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## Reduced insulin clearance contributes to the increased insulin levels after administration of glucagon-like peptide 1 in mice

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**Abstract** *Aims/hypothesis:* Glucagon-like peptide-1 (GLP-1) is known to be a potent stimulator of insulin secretion. However, whether GLP-1 also affects insulin clearance is not known. To explore this, we developed a technique to determine prehepatic insulin secretion in mice, based on deconvolution of plasma C-peptide concentrations. The estimated beta cell secretion was then related to plasma insulin levels to allow determination of clearance rate of endogenously produced insulin. *Materials and methods:* Kinetic parameters of C-peptide were estimated after i.v. injection of human C-peptide (0.8 or 3 nmol/kg) or glucose (1 g/kg), either alone or together with GLP-1 (10 nmol/kg), in anaesthetised NMRI mice. *Results:* C-peptide was distributed in two compartments (distribution volume  $11.4 \pm 0.4$  ml,  $42 \pm 2\%$  of which was in the accessible compartment). Fractional C-peptide clearance was  $8.2 \pm 0.6\%$  of the total distribution volume per minute. GLP-1 markedly enhanced prehepatic insulin secretion; more than 80% of prehepatic secretion occurred during the first minute after injection. Fractional clearance of endogenously released insulin after glucose was  $0.66 \pm 0.11 \text{ min}^{-1}$  and this was reduced to  $0.36 \pm 0.10 \text{ min}^{-1}$  by GLP-1 ( $p=0.04$ ). *Conclusions/interpretation:* It is possible to perform C-peptide deconvolution for estimating prehepatic insulin secretion in mice. GLP-1 reduces the clearance of endogenously released insulin; therefore, it may affect insulin levels by increasing prehepatic insulin secretion and by reducing insulin clearance.

**Keywords** C-peptide kinetics · GLP-1 · Glucagon-like peptide 1 · Insulin clearance · Insulin secretion · IVGTT · Mathematical modelling · Population modelling

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**Abbreviations** GLP-1: glucagon-like peptide 1

### Introduction

After its release from the beta cells, insulin is substantially degraded in the liver, which extracts approximately 50% of the secreted hormone during the first passage [1]. Insulin is also cleared in the kidneys [2]. This results in a short circulating half-life of insulin, which is only approximately 4 min in humans [1, 3]. Hence, net plasma insulin levels are dependent not only on secretion by beta cells but also to a large degree on its clearance. Factors affecting plasma insulin levels may thus achieve their goal through effects not only on beta cell secretion but also on insulin clearance. These processes can be quantified by determining C-peptide concentrations, since this peptide, unlike insulin, is not extracted in the liver [1].

Following meal ingestion, the large increase in plasma insulin is usually assumed to reflect potentiated glucose-stimulated insulin secretion by the gut incretin hormones [4]. The increase in insulin after a meal may, however, also be due to reduced insulin clearance. Thus, previous studies have demonstrated that, despite marked augmentation of the insulin response to oral glucose when compared with glucose, the increase in C-peptide levels was not augmented to the same extent [5–7]. Also, a study in perfused rat liver has shown that perfusion with portal venous effluents obtained from the isolated perfused rat intestine reduces hepatic extraction of insulin after glucose administration [8]. One of the main incretins is glucagon-like peptide 1 (GLP-1), which has a potent stimulatory action on insulin secretion [9]. GLP-1 is also of interest as a novel means for the treatment of type 2 diabetes, which may be achieved by activating GLP-1 receptors (GLP-1 mimetics) or inhibiting the inactivation of GLP-1 by inhibitors of the enzyme dipeptidyl peptidase-4 [10, 11]. Whether GLP-1 augments plasma insulin levels not only by stimulating insulin secretion but also by reducing insulin clearance has not been established.

In the present study we therefore examined this possibility in mice, in which it is known that i.v. administered GLP-1 markedly augments glucose-stimulated insulin secretion [12–14]. This observation derives from the large post-challenge increase in plasma insulin. When estimating a potential influence on clearance of endogenously released insulin, however, a more direct estimation of in vivo beta cell secretion needs to be performed. Since insulin and C-peptide are released equimolarly, a method of quantifying their release is the deconvolution of plasma C-peptide concentrations. The estimated beta cell secretion is then related to plasma insulin levels and the clearance rate can be determined. However, deconvolution of C-peptide requires evidence about C-peptide kinetics, which is currently unavailable in mice. In this study, therefore, C-peptide kinetics was assessed in mice following i.v. administration of C-peptide. Thereafter we determined the kinetics of endogenously released insulin during an IVGTT, and we used these estimates to determine insulin secretion and insulin clearance after i.v. administration of GLP-1.

## Materials and methods

### Animals

Female NMRI mice, weighing  $28.3 \pm 0.9$  g (range 20.6–40.1 g), were purchased from Taconic (Skensved, Denmark). The animals were maintained in a temperature-controlled room ( $22^\circ\text{C}$ ) on a light–dark cycle of 12 h each. Mice were fed a standard pellet diet (Lactamin, Stockholm, Sweden) and tap water ad libitum. The study was approved by the local animal ethics committee.

### Experiments

Mice were anaesthetised after a 3 h fast during the late morning hours, with an i. p. injection of midazolam (Dormicum; Hoffman-La Roche, Basel, Switzerland; 0.14 mg/mouse) and a combination of fluanisone (0.9 mg/mouse) and fentanyl (0.02 mg/mouse; Hypnorm; Janssen, Beerse, Belgium). After 30 min, a blood sample (75  $\mu\text{l}$ ) was taken from the retrobulbar, intraorbital, capillary plexus in a 100  $\mu\text{l}$  pipette which had been prerinsed in heparin solution (100 U/ml in 0.9% NaCl; Lövens, Ballerud, Denmark). Thereafter, for the C-peptide kinetics study, human C-peptide (dissolved in saline; Sigma, St Louis, MO, USA) was given i.v. over 3 s in a tail vein at the doses of 0.8 nmol/kg in eight animals and 3.0 nmol/kg in 16 mice. For the experiments on the effects of incretins on insulin dynamics, either D-glucose alone (1 g/kg,  $n=35$ ; British Drug Houses, Poole, UK) or D-glucose together with synthetic GLP-1 (10 nmol/kg,  $n=12$ ; Peninsula Laboratories Europe, St Helens, UK) was injected in a tail vein. Previous studies have demonstrated that these doses of GLP-1 are those with the maximal effect in augmenting glucose-stimulated insulin secretion in mice [12–14]. To assess basal insulin variability in the absence of any stim-

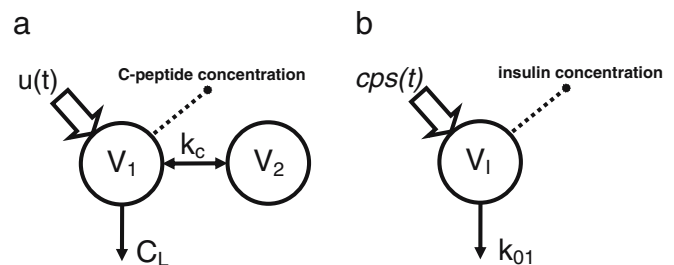
ulus, saline was given to 13 control mice. An additional three series of experiments were performed to evaluate possible effects of increasing insulin levels on insulin clearance. D-Glucose (1 g/kg) was injected i.v. in normal mice together with human insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) at 1.5 ( $n=14$ ), 3.0 ( $n=18$ ) or 6.0 nmol/kg ( $n=10$ ). Experiments with the two highest doses of insulin have been described in a previous study [13]. In all experiments, the volume load was 10  $\mu\text{l/g}$  body weight. Blood samples (75  $\mu\text{l}$  each) were taken at 1, 5, 10, 20, 30 and 50 min. Plasma was immediately separated and stored at  $-20^\circ\text{C}$  until analysis.

### Assays

Insulin concentration was determined by a double-antibody radioimmunoassay using guinea-pig anti-rat insulin antibodies,  $^{125}\text{I}$ -labelled human insulin and, as standard, rat insulin (Linco Research, St Charles, MO, USA). C-peptide was determined as either human C-peptide (in the experimental series in which human C-peptide was administered) or as mouse C-peptide (in the series in which beta cell secretion was stimulated). Human C-peptide was determined by a double-antibody radioimmunoassay using guinea-pig anti-human C-peptide antibody, human C-peptide standard and  $^{125}\text{I}$ -human C-peptide as tracer (Linco). Mouse C-peptide was determined by a double-antibody radioimmunoassay using guinea-pig anti-rat C-peptide antibody (cross-reacts to 100% with mouse C-peptide), rat C-peptide standard and  $^{125}\text{I}$ -rat C-peptide as tracer (Linco). Glucose was measured by the glucose oxidase technique.

### Glucose elimination

The rate of glucose disappearance after bolus glucose administration was evaluated as the tolerance index  $K_G$  ( $\% \text{ min}^{-1}$ ), calculated as the slope of the regression against time of the logarithmically transformed glucose concentration values from 5–20 min [14].



**Fig. 1** Two-compartment model of C-peptide kinetics (a) and one-compartment model of insulin kinetics (b). See [Materials and methods section](#) for meanings of symbols and units

## Assessment of C-peptide kinetics

The kinetics of C-peptide after its injection was described by a two-compartment model, in which the two pools represent intra- and extravascular space with irreversible loss occurring from the former. The model (Fig. 1a) is described by:

$$\frac{dx_1(t)}{dt} = -C_L \frac{x_1(t)}{V_1} + k_c \left[ \frac{x_2(t)}{V_2} - \frac{x_1(t)}{V_1} \right] + u(t) \quad (1)$$

$$\frac{dx_2(t)}{dt} = k_c \left[ \frac{x_1(t)}{V_1} - \frac{x_2(t)}{V_2} \right] \quad (2)$$

$$y(t) = \frac{x_1(t)}{V_1} \quad (3)$$

where  $x_1(t)$  and  $x_2(t)$  (pmol) are the compartmental masses of C-peptide with initial conditions  $x_1(0)=\text{dose}$ ,  $x_2(0)=0$ . The dose is either 0.8 or 3.0 nmol/kg, according to the experiment. Model parameter  $k_c$  (l/min) represents the mass transfer rate between compartments,  $C_L$  (l/min) is the C-peptide clearance rate,  $V_1$  and  $V_2$  (l) are the distribution spaces of the accessible and remote compartments, respectively. The total distribution volume is defined as  $V_{\text{tot}}=V_1+V_2$ . Variable  $y(t)$  represents the actual C-peptide plasma concentration measurements (pmol/l), while  $u(t)$  is the C-peptide input rate (pmol/min).  $V_{\text{tot}}$  was relatively stable and constant within mice (see Results section); therefore we can express  $V_1$ ,  $V_2$ ,  $C_L$ , and  $k_c$  as fractions of  $V_{\text{tot}}$ ; i.e.  $V_1=f_{\text{cv}}V_{\text{tot}}$  and  $V_2=(1-f_{\text{cv}})V_{\text{tot}}$ , where  $0<f_{\text{cv}}<1$  is the volume fraction of the accessible compartment. Similarly,  $C_L=f_{\text{cl}}V_{\text{tot}}$  and  $k_c=f_{\text{kc}}V_{\text{tot}}$ , where  $f_{\text{cl}}$  and  $f_{\text{kc}}$  (per min) are the fractions of the total volume irreversibly cleared from the accessible compartment or exchanged between the two compartments in one time unit, respectively. In summary, C-peptide kinetics is characterised by the parameters  $f_{\text{cl}}$ ,  $V_{\text{tot}}$ ,  $f_{\text{kc}}$  and  $f_{\text{cv}}$ , which are also non-linearly transformed to guarantee the fulfilment of constraints on parameter estimates and to reduce possible problems due to large inter-individual variability [15]. Individual parameter estimates for each mouse were obtained by the non-linear weighted least squares fitting based on the simultaneous analysis of all available data sets (population modelling approach) [16]. In particular, the mixed-effects model technique has been used [17], because it explicitly accounts for intra- and interindividual variation in parameters and is a more dependable approach than the traditional two-stage method of obtaining first individual parameter estimates and then analysing their relationship among animals.

## Assessment of insulin secretion and clearance during IVGTT

To characterise the average response and the interindividual variability in insulin secretion in mice during IVGTT, and to assess the effects of administration of GLP-1, beta cell insulin secretion was reconstructed by deconvolution from the measured plasma C-peptide concentration profiles. Deconvolution consists in estimating the unknown insulin secretion profile from the measured plasma concentrations, given the dynamic input–output relationship described by the kinetic parameters of C-peptide, which were estimated after C-peptide bolus experiments. This method is commonly applied to C-peptide concentration measurements instead of insulin concentration because C-peptide has no first-pass elimination through the liver and its kinetics can be conveniently assessed by exogenous bolus injection of biosynthetic C-peptide. Deconvolution is necessary in order to properly take into account the memory effect produced by the relatively slow kinetics of C-peptide, and to reconstruct average prehepatic insulin secretion during specific time intervals. In this study, we used successfully in mice the widely applied two-compartment C-peptide kinetics modelling approach proposed in humans [18], having preliminarily assessed the C-peptide kinetic parameter values proper of this species. The deconvolution of C-peptide data, together with the mixed-effects modelling analysis, provided estimates of prehepatic insulin secretion,  $\text{cps}(t)$  (pmol/min), for each mouse.

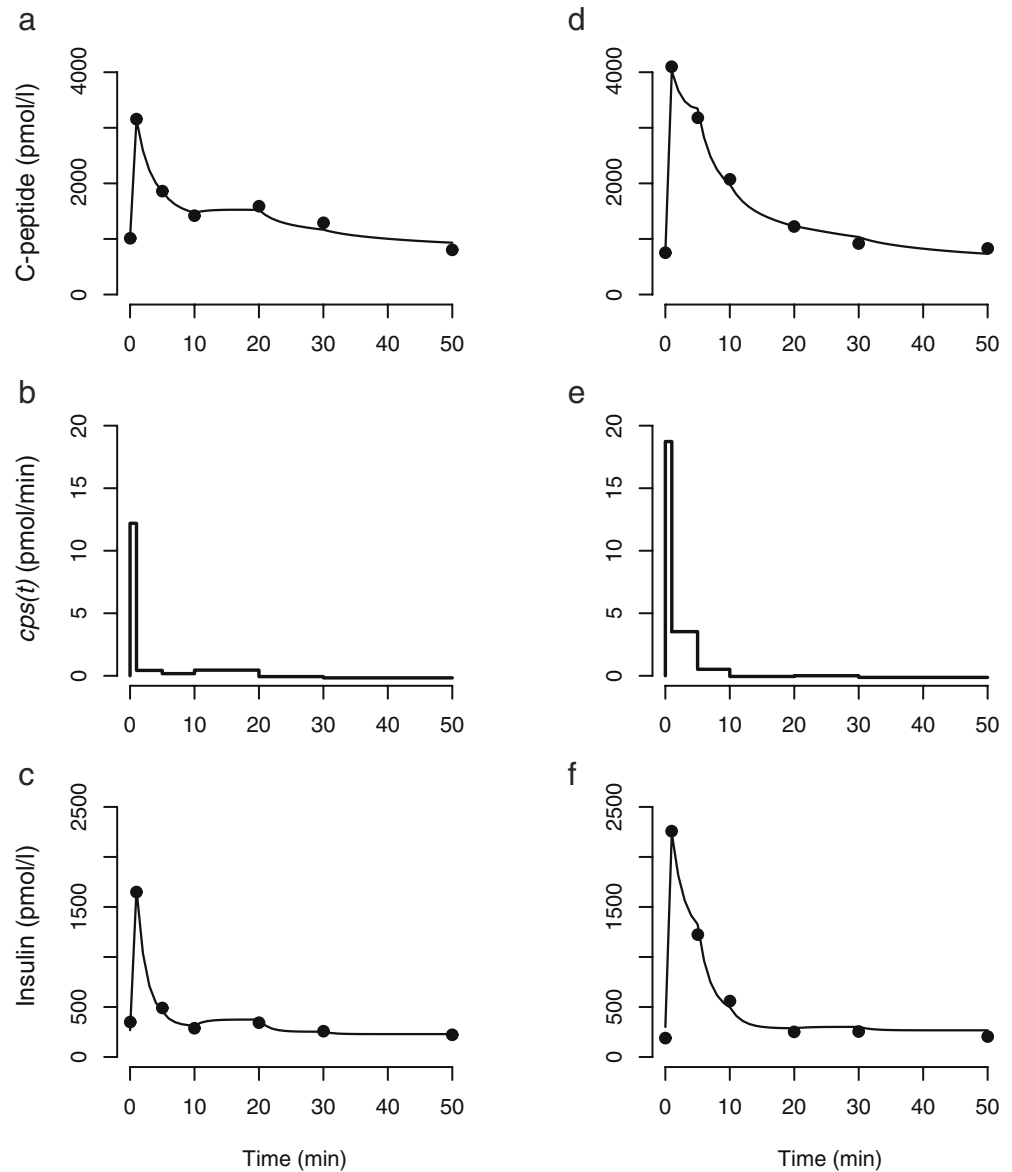
As in other studies on insulin dynamics during glucose tests (for example, [19]) a one-compartment model (volume  $V_1$ , ml) was used to describe insulin kinetics (Fig. 1b). Insulin secretion,  $\text{cps}(t)$ , determined for each mouse by deconvolution of its own C-peptide data, was used as forcing input. By fitting peripheral insulin concentration data, the value of  $k_{01}$ , which represents the fractional insulin clearance (per min), was estimated in every animal, again using non-linear mixed-effects modelling [16, 17]. In order to clarify the procedure with an example, in Fig. 2 we show two cases of C-peptide deconvolution and assessment of insulin kinetics.

Fractional insulin clearance and distribution volume following exogenous insulin administration were calculated by fitting the suprabasal insulin concentration data with the same one-compartment model (Fig. 1b), in which  $\text{cps}(t)$  was replaced with the bolus insulin dose. We assumed that endogenous insulin secretion is negligible compared to the exogenous component in these experiments.

## Statistical analysis

Data and results are reported as means $\pm$ SEM. Statistical comparisons between groups were performed by ANOVA with Bonferroni post hoc analysis. A  $p$  value less than 0.05 was considered significant.

**Fig. 2** Examples of deconvolution of C-peptide data and assessment of insulin kinetics following a bolus injection of glucose alone (**a, b, c**) or glucose plus GLP-1 (**d, e, f**). C-peptide (**a, d**) measurements (closed circles) and model predictions (continuous line) based on  $cps(t)$  (shown in **b, e**) and on the two-compartment model of C-peptide kinetics (see Fig. 1a). Insulin secretion profile,  $cps(t)$  (**b, e**), reconstructed by non-linear mixed-effects estimation. Insulin (**c, f**) measurements (closed circles) and best model fit (continuous line) in the one-compartment model (see Fig. 1b), obtained by optimally adjusting the insulin distribution volume and fractional clearance parameter, using the individually reconstructed secretion profile,  $cps(t)$ , as forcing input



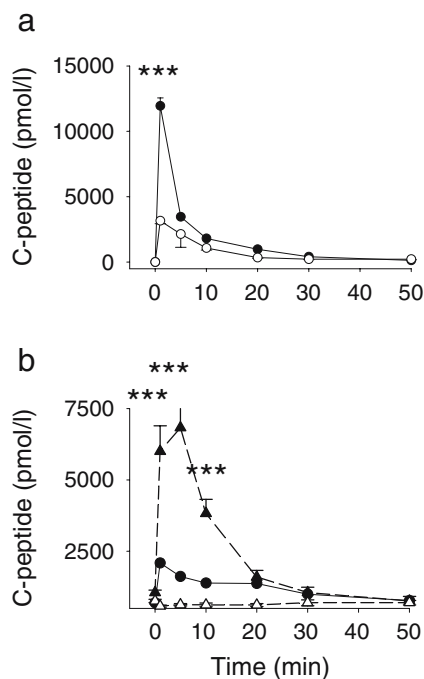
## Results

### C-peptide kinetics

Figure 3a shows the C-peptide levels after i.v. administration of human C-peptide at the two doses. After the peak at  $t=1$  min, plasma C-peptide was eliminated by a first-order elimination kinetic. The  $V_{tot}$  of C-peptide was  $11.4 \pm 0.4$  ml (range 9.0–14.6 ml; 38% of body weight) and exhibited little variability among animals (between-animal coefficient of variation was 24%) with no significant effect of body weight. Of  $V_{tot}$ ,  $42 \pm 2\%$  was in the accessible compartment. Fractional C-peptide clearance was  $8.2 \pm 0.6\%$  of  $V_{tot}$  per min. The average fractional mass transfer rate coefficient between the accessible and remote compartments ( $k_c$ ) was  $7.7 \pm 0.4\%$  of  $V_{tot}$  per min. There was no significant effect of body weight on C-peptide kinetics.

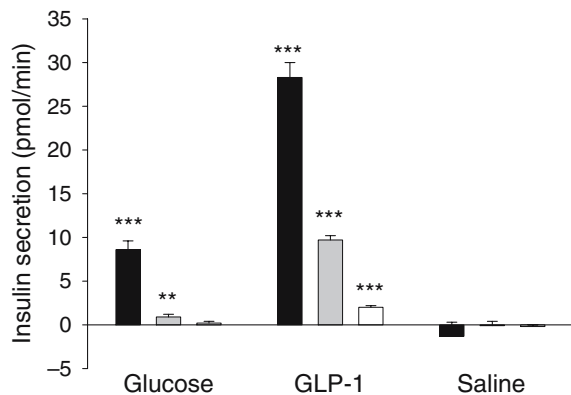
### Insulin secretory rates

C-peptide and glucose were determined following the i.v. injection of mice with either glucose alone or glucose together with GLP-1 (Fig. 3b). Glucose alone increased plasma C-peptide levels with the peak level obtained at 1 min and the addition of GLP-1 markedly augmented C-peptide levels. This was associated with increased glucose elimination rate ( $K_G = 3.73 \pm 0.22\% \text{ min}^{-1}$  with glucose alone vs  $6.77 \pm 1.06$  with GLP-1,  $p=0.0003$ ). Prehepatic beta cell insulin secretion was obtained in the intervals between blood sampling by deconvolution from the measured plasma C-peptide profiles and C-peptide kinetic parameters estimated in the ad hoc experiments. Figure 4 shows the average suprabasal insulin secretion in the three groups of animals in the first three time intervals. Glucose alone and glucose plus GLP-1 significantly increased insulin secretion above basal in the intervals 0–1 and 1–5 min, whereas only glucose plus GLP-1 also increased



**Fig. 3** **a** Plasma human C-peptide levels after i.v. administration of 0.8 nmol/kg (open circles) or 3 nmol/kg (closed circles) of human C-peptide at time  $t=0$  to anaesthetised NMRI mice ( $n=12$  in each group). **b** Plasma mouse C-peptide levels after i.v. administration of glucose (1 g/kg) alone ( $n=35$ ; closed circles) or together with GLP-1 (10 nmol/kg,  $n=12$ ; closed triangles) or saline ( $n=13$ ; open triangles) at time  $t=0$  to anaesthetised NMRI mice. Data are shown as means $\pm$ SEM. \*\*\* $p<0.001$  (probability level of random difference from baseline between groups given glucose alone vs. glucose+GLP-1)

insulin secretion in the interval 5–10 min. For all groups, however, most insulin was released during the first minute. No significant insulin secretion above baseline was evident in the intervals 10–20, 20–30 and 30–50 min (not shown).

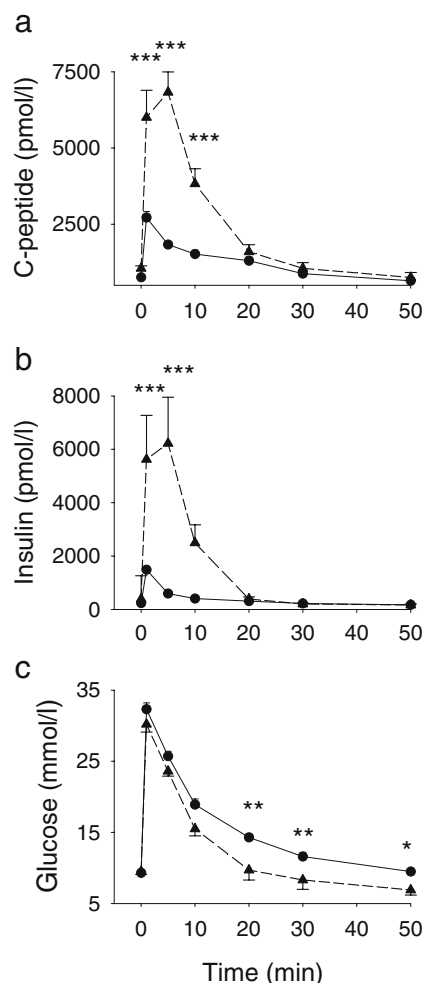


**Fig. 4** Integrated insulin secretion rate as determined from C-peptide deconvolution during three intervals: minutes 0–1 (black columns), 1–5 (grey columns) and 5–10 (white columns) after i.v. injection of glucose alone ( $n=35$ ), glucose plus GLP-1 ( $n=12$ ) or saline ( $n=13$ ) in anaesthetised NMRI mice. Data are shown as means $\pm$ SEM. \*\* $p<0.01$ ; \*\*\* $p<0.001$  (probability level of random difference from baseline)

After saline injection, insulin secretion was not significantly affected in any of the intervals.

#### Insulin clearance

In one series of experiments, insulin was also determined with C-peptide in the same mouse after administration of glucose with or without GLP-1 ( $n=12$  in each group). Figure 5 shows the concentrations measured in this series. GLP-1 also markedly enhanced insulin levels, again associated with increased glucose elimination ( $K_G$  of glucose alone experiment was in this case  $3.77\pm 0.19\%$   $\text{min}^{-1}$ ;  $p<0.02$  when compared with experiments with GLP-1). By analysing data with the insulin kinetics model, insulin distribution volume averaged 5.9 ml with no significant difference between the groups; fractional insulin clearance during the IVGTT when glucose was administered alone was  $0.66\pm 0.11 \text{ min}^{-1}$ . It was  $0.36\pm 0.10 \text{ min}^{-1}$  with GLP-1,



**Fig. 5** Plasma levels of C-peptide (a), insulin (b) and glucose (c) after i.v. administration of glucose (1 g/kg) alone ( $n=12$ ; circles) or together with GLP-1 (10 nmol/kg,  $n=12$ ; triangles) at time  $t=0$  in anaesthetised NMRI mice. Data are shown as means $\pm$ SEM. \* $p<0.05$ ; \*\* $p<0.01$ ; \*\*\* $p<0.001$  (probability level of random difference from baseline between the two groups)

**Table 1** Dose–response analysis of insulin kinetics after bolus injection of human insulin at different doses

Insulin dose (U/kg)	Number of mice	AUC <sub>insulin</sub> (nmol/l in 50 min)	Insulin clearance rate (l/min)
0.25	14	31.5±2.8	0.276±0.006
0.50	18	47.6±2.8	0.280±0.009
1.00	10	97.4±7.1	0.236±0.007

All AUC<sub>insulin</sub> values were significantly different ( $p < 0.001$ ). There were no significant differences in the clearance rates

which is a 46% reduction when compared with glucose alone ( $p = 0.04$ ). A model-independent index of insulin clearance vs. secretion was calculated as the ratio of the area under the concentration curves of insulin and C-peptide. These ratios were  $0.312 \pm 0.019$  with glucose only and  $0.589 \pm 0.090$  with GLP-1 ( $p = 0.006$ ) and support the model-based results of a slower insulin elimination rate with GLP-1.

Clearance of exogenously administered insulin at the various doses is reported in Table 1. Despite the increased levels of insulin (area under the insulin curve, AUC<sub>insulin</sub>), the clearance rate was not different for any dose. A slight reduction (borderline insignificant,  $p = 0.052$ ) was observed at the highest dose, which yielded unphysiological, high levels of insulin, far greater than those obtained with GLP-1. The insulin distribution volume averaged  $29 \pm 1\% \text{ kg}^{-1}$ , which corresponds to 8.4 ml in a 29 g mouse, with no significant differences between the groups.

## Discussion

This study examined whether GLP-1 affects the clearance of endogenously released insulin besides its well-described stimulatory action on insulin secretion in mice. To answer this, prehepatic insulin secretory rates needed to be quantified, which has never been performed previously in mice. For this purpose, we applied the C-peptide deconvolution technique, which has been used widely in humans [18]. Since it requires knowledge of C-peptide kinetics, we first evaluated C-peptide dynamics in mice after injection of human C-peptide. We found that the variations in the parameters of C-peptide kinetics within our animal population were small: for example,  $V_{\text{tot}}$  changed little and body weight had no influence in spite of the large variation among animals. Another aspect that made us confident about the correctness of our approach was that C-peptide kinetics, estimated in mice, was comparable to that previously reported in humans [20] and dogs [21]; therefore, it can be considered a valid basis for reconstructing prehepatic insulin secretion by deconvolution of C-peptide measurements.

The vast majority of insulin was released during the first minute after i.v. administration in all groups, regardless of

the stimulus to the beta cell. In fact, more than 80% was released during the first minute. Release continued in the interval 5–10 min only in the group given GLP-1 in addition to glucose. By measuring insulin and C-peptide in the same animal, it was possible to calculate the clearance rate of endogenously released insulin. This was significantly reduced with GLP-1 by 46% compared with glucose alone. Additional experiments in which increasing doses of insulin were given demonstrated that clearance of exogenously administered human insulin in mice does not change significantly when insulin levels during an i.v. test vary over a broad range from low to unphysiologically high values. Thus, despite possible changes in insulin clearance due either to different appearance routes (endogenous secretion vs. i.v. injection) or to species dissimilarity of insulin, the reduction we observed in insulin clearance with GLP-1 is unlikely to have been due to the different insulin concentrations. Our study therefore shows that, besides marked stimulation of the insulin secretory rate, a reduction in insulin clearance is observed when GLP-1 is administered. Since the distribution volume of insulin was not altered by the treatment, we conclude that the effect of GLP-1 on insulin clearance is a result of action on the distribution or metabolism of insulin or permeability to insulin. It is important to acknowledge this effect when explaining the marked increase in plasma insulin seen after administration of GLP-1.

Oral glucose and meal ingestion are known to reduce insulin clearance, probably mainly through reduced extraction of insulin in the liver [5–7]. The signal responsible for this action has never been established: GLP-1 is an obvious candidate in view of its importance as an incretin factor that is released after intake of a meal in order to augment the insulin response to food ingestion [4]. Establishing whether GLP-1 affects the clearance of endogenously released insulin is also of interest from a therapeutic point of view, since GLP-1-based therapy has been explored as a novel therapy for type 2 diabetes [10, 11]. Based on our findings, we therefore suggest that, besides stimulating insulin secretion, GLP-1 also augments plasma insulin levels by inhibiting insulin clearance.

In conclusion, we have developed methods for reconstructing insulin secretory rates by using the deconvolution of C-peptide kinetics in mice, and we have used this technique in association with measures of insulin kinetics to calculate insulin clearance in mice. Using these methods, we have demonstrated that GLP-1 reduces insulin clearance. This suggests a novel mechanism to explain the increase in plasma insulin levels obtained by GLP-1, in association with its potent stimulatory action on islet beta cells.

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