fig. 4C).

**Obese rats:** As expected, Obese exhibited higher average FFA levels than Lean during the Basal Period (↑86% vs. Lean saline, P<0.001, Fig. 3). Previous exposure to NiAc had no effect on Basal Period FFA (P>0.05, vs. saline control). As in Lean, NiAc-induced FFA lowering was completely lost in the Cont. NiAc group after 5 days of continuous, uninterrupted NiAc infusion. Thus, during the Infusion Period the FFA AUC was similar in Cont. NiAc vs. saline control (P>0.05, Fig. 4A). Intermittent NiAc dosing succeeded in retaining significant FFA lowering during the Infusion Period, with FFA AUC in the Inter. NiAc group less than saline control (P<0.001). Unlike Lean though, there was some loss of the extent of FFA AUC lowering (Inter. NiAc vs. NiAc naïve, P<0.001 Fig. 4A). Importantly, an additional group of animals studied following 11 days of intermittent NiAc (Inter. NiAc Day 11) showed that there was no further
development of tolerance (Inter. NiAc Day 11 vs. Inter. NiAc, P>0.05, Fig. 4A). Total 8h FFA AUC lowering was achieved only in the NiAc naïve group (↓36%, P<0.001 vs. saline control, Fig. 4C), while being unaffected in both intermittent NiAc groups and actually increased in the Cont. NiAc group compared to saline control (P<0.05, Fig 4C).

**Insulin and Glucose**

Plasma insulin and glucose concentration-time profiles are shown in Fig. 5. Comparisons between groups are based on analysis of AUC for insulin (Fig. 6) and glucose (Fig. 7). To satisfy homogeneity of variance assumption, insulin AUC data were expressed as percent of respective saline control values. An important objective was to assess the potential of NiAc-induced FFA lowering to improve glucose control. Indeed, compared to their respective saline infused controls, we observed a reduction in insulin AUC during the Infusion Period (Fig. 6A) in Lean NiAc naïve (P<0.01), Obese NiAc naïve (P<0.05) and Obese Inter. NiAc (P<0.05). This occurred in the absence of change in Infusion Period glucose AUC (P>0.05 for all NiAc-infused groups vs. respective saline controls, Fig. 7A), suggesting an improvement in insulin sensitivity which was particularly remarkable in the obese groups. While insulin profiles in the different groups were broadly similar in pattern to the FFA profiles described above, one difference was in the Obese animals where the lowering of insulin AUC was fully preserved at 5 days in Inter. NiAc vs. NiAc naïve groups (P>0.05, Fig. 6A), compared to FFA lowering which was partially lost at this time point (Fig. 4A). The rebound phenomenon was not just restricted to FFA. Insulin rebounds were observed in all NiAc dosed groups (Fig 5A-B) with the exception of Obese Cont. NiAc (Fig. 5B). As for FFA (described above), reduction in insulin AUC achieved during the Infusion Period tended to be cancelled during the Post Infusion Period, with the result that total 8h insulin AUC is
similar in all NiAc groups compared to respective saline control groups (Fig. 6C). NiAc succeeded in moderately lowering blood glucose AUC in the Obese NiAc naïve group (↓11%, P<0.001, Fig.7C), although this effect was not maintained with either intermittent or continuous NiAc dosing.

**Study II: effects of continuous vs. intermittent NiAc dosing on whole body insulin sensitivity in obese Zucker rats**

Whole body insulin sensitivity was assessed in anesthetized Obese animals using hyperinsulinemic-isoglycemic clamps. During the Basal Period, FFA, glucose and insulin levels were similar across all groups (Table 2), confirming complete tolerance development in the Cont. NiAc group. NiAc infusion lowered FFA and insulin in both the NiAc naïve and Inter. NiAc groups (P<0.05, Table 2) while these variables remained stable in the saline group. In saline infused animals, insulin infusion increased total plasma insulin by ~80%. Despite this, plasma FFA levels remained stationary, *i.e.* a complete loss of anti-lipolytic action of insulin and supporting the phenotype of this insulin resistant animal model (28). Importantly, acute NiAc exposure restored insulin’s ability to suppress plasma FFA levels in both NiAc naïve and Inter. NiAc groups (P<0.001 vs. saline control, Table 2). The extent of FFA suppression was similar in NiAc naïve and Inter. NiAc groups (P>0.05).

Clamps were performed at similar levels of glycemia (Table 2). Steady state glucose infusion rates (GIRs) needed to maintain basal glucose levels during the hyperinsulinemic-isoglycemic clamp are summarized in Fig. 8. In the NiAc naïve group, GIR was markedly increased compared to saline infused controls (↑92%, P<0.01). Importantly, the Inter. NiAc group also had an
elevated GIR (↑71%, P<0.05), similar in magnitude to the NiAc naïve group (P>0.05), compatible with a sustained insulin sensitization of the intermittent dosing approach. In stark contrast, upon continuous dosing, this effect was completely lost (P>0.05 vs. saline control, Fig. 8). Across all groups, GIR was negatively correlated with prevailing clamp FFA levels; individual data linear regression, r²=0.35, P<0.01 (data not shown).

**Study III: NiAc induced changes in adipose tissue gene (mRNA) expression**

Quantitative RT-PCR (Taqman) was conducted to assess whether changes in mRNA expression of adipocyte proteins could explain the differences observed in the NiAc-induced FFA lowering in Study I (Table 3). Degradation of TG to FFA predominantly involves adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL). The activity of these lipases is governed by the intracellular concentration of cAMP. Production and degradation of cAMP is controlled via adenylate cyclase and phosphodiesterase-3B (PDE3B), respectively. There was a general tendency for expression levels to be lower in Obese saline vs. Lean saline, with significantly lower levels for PDE3B (P<0.05) and GPR109A (P<0.05). In Lean rats, continuous NiAc infusion induced an increase in expression of genes promoting both FFA mobilization (ATGL, P<0.05) and FFA storage (GPR81, P<0.05) compared to saline controls. By contrast, intermittent NiAc administration had limited impact on gene expression compared to saline control. Unlike the Lean situation, continuous NiAc infusion in Obese rats had no significant impact on gene expression. Neither was there any apparent effect of intermittent dosing on the genes measured. Overall there was no evidence that a coordinated alteration in expression of genes was responsible for the tolerance development in either Lean or Obese (Table 3).
**Liver triglycerides**

Liver triglyceride (TG) content, at the end of the Infusion Period, is presented in Table 4. As expected, saline infused Obese displayed elevated liver TG content (P<0.001, Table 4) compared to Lean saline. In both Lean and Obese, NiAc (either intermittent or continuous exposure) had no significant impact on liver TG content.
DISCUSSION

The intermittent drug holiday approach succeeded in retaining the ability of a therapeutically relevant NiAc exposure to induce FFA lowering. In lean rats, following intermittent (12h on/12h off) infusions for 5 days, the acute NiAc-induced FFA suppression was completely preserved (Fig. 3 and 4). While in the obese rats there was a partial loss of FFA lowering efficacy, following 5 days intermittent dosing, importantly, this did not appear to be progressive with similar FFA lowering at 11 vs. 5 days (Fig. 3 and 4). By contrast, continuous NiAc infusion for 5 days resulted in complete return of FFA to pre-treatment levels, consistent with the findings of Oh et al. (20).

NiAc has a very short half-life in the rat (~2 min in plasma) which precludes oral dosing as a means of achieving stable and well defined exposures. Therefore, NiAc was administrated using an implantable, programmable pump infusing via the subcutaneous route. Given the importance of therapeutically relevant exposures (29), a key aspect of the current studies was that they were performed at plateau plasma NiAc concentrations of ~1µM, that in NiAc naïve animals are just sufficient to maximally suppress FFA levels (26). This was done since loss of FFA lowering might theoretically be exacerbated by sustained supramaximally effective levels of target engagement e.g. by ligand induced GPR109 desensitization and internalization (30). Oh et al. (20) previously demonstrated, in Wistar rats, complete return of FFA to pre-treatment levels during a ~10-fold higher NiAc continuous infusion rate than we have used in the current study. Based on a pharmacokinetic analysis (data not shown), and the fact that NiAc clearance exhibits saturation kinetics (26), we estimate that this infusion rate would result in plateau concentrations >20-fold above those achieved in the current study.
Markedly improved insulin sensitivity was seen in association with NiAc induced FFA lowering, either in NiAc naïve or previously intermittently dosed obese Zucker rats. Adult male obese Zucker rats exhibit extreme whole body insulin resistance associated with tissue lipid overload. Elevated FFA mobilization from adipose tissue, seen under both basal fasting as well as hyperinsulinemic clamp conditions, is an important mechanism driving this condition (28, 31). In the present study, acute suppression of circulating FFA levels by NiAc in the Obese animals was associated with reduced fasting hyperinsulinemia (Fig. 6A). Reduced insulin secretion might be explained by direct effects on the islets of either NiAc (32) or the fall in FFA level (33). However, if the effect was only to decrease insulin secretion then levels of glycemia should have increased, which was not the case (Fig. 7A). Reduced fasting insulinemia in association with normoglycemia suggests instead that NiAc enhanced whole body insulin sensitivity. Indeed, this was confirmed by the elevated GIRs needed to maintain isoglycemia during the hyperinsulinemic clamps (Fig. 8 and Table 2).

Rapid effects of acute modulation of FFA metabolism on insulin sensitivity have been previously reported. The acute blockade of β-oxidation, using etomoxir, increased insulin sensitivity in skeletal muscle and reduced gluconeogenesis (34, 35) which could enhance insulin mediated suppression of hepatic glucose output. More direct effects of NiAc per se, such as attenuated toll-like receptor 4 (TLR4) pro-inflammatory signaling (36) and changes in tissue NAD pools to reduce oxidative stress (37) have been reported. Whether these effects could explain the rapid increase in insulin sensitivity in the present study requires further investigation.
Tissue lipid loading is determined both by the acute circulating lipid levels (plasma FFA and TG), as well as, the endogenous intracellular lipid stores. The ability of plasma FFA lowering alone, to significantly impact on total tissue fatty acid utilization has been clearly demonstrated by work showing that acute NiAc administration, in the fasting state, induces a major shift from whole body fat to carbohydrate oxidation, in association with the suppression of plasma FFA (38). We were hoping to reverse lipid overload, not just by reducing acute circulating FFA, but also by lowering endogenous lipid stores via a reduction of net (24h average) FFA levels with the intermittent dosing protocol. Our quantitative AUC analysis (Fig. 4) suggests, however, that the FFA rebound is of a magnitude sufficient to cancel the acute NiAc induced FFA lowering in the intermittently dosed groups, despite the fact that this presumably only occurred once per day. Failure of the intermittent dosing strategy to lower net FFA levels over the 5 day dosing period was also indicated by the lack of lowering of hepatic TG content (Table 4); a relatively slow turnover tissue lipid pool. This may well explain why the NiAc induced enhancement in GIR was similar in the previously intermittently dosed group compared to the naïve group, since reduced peripheral lipid availability was only effected through reduction of circulating FFA, and the degree to which plasma FFA level was lowered was similar in both groups. The critical role of acute FFA levels is also evidenced by the apparent lack of amelioration of insulin resistance (HOMA-IR) in the intermittent NiAc dose group in the basal state; a period when FFA levels were not reduced (Table 2).

Our results are reminiscent of studies of the nicotinic acid analog, acipimox, in patients with type 2 diabetes. Thus in association with FFA lowering, acipimox acutely enhanced whole body insulin sensitivity in patients naïve to the drug (via a selective increase in oxidative glucose
disposal) and moreover the degree of insulin sensitization was very similar following 3 months of acipimox treatment (21). The authors of this study also observed FFA rebound, occurring between acipimox doses, and pointed to this as the probable cause of the failure of long term acipimox treatment to improve glycemia in the patients. A recent study confirmed insulin sensitization in association with FFA lowering during short, intensive acipimox therapy, despite failure to reduce lipid accumulation in skeletal muscle (39). We hypothesize that to fully realize the potential of FFA lowering on improving glucose control, net FFA lowering must be achieved and to do that the FFA rebound issue has to be solved (see below).

In contrast to the response to intermittent dosing, continuous NiAc infusion did not enhance whole body insulin action, associated with the loss of FFA lowering. There are few reports of continuous NiAc administration with which to compare. In healthy rats, FFA lowering was achieved for at least the first 5h but had completely returned to pre-treatment levels by 24h. At the 24h time point, despite similar FFA levels compared to saline infused controls, an insulin sensitizing effect was seen, provided that NiAc exposure was maintained (20). Whether this could be explained by the combined effect of acute FFA level during the clamp and a reduction in FFA AUC over the preceding 24h (both factors determining tissue lipid concentrations involved in interference of insulin signaling) cannot be answered by the available data. At some point though, prolonged exposure might have a negative effect, as shown by a study made in baboons continuously infused for 20 days (40). An important aspect of the tolerance development, not explored by the current work, is the influence of NiAc infusion duration on the minimum drug holiday length i.e. the time required to restore the acute dosing effects of NiAc on FFA lowering. This could have implications for optimal dosing design. An informative additional experimental
group would be to study a 12h drug holiday at the end of a 5-day continuous NiAc infusion to see whether acute FFA lowering and insulin sensitizing effects are restored.

Loss of FFA lowering during prolonged NiAc infusion was not associated with a shift in the balance in expression of adipose tissue genes involved in liberating vs. storing fatty acids. The current results differ in some respects from those of Oh et al. (20), who related apparent changes in lipolysis to changes in adipose tissue gene expression of Wistar rats. In particular, they observed a substantial downregulation of PDE-3B, which the authors argued could be an important mechanism responsible for the return of FFA to pre-infusion levels during prolonged NiAc infusion. NiAc induced suppression of PDE-3B expression and function has also been reported to occur in mice (41). In the lean (Sprague Dawley) rats used in the current study, we observed no significant change in PDE-3B mRNA after 5 days of continuous NiAc infusion. We cannot explain the discrepancy between our study and the previous work, although one possible cause might be that we used substantially lower NiAc doses, ~1/10th of those used in the earlier studies. Overall our analysis of several genes involved in liberation and storage of FFA in adipose tissue does not point to any involvement of expression as the mechanism for the loss of FFA lowering after 5 days of NiAc administration.

The metabolic responses to NiAc cessation are not restricted to FFA rebound and provide insight into the nature of the loss of FFA lowering during continuous NiAc infusion. To our knowledge, this is the first study to assess the detailed, time dependent response of plasma insulin and glucose in response to sudden NiAc withdrawal and reveals that in all cases where there was a FFA rebound, this was associated with an insulin rebound, with a particularly pronounced response in the obese NiAc naïve animals. This phenomenon may be the result of the well known
potentiation of glucose stimulated insulin secretion by long chain FFAs (33). However, the surprisingly modest reductions seen in plasma glucose in response to the insulin rebound, suggest that the complete explanation is likely more complex. Indeed, Vega et al. (42) suggested that NiAc induced FFA lowering might trigger a counter-regulatory response, perhaps to defend substrate supply, and NiAc has been shown to significantly alter levels of a number of hormones including glucagon and growth hormone (43). The mechanism of the loss of FFA lowering in response to continuous NiAc exposure appears to be different in the lean and obese animals. Thus in the lean animals, sudden withdrawal of NiAc after 5 days uninterrupted exposure induced a marked FFA rebound (Fig. 3 and Fig. 4B), consistent with the interpretation of Oh et al. (20) that the return of FFA to pre-infusion levels represents the net effect of preserved NiAc action in the presence of an enhanced basal rate of lipolysis. By contrast, in obese Zuckers there was absolutely no evidence of FFA rebound indicating that lipolysis had become completely tolerant to NiAc i.e. complete tachyphylaxis.

Further refinements to NiAc dosing might achieve greater lipid lowering and insulin sensitization. As discussed above, the simple intermittent protocol for infusion of NiAc has probably not succeeded in lowering average daily FFA levels, with the rebound rise in FFA occurring rapidly in response to NiAc withdrawal quantitatively cancelling the FFA lowering during NiAc infusion, resulting in no reduction of hepatic tissue lipid storage. We anticipate that further refinements of the infusion protocol or optimal timing of NiAc administration relative to food intake might mitigate this issue. Thus a programmed, more gradual decline in NiAc concentrations to terminate each infusion period might help to minimize the rebound. Alternatively, FFA rebound might be minimized if NiAc withdrawal is timed to occur in association with feeding/insulin administration. This might especially be the case if the proposal
that FFA rebound involves a counter-regulatory response (42) to defend substrate supply is correct. Whatever the specific strategy, the current experimental paradigm combining a translationally relevant preclinical disease model, chronically catheterized for repeated stress free blood sampling, the programmable/implantable pump to deliver nicotinic acid with its desirable properties of high potency, high solubility and rapid clearance, provides a useful experimental paradigm to explore alternative protocols in order to reveal the potential of FFA lowering to improve glucose control.

How do we resolve our data with the general view that NiAc dosing in man worsens glucose control? Current study limitations, either species differences or the relatively short duration compared to the long-term treatments in the clinic, might provide trivial explanations. However, we suggest that the timing of NiAc administration and assessment of FFA level, glucose control or insulin sensitivity may be critical. In particular, we assessed insulin sensitivity in the presence of circulating levels of NiAc sufficient to suppress FFA. This differs from almost all clinical reports where assessments are made when circulating NiAc levels are likely to be very low (overnight fasting, last dose taken at bedtime the night before) e.g. when NiAc was orally dosed 12h before clamp studies, FFA rebound was associated with insulin resistance (25). We are not the first to suggest the potential impact of timing on niacin therapy. Usman et al. (44) proposed that giving ER niacin at meal time, instead of bed time, could improve triglyceride lowering. Based on the current results it seems reasonable that this strategy might also improve glucose handling following mixed meal ingestion.

An intermittent NiAc exposure profile equivalent to the one used in the present study, with stable and sufficient NiAc levels to suppress FFA 12h/day, has to our knowledge not been applied in the
clinic. This is important because different profiles may have different effects on average FFA levels. Crystalline NiAc dosing seems to raise net plasma FFA levels, due to marked rebound between doses, and induces insulin resistance (25, 38). ER formulations might do better but reported effects on glucose control are generally either neutral or negative (16, 17, 42, 45). This could result from a failure of the ER formulations to lower FFA, either on average or at the time point of glucose control assessment. Several factors probably prevent ER formulations from reducing average FFA levels. 1) Dosing has not been designed to lower FFA, rather the goal has been to ameliorate dyslipidemia via effects now understood to be independent of the FFA lowering mechanism (46). 2) While the ER formulation prolongs plasma NiAc exposure, maximum plasma NiAc levels are reduced compared to equivalent crystalline formulation doses (47), likely resulting in poor FFA suppression over a large fraction of the day (based on the relationship between circulating NiAc levels and FFA suppression, (23)). 3) In addition, clinical dosing is also associated with significant FFA rebound (e.g. (42)).

In conclusion, an intermittent nicotinic acid dosing strategy succeeded in retaining FFA lowering and improving insulin sensitivity in obese Zucker rats. While these data suggest that FFA lowering is sufficient to improve insulin sensitivity, further refinements to the administration regime should be explored to more profoundly reverse lipid-overload induced insulin resistance.
ACKNOWLEDGMENTS

We thank Simonetta Wallin for performing the mRNA analysis, Therese Hagstedt for surgery assistance, Charlotte Lindgren for assay setup assistance (liver TG and plasma insulin) and Kristina Wallenius as a valuable discussion partner (all above-mentioned persons were at the time full time employees at AstraZeneca R&D, Mölndal, Sweden).

DISCLOSURES

N.D.O., P.T. and A.K. are full time employees and J.G. and T.K. are former employees at AstraZeneca R&D, Mölndal, Sweden.

AUTHOR CONTRIBUTIONS

T.K., J.G. and N.D.O. contributed to the formation and design of the research; T.K. performed the experiments and analyzed the data; P.T., A.K. and N.D.O. assisted in the experiments; T.K., J.G. and N.D.O. interpreted the results of the experiments; T.K. and N.D.O. drafted the manuscript; T.K., J.G. and N.D.O. edited and revised the manuscript.
REFERENCES


glycemic control in patients with diabetes and peripheral arterial disease: The ADMIT study: A randomized trial. *JAMA* **284**: 1263-1270.


increased circulating fatty acids and fat oxidation but not muscle lipid content. *Metabolism* **52**: 699-704.


Figure captions

Fig. 1. A) Nicotinic acid (NiAc) and saline infusion profiles across Study I-III. Black (■ NiAc) and open (□ Saline) bars represent time periods of constant rate infusions during day 1-5. B) Terminal protocol for Study I (NiAc induced FFA lowering) and III (NiAc induced changes in adipose tissue gene expression). C) Terminal protocol for Study II (hyperinsulinemic-isoglycemic clamps).

Fig. 2. Plasma NiAc concentration in Lean (left) and Obese (right) with NiAc (0.17 µmol min⁻¹ kg⁻¹) given acutely (NiAc naïve, n=7/group) or following 5 days continuous (Cont. NiAc, Lean n=4, Obese n=8) or intermittent (Inter. NiAc, Lean n=4, Obese n=9) or 11 days intermittent (Inter. NiAc Day 11, Obese n=4) dosing. The black horizontal bar (▬) represent the period of acute NiAc/saline infusion. Data presented as mean ± SE.

Fig. 3. Plasma FFA concentration in Lean (left) and Obese (right) following infusion of saline (Lean n=5, Obese n=12) or NiAc (0.17 µmol min⁻¹ kg⁻¹) given acutely (NiAc naïve, n=7/group) or following 5 days continuous (Cont. NiAc, Lean n=4, Obese n=8), intermittent (Inter. NiAc, Lean n=4, Obese n=9) or 11 days intermittent (Inter. NiAc Day 11, Obese n=4) dosing. The black horizontal bar (▬) represent the period of acute NiAc/saline infusion. Data presented as mean ± SE.
Fig. 4. Plasma FFA AUCs during the Infusion Period (A), Post Infusion Period (B) and the sum of these two periods (C) in Lean (□) and Obese (■). *P<0.05, ***P<0.001 vs. Lean Saline; †P<0.05, ††P<0.01 vs. Lean NiAc naïve; ‡P<0.05 vs. Lean Cont. NiAc; +P<0.05, ++P<0.01, P<0.001 vs. Obese Saline; ⧫P<0.05, ⧭P<0.01, ⧮P<0.001 vs. Obese NiAc naïve; ¥¥P<0.01, ¥¥¥P<0.001 vs. Obese Cont. NiAc. Data presented as mean ± SE.

Fig. 5. Plasma insulin (A, B), and glucose (C, D) concentration in Lean (left column) and Obese (right column) following infusion of saline (Lean n=5, Obese n=12) or NiAc (0.17 µmol min⁻¹ kg⁻¹) given acutely (NiAc naïve, n=7/group) or following 5 days continuous (Cont. NiAc, Lean n=4, Obese n=8) or intermittent (Inter. NiAc, Lean n=4, Obese n=9) or 11 days intermittent (Inter. NiAc Day 11, Obese n=4) dosing. The black horizontal bar (▬) represent the period of acute NiAc/saline infusion. Data presented as mean ± SE.

Fig. 6. Plasma insulin AUCs during the Infusion Period (A), Post Infusion Period (B) and the sum of these two periods (C) in Lean (□) and Obese (■). **P<0.01 vs. Lean Saline; +P<0.05, +++P<0.001 vs. Obese Saline. Data presented as mean ± SE.

Fig. 7. Plasma glucose AUCs during the Infusion Period (A), Post Infusion Period (B) and the sum of these two periods (C) in Lean (□) and Obese (■). ***P<0.001 vs. Lean Saline; ++P<0.01 vs. Obese Saline; ⧫P<0.05 vs. Obese NiAc naïve. Data presented as mean ± SE.

Fig. 8. Glucose infusion rate (GIR) at clamp steady state in Obese. +P<0.05, ++P<0.01 vs. saline; ¥¥P<0.01, ¥¥¥P<0.001 vs. Cont. NiAc. Data presented as mean ± SE (n=6/group).
Table 1. Genes with corresponding primers and probes used for the mRNA expression analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe (5'FAM-3'TAMRA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>36B4</td>
<td>AAATCTCCAGAGGTAC</td>
<td>GCTGGCTCCCACCTTGT</td>
<td>TGAGCGATGTGCAGCTGA</td>
</tr>
<tr>
<td></td>
<td>CATTGAAATC</td>
<td>CT</td>
<td>TAAAGAC</td>
</tr>
<tr>
<td>ATGL</td>
<td>CACCCACACAGATCCA</td>
<td>CCGGGTGAGCGAGAA</td>
<td>CCTCCACCTTGCTGAGA</td>
</tr>
<tr>
<td></td>
<td>TCTG</td>
<td>T</td>
<td>ACCA</td>
</tr>
<tr>
<td>HSL</td>
<td>AGACGGGCTCTAGTG</td>
<td>AACTCTGGGCTATGGA</td>
<td>AAGTCCCTCTTACGAGG</td>
</tr>
<tr>
<td></td>
<td>GACT</td>
<td>GAATC</td>
<td>TGGC</td>
</tr>
<tr>
<td>PLIN1</td>
<td>TCCGAAGCGCCAGGA</td>
<td>CCCGAAACCTTTCAG</td>
<td>AGCATCAGCGCTGCCATT</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>AAGTG</td>
<td>GCAA</td>
</tr>
<tr>
<td>PDE-3B</td>
<td>GCACCATGCAGTCTG</td>
<td>GGGTACATGGCTGAA</td>
<td>TGGTGCTTTCCACGCCC</td>
</tr>
<tr>
<td></td>
<td>AGAA</td>
<td>GAATTCA</td>
<td>CA</td>
</tr>
<tr>
<td>GPR109A</td>
<td>ACTGGAGGTTGAGGA</td>
<td>CGGTTCATGCAACAT</td>
<td>CCGTGCGGTGATGCTC</td>
</tr>
<tr>
<td></td>
<td>GCAT</td>
<td>GA</td>
<td></td>
</tr>
<tr>
<td>GPR81</td>
<td>CCCCTCTTCCCCAAA</td>
<td>TGGGCGTCTGGGTTCA</td>
<td>TACGCAAGCTCAAAATC</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td></td>
<td>CGCAGC</td>
</tr>
</tbody>
</table>

Reference gene (36B4), adipocyte triglyceride lipase (ATGL), hormone sensitive lipase (HSL), perilipin (PLIN1), phosphodiesterase 3B (PDE3B), nicotinic acid receptor (GPR109A), lactate receptor (GPR81)
Table 2. Plasma FFA, glucose, total (endogenous + human) insulin and human insulin during the Basal Period, Pre-Clamp and at Clamp time periods in obese Zucker rats.

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>NiAc naïve</th>
<th>Cont. NiAc</th>
<th>Inter. NiAc</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FFA (mM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal Period</td>
<td>1.30±0.06</td>
<td>1.31±0.15</td>
<td>1.22±0.08</td>
<td>1.32±0.04</td>
</tr>
<tr>
<td>Infusion Period</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-Clamp</td>
<td>1.39±0.08</td>
<td>0.94±0.12++</td>
<td>1.18±0.07</td>
<td>0.77±0.08++</td>
</tr>
<tr>
<td>Clamp</td>
<td>1.24±0.10</td>
<td>0.52±0.07+++</td>
<td>0.94±0.05</td>
<td>0.42±0.07+++</td>
</tr>
<tr>
<td><strong>Glucose (mM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal Period</td>
<td>7.10±0.36</td>
<td>7.32±0.52</td>
<td>6.98±0.29</td>
<td>8.82±0.97</td>
</tr>
<tr>
<td>Infusion Period</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-Clamp</td>
<td>6.61±0.99</td>
<td>7.62±1.26</td>
<td>6.19±0.89</td>
<td>7.94±1.29</td>
</tr>
<tr>
<td>Clamp</td>
<td>7.63±0.47</td>
<td>8.53±0.67</td>
<td>7.07±0.26</td>
<td>9.26±0.72</td>
</tr>
<tr>
<td><strong>Total insulin (nM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal Period</td>
<td>3.00±0.56</td>
<td>3.46±0.86</td>
<td>4.46±0.55</td>
<td>3.15±0.55</td>
</tr>
<tr>
<td>Infusion Period</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-Clamp</td>
<td>2.99±0.57</td>
<td>1.22±0.33+++</td>
<td>4.08±0.71</td>
<td>1.05±0.13+++</td>
</tr>
<tr>
<td>Clamp</td>
<td>5.43±0.74</td>
<td>5.45±1.11</td>
<td>8.27±0.80</td>
<td>4.03±0.37+++</td>
</tr>
<tr>
<td><strong>Human insulin (nM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>2.12±0.28</td>
<td>1.96±0.20</td>
<td>2.85±0.44</td>
<td>1.89±0.25</td>
</tr>
<tr>
<td><strong>HOMA-IR (% of Saline)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal Period</td>
<td>100±21</td>
<td>111±23</td>
<td>143±18</td>
<td>120±14</td>
</tr>
</tbody>
</table>

P<0.05, ++P<0.01, +++P<0.001 vs. saline; ¥P<0.05, ¥¥P<0.01, ¥¥¥P<0.001 vs. Cont. NiAc. Measurements were made either in the 30 min period preceding NiAc/saline infusion (Basal Period) and during the NiAc/saline Infusion Period, either prior to clamp (Pre-Clamp) or during clamp (Clamp). In the Basal and Pre-Clamp periods, Total insulin=endogenous (rat) insulin; in the Clamp Phase, Total Insulin=Endogenous + Human insulin achieved by use of two RIAs (see Methods and Materials). Data presented as mean ± SE (n=6/group).
Table 3. Lean and Obese epididymal adipose tissue gene (mRNA) expression following 5 days saline, continuous (Cont. NiAc) or intermittent (Inter. NiAc) NiAc administration.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ATGL</td>
<td>100±25</td>
<td>66±12</td>
<td>168±17*</td>
<td>50±9</td>
<td>105±19</td>
<td>50±8</td>
</tr>
<tr>
<td>HSL</td>
<td>100±17</td>
<td>61±11</td>
<td>140±13</td>
<td>53±10</td>
<td>87±13‡</td>
<td>48±7</td>
</tr>
<tr>
<td>PLIN1</td>
<td>100±26</td>
<td>110±20</td>
<td>153±13</td>
<td>98±20</td>
<td>105±22</td>
<td>89±14</td>
</tr>
<tr>
<td>PDE3B</td>
<td>100±9</td>
<td>68±11*</td>
<td>125±4</td>
<td>56±8</td>
<td>101±7</td>
<td>61±5</td>
</tr>
<tr>
<td>GPR109A</td>
<td>100±29</td>
<td>35±5*</td>
<td>144±17</td>
<td>27±4</td>
<td>58±15‡‡‡</td>
<td>19±2</td>
</tr>
<tr>
<td>GPR81</td>
<td>100±20</td>
<td>65±12</td>
<td>171±24*</td>
<td>54±8</td>
<td>81±13‡‡</td>
<td>43±7</td>
</tr>
</tbody>
</table>

Adipocyte triglyceride lipase (ATGL), hormone sensitive lipase (HSL), perilipin 1 (PLIN1), phosphodiesterase 3B (PDE3B), nicotinic acid receptor (GPR109A), lactate receptor (GPR81). Lean (n=6/group), Obese (n=7-8/group); *P<0.05 vs. Lean Saline; ‡P<0.05, ‡‡P<0.01, ‡‡‡P<0.001 vs. Lean Cont. NiAc. Data expressed as % of Lean saline and presented as mean ± SE (normalized to reference gene 36B4).
**Table 4.** Lean and Obese liver triglyceride content following 5 days continuous or intermittent saline or NiAc dosing.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Liver TG (g/100g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lean</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>6</td>
<td>1.30 ± 0.23</td>
</tr>
<tr>
<td>Cont. NiAc</td>
<td>6</td>
<td>1.28 ± 0.20</td>
</tr>
<tr>
<td>Inter. NiAc</td>
<td>6</td>
<td>0.83 ± 0.22</td>
</tr>
<tr>
<td><strong>Obese</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>8</td>
<td>6.79 ± 0.63***</td>
</tr>
<tr>
<td>Cont. NiAc</td>
<td>8</td>
<td>8.26 ± 1.01</td>
</tr>
<tr>
<td>Inter. NiAc</td>
<td>8</td>
<td>6.40 ± 1.05</td>
</tr>
</tbody>
</table>

***P<0.001 vs. Lean saline. Data presented as mean ± SE.
Fig. 1

A) Saline
NiAc naïve
Continuous NiAc
Intermittent NiAc

NiAc  Saline

Time (days)

Mini-pump surgery 1 pm Acute exp. time = 0 hrs

Acute Experiments
Study I (Panel B)
Study II (Panel C)
Study III (Panel B)

B) Basal Period Infusion Period Post Infusion Period

Day 5

Exp. time (hrs)

Study III:
Tissue sampling

C) Basal Period Pre Clamp Clamp

Exp. time (hrs)
Fig. 2
Fig. 3

[Graph showing FFA (mM) over time for Lean and Obese groups with different conditions: Saline, NiAe naïve, Cont. NiAe, Inter. NiAe, Inter. NiAe Day 11.]
Fig. 4

(A) Infusion Period
(B) Post Infusion Period
(C) Infusion + Post Infusion Periods

Legend:
- Lean
- Obese
Fig. 5
Fig. 6
Fig. 7

[Graph showing Glucose AUC (mM h) during Infusion Period, Post Infusion Period, and Infusion + Post Infusion Periods for Lean and Obese groups with statistical significance indicated by asterisks.]
Fig. 8

![Bar chart showing GIR (μmol/min/kg) for different groups: Saline, NiAc naïve, Control NiAc, and Inter. NiAc.](image)

- Saline: 25 ± 5 μmol/min/kg
- NiAc naïve: 45 ± 10 μmol/min/kg
- Control NiAc: 10 ± 3 μmol/min/kg
- Inter. NiAc: 40 ± 7 μmol/min/kg

Significance levels:
- ++ for NiAc naïve compared to Saline
- + for Inter. NiAc compared to NiAc naïve