Diabetologia (2011) 54:1502–1506
DOI 10.1007/s00125-011-2111-y

SHORT COMMUNICATION

Unconditioned and conditioned effects of intranasally administered insulin vs placebo in healthy men: a randomised controlled trial

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Received: 26 September 2010 / Accepted: 11 February 2011 / Published online: 2 April 2011

Abstract
Aims/hypothesis In humans, the intranasal route allows insulin to reach the brain while maintaining peripheral euglycaemia. Our aims were to examine acute (unconditioned) effects of central insulin on normal-range blood glucose and hormones in men, and to find out whether the effects of intranasal insulin can be learnt via classical conditioning.
Methods In a randomised controlled trial, 32 healthy normal-weight men (mean age 24.2 [SEM 0.5], mean BMI 22.4 [0.3]) received a conditioned stimulus (CS) and six administrations of either soluble H-insulin 100 (20 U [0.2 ml]; group 1; $n=16$) or vehicle (0.2 ml; group 2; $n=16$) on day 1. The CS was the tarry smell of *meta*-cresol (used as a stabilising vehicle in many insulin preparations and placebos). On day 2, all participants received the CS and six administrations of placebo. Participants and experimenters were blinded to group assignment. Sixteen individuals were randomised to and analysed in each group. Participants were sequentially numbered for group allocation. The main outcome measures were blood glucose and insulin, expressed as cumulative difference-from-baseline changes.
Results While maintaining euglycaemia, intranasal insulin induced an increase of peripheral insulin on day 1 (group 1, 17.78 [21.88] pmol/l vs group 2, −10.24 [9.42] pmol/l), and also on day 2 when the CS was given with placebo (group 1, 12.53 [5.57] pmol/l vs group 2, −5.51 [6.16] pmol/l). Moreover, a moderate reduction of blood glucose on day 1 (group 1, −0.54 [0.36] mmol/l vs group 2, 0.58 [0.48] mmol/l) was obtained (all p values <0.05). There were no adverse side effects.
Conclusions/interpretation The unconditioned blood glucose decrease on day 1 and the unconditioned and conditioned increases of peripheral insulin are indicative of brain–pancreas cross-talk. The conditionability of the hormonal responses reveals new applications for intranasal insulin.

Trial registration: DRKS00000537 http://apps.who.int/trialsearch/
Funding: Deutsche Forschungsgemeinschaft DFG STO 323/1-1 and 1-2.

Keywords Blood glucose · Brain · Classical conditioning · Intranasal insulin · Peripheral insulin

Abbreviations
CR Conditioned response
CS Conditioned stimulus
NPY Neuropeptide Y
US Unconditioned stimulus

Introduction

In humans, the intranasal route is an effective pathway for insulin to enter the brain while maintaining euglycaemia

Electronic supplementary material The online version of this article (doi:10.1007/s00125-011-2111-y) contains supplementary material, which is available to authorised users.

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and normal insulin levels in peripheral blood [1]. This allows assessment of the direct effects of insulin within the brain. Central insulin has been shown to affect food intake, body weight and memory [2–4]. We here address the effects of insulin acting in the brain on normal-range blood glucose and hormones, and the conditionability of these effects in humans.

Classical (Pavlovian) conditioning is a learning process that is mediated by the central nervous system and that is based on associating a previously neutral stimulus with an unconditioned stimulus (US). Through its association with the US, the neutral stimulus becomes a conditioned stimulus (CS), which is able to elicit the conditioned response (CR) that often resembles the unconditioned response. Specifically, after pairings of a CS (neutral odour) with peripheral insulin administration (US), rats responded to the CS plus placebo injection with a vagally mediated increase of insulin (that was blocked by vagotomy or atropine), and with a consequent reduction in blood glucose [2, 5, 6]. Moreover, increasing insulin action locally within the brain activated a vagal reflex to the pancreas causing increased insulin secretion [2, 6]. The relevant stimulus for classical conditioning of insulin effects is insulin afferently reaching central insulin receptors [7]. Therefore, the intranasal administration of insulin constitutes an ideal paradigm in humans to validate this brain–pancreas cross-talk by demonstrating classical conditioning of central insulin effects. No such experiments have previously been reported.

In a between-group placebo-controlled design we looked for the acute and the classically conditioned effects of having received intranasal insulin on blood insulin and glucose. We anticipated a neurally mediated increase in insulin and a decrease in glucose within the euglycaemic range.

Methods

Participants Participants were 32 healthy students (16 per group). Mean age (±SEM) was 24.0±0.7 years in group 1 and 24.4±0.7 years in group 2. BMI was within the normal range (group 1, 22.3±0.4 kg/m^2; group 2, 22.4±0.4 kg/m^2). Exclusion criteria were diabetes mellitus, allergies, chronic and/or acute rhinitis, anatomic deviations of the nose, endocrine, neurological and cardiovascular diseases, substance abuse, current medication and treatment and smoking. The study was approved by the local ethics committee (Heinrich-Heine-University, Duesseldorf, Germany), and fulfilled the criteria of the Declaration of Helsinki.

All participants took part in a screening session where they gave their written informed consent, and underwent a physical examination and blood sampling. Of the 50 candidates screened, 32 were randomised (see electronic supplementary material [ESM] Fig. 1).

Intranasal substances The following substances were administered in the doses described. The intranasal insulin preparation was H-insulin U 100, Insuman Rapid, Hoechst, Frankfurt, Germany, containing *meta*-cresol (2.63 mg/ml). The placebo preparation was HOE31, Hoechst, containing *meta*-cresol (2.63 mg/ml). The precision air pumps used to administer insulin (Mistette MK Pump II, GL18) were manufactured by MeadWestvaco Calmar, Hemer, Germany.

Research design A two-group between-subject (randomised controlled trial) design was conducted under double-blind conditions.

Groups differed only by the substance that was intranasally administered on day 1 of the two-session experiment. On day 1 (acquisition), group 1 received soluble insulin intranasally, 20 U (= 0.2 ml) per application, 10 U per nostril (sequence right/left counterbalanced). Group 2 received the vehicle solution without insulin, 0.2 ml per administration, 0.1 ml per nostril. Six intranasal applications (separated by 15 min) were administered. Neither solution contained absorption enhancers. In classical conditioning terms, insulin on day 1 functioned as the US. The CS was the tarry smell of *meta*-cresol (used as a stabilising vehicle) which was presented along with the insulin or vehicle on day 1. On day 2 (test), both groups received the CS and six administrations of placebo.

Participants were instructed to breathe regularly, to avoid any sneezing, and to bend their necks by about 45° during and 60 s after the administration of the nasal spray.

Procedure On both days 1 and 2, participants had to fast for 12 h. Sessions were scheduled on two consecutive days.

Sessions started at about 08:30 hours. After a 60 min baseline, insulin or placebo was intranasally administered every 15 min (from 0 to 75 min) in the post-baseline phase, which lasted for 120 min (Fig. 1).

Fourteen venous blood samples were obtained, seven were larger (‘B’ in Fig. 1) and used for assessing glucose as well as several hormones (insulin, leptin, catecholamines and cortisol), and another seven were smaller (‘b’) and used for glucose only.

Dependent variables Blood glucose was determined from venous haemolysed blood samples using an EPOS 5060 analyser (Eppendorf, Hamburg, Germany). Serum insulin was measured with a commercially available microparticle enzyme immunoassay as previously reported [7, 8]. For leptin, catecholamines and cortisol see ESM.

Data reduction and analysis Data are expressed as means ± SEM. As there was baseline variation, differences-from-baseline levels were determined. Hormone baseline was the −15 min value, and glucose baseline was the mean of

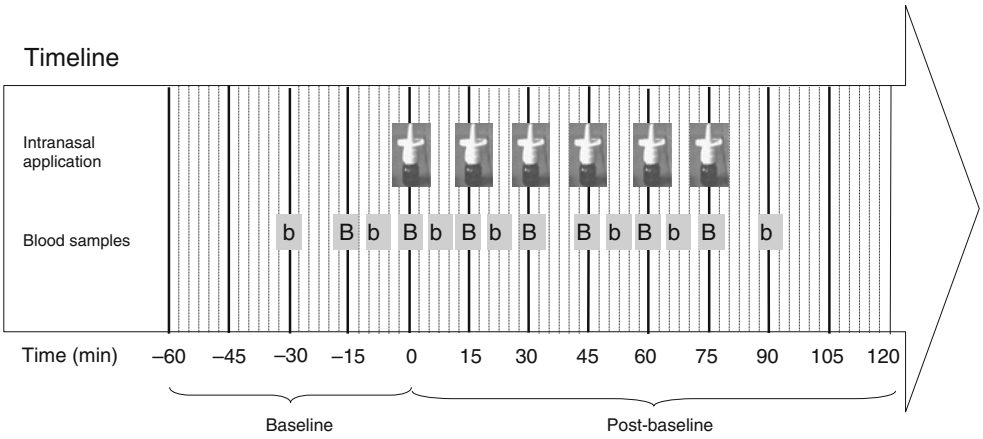

Fig. 1 Timeline of each session. Smaller (20 μl) blood sampling, 'b' mainly every 7.5 min, was for the determination of blood glucose; larger (10 ml) blood sampling, 'B' at −15, 0, 15, 30, 45, 60 and 75 min, was for the determination of insulin, leptin, epinephrine, norepinephrine and cortisol. Experiments were performed between around 08:30 hours and 11:30 hours (each two participants separated by a 22 min lag). The baseline lasted 60 min (min −60 to 0); the post-baseline phase lasted for 120 min

the three pre-nasal administration values. Six difference-from-baseline values ([min 0, 15, 30, 45, 60, 75]−baseline) were calculated for the hormones, and 11 for blood glucose. The single difference-from-baseline values were summed, yielding the cumulative change. To reduce the impact of outliers, and because several variables were not normally distributed, all inter-group comparisons were calculated by a non-parametric test for rank-scaled data,

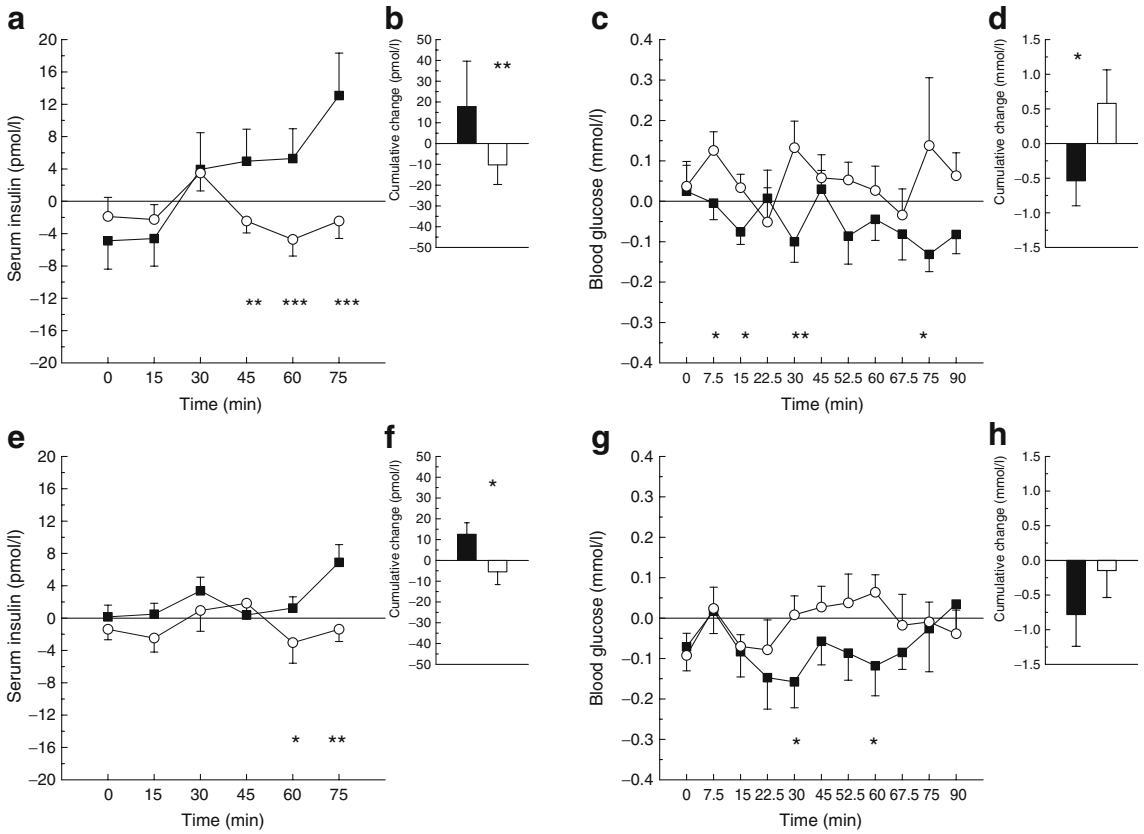

Fig. 2 **a**, **b**, **e**, **f** Changes of insulin (pmol/l) relative to the mean baseline level, at time points and cumulative, on day 1 (acquisition) (**a**, **b**) and day 2 (test) (**e**, **f**) in group 1 and group 2. Fasting insulin levels in group 1 and group 2 were comparable at day 1 (group 1, 38.5±4.5 pmol/l; group 2, 37.1±4.0 pmol/l; p=0.955, two-sided) and at day 2 (group 1, 33.0±2.9 pmol/l; group 2, 34.1±2.6 pmol/l; p=0.734). On day 1, cumulative change, effect size d=0.42. On day 2, cumulative change, d=0.77. **c**, **d**, **g**, **h** Changes in blood glucose (mmol/l) relative to baseline, at time points and cumulative, on day 1 (**c**, **d**) and day 2 (**g**, **h**) in group 1 and in group 2. Fasting glucose levels were comparable for groups 1 and 2 on day 1 (group 1, 4.5±0.08 mmol/l; group 2, 4.3±0.08 mmol/l; p=0.163, two-sided). On day 2 there was a tendency for a higher level in group 1 (4.7±0.09 mmol/l) than group 2 (4.5±0.07 mmol/l; p=0.073, two-sided). Day 1, cumulative change, d=0.66. Day 2, cumulative change, d=0.37. Results of the Mann–Whitney U tests are shown as *p<0.05, **p<0.01, ***p<0.001. The inter-group comparisons of the single-measurement time points are post hoc tests. Group 1, black squares/bars; group 2, white circles/bars

the Mann–Whitney U test (SPSS). Levels for significance were set at $p<0.05$. Additional post hoc U tests were calculated for single time-point comparisons.

Results

Insulin Cumulative insulin significantly increased after intranasal insulin on day 1 in group 1, but decreased below baseline in group 2 ($U=54.5$, $p=0.003$) (Fig. 2b), also evident in single measurement time points (Fig. 2a). On day 2, where any difference between groups might reflect a CR, a similar pattern was observed: a cumulative increase in insulin in group 1 and a moderate decrease in group 2 ($U=77$, $p=0.027$; Fig. 2f). The effect size on day 2 ($d=0.77$) actually exceeded that on day 1 ($d=0.42$).

Blood glucose As expected, blood glucose remained euglycaemic during intranasal treatment. On day 1, group 1 had a significant cumulative decrease from baseline compared with an increase in group 2 ($U=83$, $p=0.045$, $d=0.66$; Fig. 2d). The same pattern was repeated on day 2 (Fig. 2h), although the difference did not reach significance ($U=100.5$, $p=0.150$, $d=0.37$).

Epinephrine On both days, epinephrine levels decreased in group 1 compared with group 2 (described in detail in the ESM).

Discussion

We found an increase of peripheral insulin, both when insulin was administered intranasally on day 1 and on the subsequent day when only vehicle was given in the presence of the CS, while blood glucose remained within the euglycaemic range. This strongly implies that the increase of insulin reflects an underlying conditioned response caused by a neurally mediated signal from the brain to the pancreas [2, 6]. The temporal manifestation of the effect is consistent with the maximum increase of insulin in the cerebrospinal fluid at 30 min after intranasal administration [1].

To explain the increased plasma insulin in group 1, we favour the conclusion that intranasal insulin accesses the brain—presumably the hypothalamus—binds to receptors there, and elicits the neural reflex to the pancreas. After this occurred repeatedly (six presentations associated with the CS on day 1), presentation of the CS on day 2 induced the same neurally mediated response. We consider it unlikely that on day 1 some of the intranasally administered insulin leaked into the blood, or passed through the nasal mucosa. For one, an insulin increase occurred on day 2, and it was even larger in magnitude than that on day 1, and it was also larger than that observed in our previous conditioning experiments using intravenously injected insulin [7, 8]. Further, for leakage from the brain into the blood extremely high amounts of insulin must be administered, and passage through the mucosa would need absorption enhancers. Of eight published studies in humans where acute intranasal insulin was administered, only one found an increase in peripheral insulin [9], one a decrease of blood glucose [10], and one both changes [11]. In order to allow a closer examination of relatively small changes and to describe the intraindividual changes that may have been missed by others, we calculated the difference-from-baseline values for each individual instead of absolute values, and measured blood glucose and insulin more frequently.

Our data showing an unconditioned and conditioned reduction of epinephrine (included in the ESM) suggest that intranasally administered insulin might also act on hypothalamic neuropeptide Y (NPY). As central actions of insulin are mediated in part via its antagonistic effects on NPY synthesis [2], and as NPY increases sympathetic activity, antagonising NPY should result in reduced sympathetic activity manifesting in epinephrine reductions.

To summarise, our data provide the first evidence for conditioning of serum insulin levels after intranasal insulin. As circulating insulin reaches the human brain under physiological conditions by a receptor-mediated saturable transport process via the endothelial cells, the insulin-conditioning effect we obtained might also imply that environmental signals that reliably predict insulin may trigger pancreatic insulin secretion within the physiological range. Thus, our results also suggest that learning processes may be evaluated as a novel tool for therapeutic purposes in eliciting insulin-like metabolic effects. The acute and the conditioned actions of insulin in the brain make centrally acting insulin an important candidate for explaining and modifying metabolic (dys)regulation.

Acknowledgements This work was supported by a research grant to U. Stockhorst from the Deutsche Forschungsgemeinschaft (German Research Foundation), DFG STO 323/1-1, and finalised during DFG ST0 323/1-2. The valuable support of Y. Schottenfeld-Naor and A. Hübinger during the study and the assistance of A. Czekalla, G. Korthaus as well as W. Mohné in performing the biochemical analyses is gratefully acknowledged.

Duality of interest The authors declare that there is no duality of interest with this manuscript.

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