

Orally Administered Whey Proteins Have Comparable Effect on C-peptide Secretion in Healthy Subjects as Standard C-peptide Stimulation Tests

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Short title: The Effect of Whey Proteins on C-peptide Secretion

Summary

Our study compared total C-peptide secretion after administration of whey proteins and whey proteins in combination with glucose with results of classical tests assessing beta cell function in the pancreas of healthy individuals. Eight young, healthy (7 males, 1 female; aged 20 – 26 years), non-obese (BMI: 17 – 25.9 kg/m²) participants with normal glucose tolerance underwent six C-peptide secretion tests. Three secretion tests measured C-peptide response to orally administered substances: whey proteins only (OWT), whey proteins with glucose (OWGT) and glucose only (OGTT); while three secretion tests measured C-peptide response to intravenously administered substances: arginine (AST), glucagon (GST) and glucose (IVGTT). OWT stimulated a greater (93%, $p < 0.05$) C-peptide response than AST and a 64% smaller response ($p < 0.05$) than OGTT. OWT also showed lower variability ($p < 0.05$) in C-peptide responses compared to OWGT and OGTT. The greatest total C-peptide response was induced by OWGT (36% higher than glucose). OWT consistently increased C-peptide concentrations with lower individual variability, while insignificantly increasing glucose levels. Results of this study suggest that both dietology and beta-cells capacity testing could take advantage of the unique property of whey proteins to induce C-peptide secretion.

Key words

Whey proteins • Pancreatic beta cell capacity tests • C-peptide • Blood glucose • Healthy

Introduction

It has been well established that ingested dietary proteins are the physiological stimuli for insulin secretion and have potential to decrease the plasma glucose levels in healthy individuals (Floyd *et al.* 1966). The magnitude of the insulin response to ingestion of different doses and types of proteins varies; however, whey proteins have unique insulinitropic property (Nilsson *et al.* 2004, Pal and Ellis 2010).

Krezowski *et al.* (1986) showed that, in healthy individuals, an acute oral administration of 50 g of protein from lean beef in combination with glucose resulted in a non-synergic increase in serum insulin and C-peptide release - the incremental area under the curve (iAUC) was 28% higher than for glucose alone - and reduced the total postprandial glucose response compared to equal amounts of glucose. An orally administered dose of protein given alone elicited a modest increase in insulin secretion (iAUC = 28% of the glucose response) and had almost no influence on fasting glucose levels. A similar metabolic response was observed in a study by Nilsson *et al.* (2004) after administration of 18.2 g of whey proteins in combination with carbohydrates in healthy subjects. The insulin iAUC for whey proteins plus a glucose mixture was 90% higher than the insulin iAUC of glucose alone. A study by Akhavan *et al.* (2010) focused primarily on the effect of pure whey proteins on food intake and pre- and post-meal satiety. However, this study did not examine the effect of whey proteins on insulin secretion over a sufficiently long period of time (Polonsky *et al.* 1988) for a complete evaluation of the total insulin secretion response following an orally administered stimulus.

Therefore we decided to evaluate the effect of pure whey proteins on C-peptide secretion (Polonsky and Rubenstein 1984) relative to C-peptide secretion after glucose only. Furthermore, we focused on the comparison between total C-peptide secretion after pure whey proteins administration and total C-peptide secretion after whey proteins administration in combination with glucose, relative to other insulin secretion tests. These tests are based on intravenous administration (Chaillous *et al.* 1996) of glucose, arginine and glucagon and the oral administration of glucose

(Small *et al.* 1985). These tests are currently used in research studies rather than in clinical practice. Since the tests ascertain the maximum C-peptide secretion capacity of the subject's pancreatic beta cells (Chaillous *et al.* 1996), these data can be used to assess the insulinotropic effect of whey proteins and its possible use in clinical practice.

Subjects and methods

Subjects

Eight healthy young non-obese (body mass index (BMI), range: 17 – 25.9 kg/m²) Caucasian subjects (7 men and 1 woman aged 20 – 26 years) were enrolled in this study. None of the participants were taking any drugs or supplements known to interfere with insulin secretion or carbohydrate metabolism. All subjects were in generally good health, with no signs or symptoms of any acute or chronic diseases and no indications of lactose malabsorption.

After evaluation, participants were found to have no disorders of glucose metabolism (based on standard diagnostic criteria expressed in the ADA Expert Committee report (Genuth *et al.* 2003)). Five participants (1 female and 4 males) had BMIs under 24.9 kg/m²; the other three males had BMIs (range) of 25.4 – 25.9 kg/m². None of the subject had any signs of insulin resistance or any condition associated with insulin resistance (e.g. hypertension, dyslipidemia, polycystic ovary syndrome or acanthosis nigricans); all participants had normal insulin sensitivity index $463 \pm 22 \text{ ml min}^{-1} \text{ m}^{-2}$ (Mari *et al.* 2001), which correlated well with the homeostasis model assessment HOMA index (Matthews *et al.* 1985).

The study protocol was designed in accordance to the principles of the Helsinki Declaration and received prior approval by the Ethics Committee of the Third Faculty of Medicine, Charles University, Prague, Czech Republic. All participants were fully informed and gave written consent.

Tests

We used a controlled cross-over study design to evaluate the effect of whey proteins on the concentration of serum C-peptide and plasma glucose. All participants were tested on 6 different occasions in random order within a one month period. The tests were carried out in the research unit of the Second Department of Internal Medicine at University Hospital Královské Vinohrady, Prague, Czech Republic.

We used three *intravenous* tests to evaluate C-peptide secretion (Chaillous *et al.* 1996): the

glucose-dependent arginine-stimulation test (AST), the glucagon stimulation test (GST) and the intravenous glucose tolerance test (IVGTT). For the AST, 5g of arginine in 50 ml of 0.9% saline (Ardeapharma, Ševětín, Czech Republic) were injected as a bolus within 45 sec. In case of the GST, 2mg of glucagon (Novo Nordisk, Bagsvaerd, Denmark) in 60 ml of 0.9% saline were injected as a bolus within 60 sec. Finally for the IVGTT; bolus of 0.5g per kg of body weight of glucose (using Glucose-30 solution, B/Braun, Melsungen, Germany) was injected within 3 min.

The other three tests for C-peptide secretion evaluation were based on the *oral* administration of glucose alone (oral glucose tolerance test (OGTT)), whey proteins alone (oral whey proteins test (OWT)) and whey proteins in combination with glucose (oral whey proteins in combination with glucose test (OWGT)). Substances used in these tests were administered in the form of drinks and were consumed within 3 min. The OGTT was performed using a 75 g oral glucose load (Glukopur; Natura, Hamry u Havlíčkova Brodu, Czech Republic) in 300 ml of water (Small *et al.* 1985). OWT substance consisted of 40 g of flavored whey proteins (Volactive UltraWhey80 (WPC80); DVN, Hoogeveen, the Netherlands) in 300 ml water and OWGT substance consisted of 40 g of flavored whey proteins (Volactive UltraWhey80 (WPC80); DVN, Hoogeveen, the Netherlands) and 72.6 g of glucose (Glukopur; Natura, Hamry u Havlíčkova Brodu, Czech Republic) in 300 ml water. OWGT had an equivalent amount of carbohydrate (75 g) as OGTT. OGTT and IVGTT results were used as reference values for their respective category (oral vs. intravenous).

All the tests were performed regularly at 8 a.m. after an overnight fast (at least 12 h), except water. Before the tests, participants had unrestricted stable diets (at least 150 g of carbohydrate per day); a minimum of 3 days/week of physical activity was encouraged. Participants were non-smokers and agreed to abstain from alcohol and coffee consumption during the study period. All participants underwent all 6 tests.

A peripheral venous catheter connected with a three-way stopcock was inserted into the antecubital vein using aseptic technique. This set-up enabled intravenous access for the purpose of intravenous stimulation tests and for obtaining samples of venous blood; it also ensured

standardized sampling conditions. After stopcock adjustment the same catheter was used for bolus injections and sampling (Gottsäter *et al.* 1992). The catheter was flushed with 0.9% saline after each blood sample and after administration of intravenous C-peptide stimulants (arginine, glucagon, glucose). The participants rested quietly on beds during the tests.

Whey proteins

The study employed whey protein concentrate 80 (Volactive UltraWhey80 (WPC80); DVN, Hoogeveen, the Netherlands) from a sweet cheese whey source containing 81% high-quality nutritional whey proteins, 6% lactose, 6% fat, 4% moisture and 3% minerals. 100 g of WPC80 had an energy value of 1549 kJ or 362 kcal. Average proportion of branched chain amino acids (BCAA) in the whey proteins was (according to the manufacturer) 23%; however our analysis using capillary electrophoresis and the HP3DCE system (Agilent Technologies, Waldbronn, Germany) showed a BCAA percentage of only 17.5%.

Powdered whey proteins were administered as a protein drink flavored with 0.05 g of low-calorie aspartame (NutraSweet: The NutraSweet Company, Chicago, USA), 0.4 g of strawberry aroma AFFRAI 0005 (RJP International, Prague, Czech Republic) and 0.4 g of vanilla extract (RAPS-CZ, Prague, Czech Republic).

Blood samples

Fasting values of plasma glucose and serum C-peptide were taken 10 min after the catheter was inserted and 10 min before administration of secretagogues (time: -10). For the intravenous agents the first venous blood samples were obtained immediately after intravenous stimuli administration (time: 0) (Ward *et al.* 1984). For the oral tests, the first blood sample was taken at 30 minutes; additional samples were taken at 60, 90, 120, 150 and 180 minutes. For the AST and GST, the sampling times were 1 min, 3 min, 5 min, 7 min, 10 min, 20 min and 30 min after administration; for the IVGTT, sampling times were 1 min, 3 min, 5 min, 7 min, 10 min, 30 min, 60

min and 120 min after administration.

Venous blood samples of glucose and C-peptide were collected in a closed blood collection system (S-Monovette; Sarstedt, Nümbrecht, Germany). Glucose was drawn into tubes containing sodium fluoride, while blood for C-peptide testing was drawn into sample tubes without additives.

Blood samples for blood glucose determination were processed immediately after being drawn by the Department of Biochemistry and Pathobiochemistry, Third Faculty of Medicine and University Hospital Královské Vinohrady, Charles University in Prague, Czech Republic. Blood samples for C-peptide determination were immediately cooled on ice to 21 °C and then centrifuged for 15 min at 3000 G (Jouan BR4i, DJB Labcare, Buckinghamshire, England). The samples then were frozen at –80 °C for 32 days until actual laboratory analysis.

Plasma glucose concentrations were determined using the hexokinase method (Passey *et al.* 1977) by a Konelab Glucose analyzer (Thermo Fisher Scientific Oy, Vantaa, Finland). The detection limit was 0.1 mmol/l.

Serum C-peptide concentrations were analyzed using a 2-site immunochemiluminometric assay (Kao *et al.* 1992) by an Immunolite 2000 immunoassay system (Diagnostic Products Corporation, Las Angeles, California, USA). The analytical range of the assay was 0.17–2.32 nmol/l and the lower limit of sensitivity was 0.017 nmol/l. The inter- and intra-assay coefficients of variation (CVs) were less than 6%.

Statistics

To quantify the plasma glucose and the serum C-peptide responses relative to the test substances, the incremental area under the curve (iAUCs) was calculated using the trapezoidal rule and using the initial fasting value of the respective metabolite as the baseline (i.e. blood sample $t = -10$ min). To allow fair comparisons among tests with different durations, iAUCs were further normalized by the test duration (iAUCs/min) according to Greenbaum *et al.* (2008). The statistical comparison between the total C-peptide response and plasma glucose level, relative to the reference

was carried out using the two-sided t-test. Comparisons of C-peptide response variability between stimulation tests were carried out by a likelihood ratio test employing coefficients of variation described by Verrill and Johnson (2007).

All data in the text are presented as means $\pm$ S.E.Ms. All statistical hypotheses were tested at significance level 0.05 using MATLAB software (2011b, Mathworks, Natick, MA, USA).

Results

C-peptide response (Fig. 1)

The average fasting concentration of serum C-peptide was (mean $\pm$ S.E.M.) 0.43 ± 0.02 nmol/l. All oral tests resulted in a similar shape for the C-peptide response curve, with a peak at 30 minutes. In case of OGTT (reference), the C-peptide concentration peaked at 1.61 ± 0.39 nmol/l above baseline. The result of OWT and OWGT, relative to OGTT, was lower by 57% and higher by 63%, respectively. Subsequent concentration decreases reached baseline within 120 – 180 min (OWT and OGTT) and after 180 minute (OWGT). Individual C-peptide concentrations differences were lower ($p < 0.05$) at 60 and 90 min in case of OWT than in case of OGTT. Following OWGT, the C-peptide concentration at 30 min ($p < 0.05$) was the only one, which was higher than OGTT.

IVGTT (reference) resulted in a peak C-peptide concentration of 1.56 ± 0.17 nmol/l above baseline, which occurred 1 min after administration and lasted for 30 min. AST peaked at value lower by 57% (3 min) compared to IVGTT, while GST produce almost (6% lower) the same maximum concentration as IVGTT. Subsequent concentration decreases reached baseline before 20 min (AST), after 30 minute (GST) and after 120 min (IVGTT). Individual C-peptide concentrations differences were lower ($p < 0.05$) during the whole test period in case of AST compared to IVGTT. GST managed to maintain similar concentrations as IVGTT except for sample times 0, 1 and 30 min, where it was lower ($p < 0.05$) than IVGTT.

Glucose response (Fig. 2)

The average fasting plasma glucose concentration (baseline) was (mean $\pm$ S.E.M.) 4.33 ± 0.06 mmol/l. Performing OWGT and OGTT induced similar glucose response shaped curves over 60 min with peak concentrations (from baseline) occurring at 30 min. The values steadily decreased until fasting concentrations were reached (between 90 – 120 min). OGTT showed glucose concentration peak at 2.25 ± 0.24 mmol/l above baseline. Compared to OGTT, the glucose response was 25% lower after OWGT. OWT lowered glucose concentrations below fasting values for the

entire 120 min observation period, the negative peak concentration value occurred at 60 min and was 13% lower than the fasting glucose value. Individual plasma glucose level differences were lower ($p < 0.05$) at 30 and 60 min in case of OWT relative to OGTT result.

The peak plasma glucose concentration, in case of IVGTT, was 16.23 ± 0.92 mmol/l above baseline and occurred immediately after administration. AST and GST showed a peak value that was 97% lower (7 min) and 88% lower (20 min), respectively, relative to IVGTT result. Subsequent concentration decreases reached baseline between 10 and 20 min (AST), after 30 minutes (GST) and between 60-120 min (IVGTT). Individual plasma glucose concentrations were lower ($p < 0.05$) during the whole test period of both AST and GST relative to IVGTT.

iAUC/min - C-peptide response (Fig. 3)

Average C-peptide iAUC/min was (mean $\pm$ S.E.M.) 1.27 ± 0.19 nmol/l and 1.00 ± 0.10 nmol/l for OGTT and IVGTT, respectively. OWT induced an iAUC/min value 64% lower ($p < 0.05$) than OGTT (reference), as opposed to OWGT, that reached an iAUC/min value 36% higher (compared to OGTT (reference)). Furthermore for OWT, the variability of the iAUC/min C-peptide response was lower ($p < 0.05$) than in case of OGTT (reference) as well as in case of OWGT. For the intravenous tests, the iAUC/min was 76% lower ($p < 0.05$) for the AST and 9% higher for the GST compared to IVGTT (reference). When comparing AST and OWT, OWT resulted in an iAUC/min value that was 93% higher than AST.

iAUC/min - glucose response (Fig. 4)

Average glucose iAUC/min was (mean $\pm$ S.E.M.) 1.04 ± 0.13 mmol/l and 4.08 ± 0.22 mmol/l for OGTT and IVGTT, respectively. OWT induced an iAUC/min value 71% lower ($p < 0.05$) than oral OGTT (reference), a similar situation was also true for OWGT, which reached an iAUC/min value that was 32% lower ($p < 0.05$). Relative to the intravenous tests, the iAUC/min was lower for both the AST (94%; $p < 0.05$) and GST (63%; $p < 0.05$) compared to IVGTT (reference).

Test tolerance by participants

OGTT caused intermittent nausea in 25% of participants and 50% of participants experienced tachycardia during IVGTT. GST caused intermittent nausea, tachycardia and tachypnea in 100% of participants. OWT, OWGT and AST produced no observed side effects.

Discussion

The results of this study showed that acute oral administration of whey proteins alone and in combination with glucose in healthy participants leads to an increase in total serum C-peptide concentration. These results were comparable with results of other methods often used for the assessment of normal C-peptide secretory responses to oral and intravenous stimuli. The advantage of using these tests is that they allow accurate characterization of both fasting and stimulated C-peptide concentrations and are thus able to detect (early) a progressive loss of function of pancreatic islet beta cells and the subsequent decrease in the maximal capacity of C-peptide secretion. This is important since changes in stimulated C-peptide levels are more obvious than changes in fasting C-peptide levels. The decrease of C-peptide secretion capacity rapidly leads to development of glucose intolerance, caused by an inadequate increase in insulin secretion in relation to insulin resistance (Ahrén and Larsson 2002). Therefore early discovery of C-peptide secretion impairment could be of an importance for the timely introduction of preventive and therapeutic interventions. Faerch et al. (2009) reported that impairment usually starts several years before it is possible to classify individuals as abnormally hyperglycemic either in the fasting or post-challenge state.

A consistent increase in the total C-peptide concentration (iAUC/min) was perceived in case of OWT, while postprandial plasma glucose level remained almost unchanged in comparison with the other tests (with the exception of the AST). Our data shows, that after administering 40 g of pure whey proteins (i.e. performing OWT), the iAUC/min for postprandial plasma glucose level (0.30 mmol/l) was comparable with the iAUC/min after intravenous administration of 5 g arginine (0.23 mmol/l), which is an important feature of non-hyperglycemic tests that evaluate glucose mechanisms in individuals with glucose tolerance disorders. However, we observed different values for the iAUC/min C-peptide response. Whey proteins alone stimulated C-peptide secretion to a higher iAUC/min (0.46 nmol/l; $p < 0.05$) than arginine (0.24 nmol/l).

Whey proteins alone are therefore able to increase C-peptide secretion more than the stimulated plasma glucose level indicates which is consistent with the findings of Nilsson et al.

(2004). Nilsson et al. (2004) further demonstrated that the major reason for the C-peptide secretion increase seems to be the activation of incretin hormones (glucose-dependent insulinitropic polypeptide (GIP), and glucagon-like peptide-1 (GLP-1)); together with the actual rise of plasma amino acid concentrations (especially BCAA), that could have a direct effect on insulin secretion of beta-cells.

Results of this study also showed that whey proteins administered in combination with glucose (as in case of OWGT) stimulated the highest iAUC/min C-peptide response of all tests. OWGT thus preferentially assessed the total C-peptide response of all tests in healthy individuals, while the iAUC/min of glucose concentrations were significantly reduced compared with results using an equivalent amount of glucose. Decreases in plasma glucose concentrations associated with OWGT are a direct physiological consequence of hyper-insulinemia (Floyd *et al.* 1963), which causes the consumption of glucose in peripheral tissues and reduction of endogenous glycogenolysis, resulting in increased glycogen concentrations to avoid hypoglycemia (Gannon and Nutall 2010).

In summary, we examined the properties of whey proteins as a C-peptide secretagogue using established C-peptide stimulation tests. Whey proteins alone consistently increased total C-peptide concentration, without increasing postprandial plasma glucose levels. Forty grams of whey proteins combined with 72.6 g of glucose induced total C-peptide secretion that was 36% higher than an equivalent amount of glucose, while maintaining significantly lower levels of plasma glucose. Results of this study suggest that both dietology and C-peptide secretion testing might take advantage of the unique property of whey proteins.

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Figures

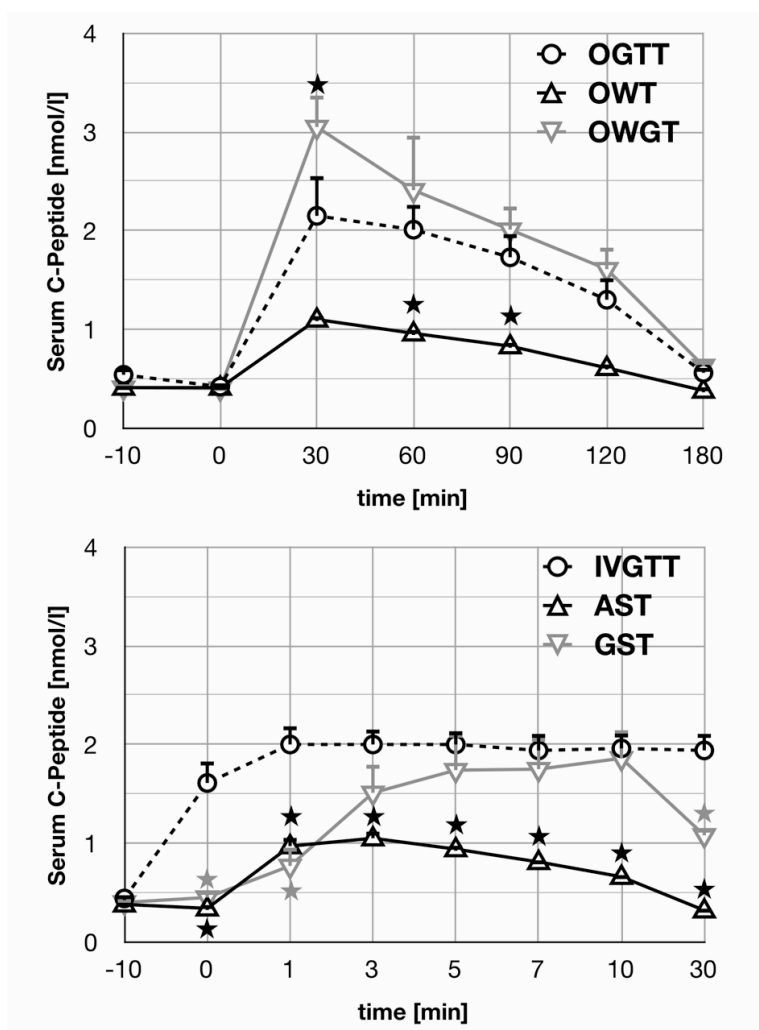


Fig. 1: Mean ($\pm$ S.E.M.) values of serum C-peptide concentration in response to orally administered tests - OGTT (reference), OWT and OWGT - and intravenous tests - IVGTT (reference), AST and GST in healthy subjects ($n = 8$). * indicates values significantly different from the reference ($p < 0.05$).

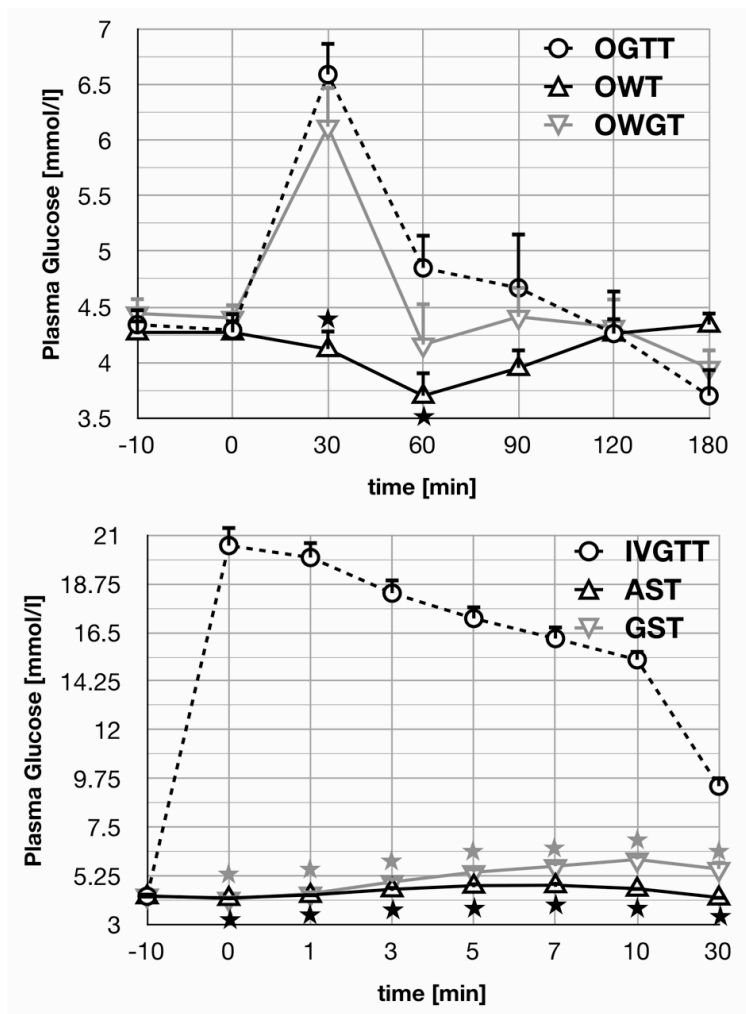


Fig. 2: Mean ($\pm$ S.E.M.) values of plasma glucose concentration in response to orally administered tests - OGTT (reference), OWT and OWGT - and intravenous tests - IVGTT (reference), AST and GST in healthy subjects ($n = 8$). * indicates values significantly different from the reference ($p < 0.05$).

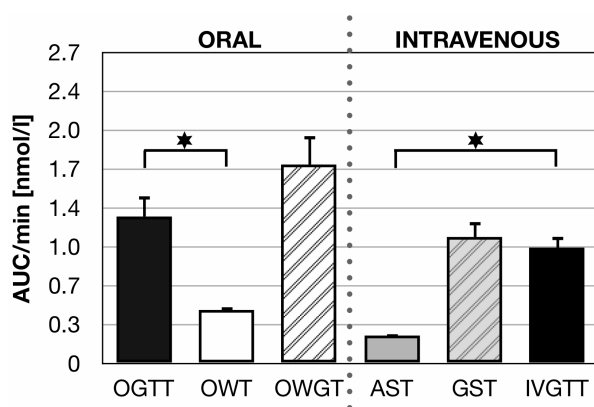


Fig. 3: Mean ($\pm$ S.E.M.) incremental areas under the curve per minute (iAUC/min) for serum C-

peptide concentration for all tests in healthy subjects (n = 8). Values from OGTT (black) were taken as a reference for orally administered tests - for intravenous tests the reference was IVGTT values (black). * indicates values significantly different from reference (p < 0.05).

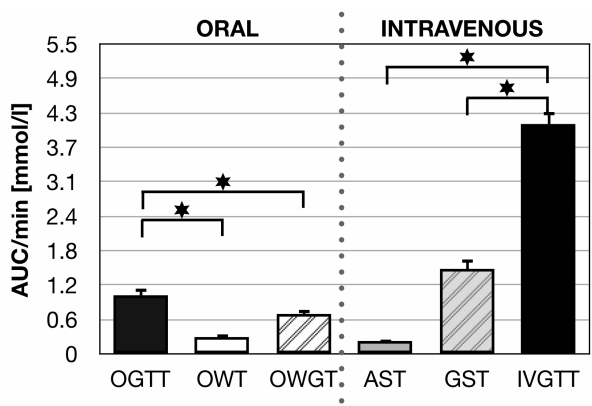


Fig. 4: Mean ($\pm$ S.E.M.) iAUC/min for plasma glucose concentration for all tests in healthy subjects (n = 8). Values from OGTT (black) were taken as a reference for orally administered tests - for intravenous tests, the reference was IVGTT values (black). * indicates values significantly different from the reference (p < 0.05).