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Prolonged increase of plasma non-esterified fatty acids fully abolishes the stimulatory effect of 24 hours of moderate hyperglycaemia on insulin sensitivity and pancreatic beta-cell function in obese men

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Abstract

Aims/hypothesis. A prolonged increase of plasma NEFA impairs acute glucose-stimulated insulin secretion (GSIS) in vitro and in vivo. Our study therefore examined the combined effect of increased plasma NEFA and glucose on GSIS in humans.
Methods. We examined GSIS on four occasions in eight obese men during a 10 mmol/l hyperglycaemic clamp and after a 24-h infusion of (i) normal saline, (ii) intralipid and heparin to raise plasma NEFA about two-fold above basal, (iii) 20% dextrose to raise plasma glucose to about 7.5 mmol/l and (iv) intralipid and heparin combined with 20% dextrose to raise plasma NEFA and glucose.
Results. In study (iii) insulin sensitivity was about 20% greater than in study (i) and the disposition index was about 50% higher. Insulin sensitivity tended to be lower in study (ii) whereas the disposition index was lower than in study (i), confirming previous observations. The combination of increased plasma NEFA and glucose (study iv) reduced insulin sensitivity in comparison with study (i) and completely abolished the increase in insulin sensitivity and disposition index seen in study (iii), but did not reduce the latter to a lower value than that in the saline control study (study i).
Conclusions/interpretation. We showed that a prolonged increase of plasma NEFA completely abolishes the stimulatory effect of moderate hyperglycaemia on insulin sensitivity and beta-cell function in obese humans. This suggests that previous observations, showing that a prolonged increase of plasma NEFA impairs pancreatic beta-cell function, also apply to the hyperglycaemic state. [Diabetologia (2004) 47:204–213]

Keywords Pancreatic beta cell · Disposition index · NEFA

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Abbreviations: GSIS, glucose-stimulated insulin secretion · SAL, saline control study · IH, intralipid heparin study · GLU, hyperglycaemia study · GLU-IH, hyperglycaemia, intralipid and heparin study · ISR, insulin secretion rate · S_I, insulin sensitivity index · DI, disposition index · ANCOVA, analysis of covariance

Type 2 diabetes is a polygenic disease involving defective insulin secretion and peripheral insulin resistance [1, 2]. An early characteristic of the defect in insulin secretion is selective impairment of glucose-stimulated insulin secretion (GSIS) [3, 4]. Insulin resistance in insulin target tissues generally does not cause diabetes without concomitant beta-cell dysfunction [5, 6].

The defect in beta-cell function is progressive. It has been proposed that glucose toxicity is an important cause of the deterioration of pancreatic beta-cell function in patients with Type 2 diabetes. The impairment of GSIS induced by chronic hyperglycaemia has been studied in animal models and in humans [7]. Rats made hyperglycaemic with intravenous glucose infusions [8] or by reducing pancreatic beta-cell mass

[9, 10] have selective beta-cell glucose hypo-responsiveness. Reversal of hyperglycaemia with phlorizin [11, 12] or insulin infusion [13, 14] restores glucose-induced insulin secretion. In patients with Type 2 diabetes, reduction of mean fasting glucose concentrations to the normal range by infusion of insulin improves glucose-induced first-phase insulin responses [15]. Thus, there is evidence that a cause and effect relationship exists between chronic hyperglycaemia and beta-cell hypo-responsiveness, although pancreatic glucose toxicity in response to a prolonged intravenous glucose infusion has only previously been documented in healthy humans when plasma glucose concentrations between 9 and 12 mmol/l were maintained for 68 hours [16]. In contrast, hyperglycaemia of a lesser degree or duration actually enhanced GSIS [17, 18, 19].

Over the past decade, several investigators have focused on the possible role of plasma NEFA, which are often increased in states of insulin resistance, in selectively desensitising the beta cell to glucose [20]. Epidemiological studies have shown that increased plasma NEFA concentrations are a risk marker for the long-term development of glucose intolerance and progression to Type 2 diabetes [21, 22]. In vitro studies in rats and in human islets have generally shown that prolonged exposure (>24 h) to fatty acids impairs GSIS [23, 24]. Results from in vivo human studies have been somewhat controversial, since some, but not all have shown that prolonged increases of NEFA impair GSIS [23].

Since glucotoxicity and lipotoxicity exert adverse effects on beta-cell function and contribute to the progressive deterioration of glucose homeostasis in Type 2 diabetes, the concept of 'glucolipotoxity' has recently been proposed [25, 26]. This concept tries to explain the synergistic effects of a combined chronic increase of glucose and NEFA on the impairment of pancreatic beta-cell function, and some investigators have proposed that glucotoxicity is a prerequisite for lipotoxicity [25, 26]. The primary purpose of our study, therefore, was to investigate the effect on GSIS in humans of a modest prolonged (24-h) increase of NEFA and glucose, alone or together, and to compare it to the effects of a saline control.

Subjects and methods

Subjects. Eight obese but otherwise healthy men participated in the study. Only one had a positive family history of Type 2 diabetes in first-degree relatives (mother), and he had normal glucose tolerance. Diabetes was ruled out by assessing fasting plasma glucose concentrations at every study visit (i.e. on four separate occasions) and by doing a standard 2-h OGTT on a separate occasion. Two patients had impaired glucose tolerance according to the 2-h 75 g OGTT. One of these two had a fasting plasma glucose concentration of 5.7 mmol/l with a 2-h post-challenge concentration of 10.6 mmol/l. The second had a fasting glucose concentration of 5 mmol/l with a 2-h post-challenge value of 9.4 mmol/l. All participants underwent all four studies in random order, with each study carried out 6 to 8 weeks apart. The mean age was 48±3 years (means ± SEM) and BMI was 33±1 kg/m^2 (range 29–38 kg/m^2). Body weight remained stable in all individuals for the duration of the study. We studied obese persons because we had previously shown that they are more susceptible than lean persons to the impairment of GSIS induced by increased NEFA [27]. None of the subjects was taking any medication or had any known systemic illness. Informed, written consent was obtained from all participants in accordance with the guidelines of the Human Subjects Review Committee of the University Health Network, University of Toronto. Studies were carried out in accordance with the Declaration of Helsinki as revised in 2000 (http://www.wma.net/e/ethicsunit/pdf/intl_response_helsinki.pdf).

Experimental protocols

Subjects were admitted to hospital on day 1 in the Metabolic Investigation Unit (MIU) of the Toronto General Hospital for each of the four studies [referred to below as SAL (saline control study), IH (intralipid heparin study), GLU (hyperglycaemia study) and GLU-IH (hyperglycaemia, intralipid and heparin study)]. During this time they received intravenous infusions for 24 h, followed on day 2 by testing of GSIS as outlined below. Subjects were assigned to each of the four studies in random order, and each study was carried out 6 to 8 weeks apart. During each admission for intravenous infusions subjects received an isocaloric diet with 50% energy derived from carbohydrate, 30% fat, and 20% protein. Prior to testing of GSIS on day 2, they fasted overnight for 12 h. Blood samples were taken at 08.00, 12:00 and 16:00 hours on day 1 (before meals) and at 08.00 hours on day 2 of the study.

Saline control study. After a 12-h overnight fast, an intravenous catheter was inserted in each forearm, one for infusion and one for blood sampling. The arm with the sampling catheter was kept in a heating blanket (~65°C) to arterialise venous blood. After a fasting baseline blood sample was drawn, normal saline was infused at a rate of 50 ml/h, starting at 08.00 hours, and continued for 24 hours before and during the subsequent intravenous glucose infusion.

At 08.00 hours on day 2, after a 12-h overnight fast and while the normal saline infusion was continued, participants underwent a hyperglycaemic clamp according to a modified De Fronzo method [28], to test GSIS. Plasma glucose concentrations were maintained at 10 mmol/l for two hours. Samples were drawn to measure blood glucose at 5-min intervals during the clamp. During the last 30 min of the clamp, samples for plasma insulin and C-peptide concentrations were drawn at 5-min intervals, while samples for plasma NEFA and triglyceride concentrations were taken at 10-min intervals. Samples for triglyceride and NEFA analysis were collected into chilled EDTA tubes on ice. The EDTA tubes contained the lipase inhibitor tetrahydrolipstatin (30 µg/ml blood) (Hoffman-La Roche, Mississauga, Ont., Canada), to prevent ongoing lipolysis of the samples in the test tubes.

Intralipid heparin study. This study protocol was identical to the SAL, except that, instead of normal saline, subjects received a continuous infusion of intralipid (20% solution at 40 ml/h; Baxter, Toronto, Ont, Canada) and heparin (Organon, Toronto, Ont., Canada) (250 U/h) in order to raise plasma NEFA concentrations for 24 h before and during the assess-

ment of pancreatic insulin secretion as described previously [29]. Intralipid is a sterile fat emulsion (20% soybean oil, 1.2% egg phospholipids and 2.25% glycerin in water) containing predominantly polyunsaturated fatty acids.

Hyperglycaemia study. This was identical to the other study protocols, except that, instead of normal saline or intralipid and heparin, participants received a continuous infusion of 20% dextrose solution at 150 ml/h to moderately raise blood glucose concentrations for 24 h before and during the assessment of GSIS. Plasma electrolytes were measured every eight hours, and potassium was added to the glucose infusion as needed to maintain normal plasma concentrations. The 24-h glucose infusion period was not in the strict sense a hyperglycaemic clamp, as patients were allowed to eat three meals a day and therefore had postprandial blood glucose excursions. In addition, the glucose infusion was given at a fixed rate in all participants and was not adjusted to achieve a predetermined concentration of hyperglycaemia. The aim was simply to create a condition of increased blood glucose concentrations during this time.

Hyperglycaemia, intralipid and heparin study. In this last protocol, subjects received an intravenous infusion of intralipid and heparin (as in the IH), as well as 20% dextrose solution (as in the GLU) for 24 h prior to and during the testing of pancreatic beta-cell function on day 2.

Laboratory methods

Glucose was analysed enzymatically at the bedside using a Beckman Glucose Analyzer II (Beckman Instruments, Fullerton, Calif., USA). Insulin was measured by radioimmunoassay using a double antibody separation method (kit supplied by Pharmacia Diagnostic, Uppsala, Sweden) with an intra- and inter-assay coefficient of variation of 5.8% and 11.2% respectively. C-peptide was measured by a double C-peptide radioimmunoassay (kit supplied by Diagnostic Products, Los Angeles, Calif., USA). The intra- and inter-assay coefficients of variation for this assay are 4.1% and 15.1% respectively. The samples for all studies in the same patient were assayed simultaneously with the same kit for insulin and C-peptide. NEFA was measured using a colorimetric method (kit supplied by Wako Industrials, Osaka, Japan). Triglycerides were measured as esterified glycerol, using an enzymatic colorimetric kit (Boehringer Mannheim Diagnostica, Mannheim, Germany). Free glycerol was eliminated from the sample in a preliminary reaction, followed by enzymatic hydrolysis of triglyceride with subsequent colorimetric measurement of the liberated glycerol.

Calculations

Estimation of insulin secretion rate. The pancreatic insulin secretion rate (ISR) was calculated from peripheral plasma C-peptide concentrations by deconvolution using a two-compartment mathematical model with standard parameters for C-peptide distribution and metabolism as described previously [30]. Although it would have been ideal to measure individual C-peptide kinetic parameters, C-peptide is unfortunately no longer commercially available for in vivo use in humans. The use of standard parameters for C-peptide clearance and distribution results in insulin secretion rates which differ in each subject by only 10 to 12% from those obtained with individual parameters, and there is no systematic over- or underestimation of insulin secretion [30]. Acute increases of NEFA concentrations do not affect the clearance of C-peptide in humans [31].

Insulin clearance. Clearance of endogenous insulin was calculated by dividing the mean ISR by the mean serum insulin during the last 30 min of the hyperglycaemic clamp [32].

Insulin sensitivity index and disposition index. The insulin sensitivity index (S_I) was calculated only for the last 30 min of the hyperglycaemic clamp studies according to the following formula:

$$S_{Iclamp} = Ginf/(Ins \times Glu)$$

where Ginf is the glucose infusion rate, Ins the plasma insulin concentration and Glu the plasma glucose concentration during the last 30 min of the clamp. S_I is reported in units of dl/kg·min per μU/ml. To calculate S_I we used the absolute values of Ginf, insulin and glucose, rather than their differences from basal, as their basal values were close to the clamp values in some patients in the GLU-IH study and the differences would therefore have been close to 0, increasing the variability of the calculated S_I. If the differences from basal had been used to do the S_I calculations, this would not have changed our conclusions. No correction was made for urine glucose loss, which was assumed to be negligible because plasma glucose concentrations were below the renal tubular glucose threshold at all times. Moreover, previous studies in our laboratory have shown that prolonged increases of plasma NEFA concentrations using intralipid and heparin intravenous infusions do not change urinary glucose excretion [33]. The disposition index (DI), which has been used as an index of insulin secretion corrected for the ambient degree of insulin resistance [34], was then calculated as the product of S_I and ISR. DI is given in arbitrary units ($l^2 \cdot kg^{-1} \cdot min^{-2}$).

Statistical analysis

The data are expressed as means ± SEM. Plasma glucose, insulin, NEFA and triglyceride concentrations at 08.00 hours on day 1 of the study were compared with the corresponding concentrations on day 2 of the study by using two-way ANOVA followed by Tukey's *t* test. Data obtained in the basal state and during the last 30 min of the clamp studies were compared using two-way ANOVA for repeated measures, followed by Tukey's *t* test. As the morning plasma glucose concentrations were different between the four studies prior to testing of GSIS on day 2, a second analysis of the data was done with correction for this difference using analysis of covariance (ANCOVA). The results of the analyses were the same whether or not the different plasma glucose concentrations were taken into account. The results reported are those obtained without taking the plasma glucose concentrations into account. A p value of less than 0.05 was considered to be significant. Calculations were performed with SAS software (Statistical Analysis System, Cary, N.C., USA).

Results

Fasting plasma glucose and insulin concentrations of the four infusion protocols prior to study of GSIS on day 2 (Table 1). As expected, plasma glucose increased in the GLU and the GLU-IH studies after the glucose and glucose + intralipid and heparin infusions began. Before GSIS analysis on day 2 plasma glucose concentrations in the GLU study were moderately in-

Table 1. Plasma glucose and insulin during the four infusion protocols for 24 h prior to the study of GSIS on day 2

	Glucose (mmol/l)				Insulin (pmol/l)			
	SAL	IH	GLU	GLU-IH	SAL	IH	GLU	GLU-IH
Day 1								
08:00 hours	5.4±0.1	5.4±0.1	5.2±0.2	4.8±0.2	157±61	78±14	71±14	88±11
12:00 hours	5.4±0.4	5.5±0.2	6.6±0.7	8.0±1.1	214±76	184±48	803±212	811±183
16:00 hours	5.4±0.3	5.6±0.2	7.3±0.5	9.9±0.7	301±82	267±73	758±191	936±216
Day 2								
8:00 hours	5.5±0.3	5.7±0.1	7.6±0.3	9.6±0.4	85±12	94±13	352±76	761±198
p-value[a]	NS	0.5	<0.01	<0.01	NS	NS	<0.01	<0.01
p-value[b]	NA	NS	<0.01	<0.01	NA	NS	<0.01	<0.01

Data are presented as means ± SEM. *p*-value[a] refers to the comparison of the 08:00 hours value on day 2 with the 08:00 value on day 1 in that particular study (statistics by ANOVA); *p*-value[b] refers to comparison of the 08:00 hours value on day 2 in IH, GLU, and GLU-IH with the 08:00 value on day 2 in SAL (statistics by ANOVA), Please note that the 12:00 and 16:00 hours samples on day 1 were not fasting samples. GSIS, glucose-stimulated insulin secretion; SAL, saline infusion study; IH, intralipid and heparin infusion study; GLU, glucose infusion study; GLU-IH, intralipid and heparin and glucose infusion study (see Methods); NA, not applicable

Table 2. Plasma triglyceride and NEFFA concentrations during the four infusion protocols for 24 h prior to the study of GSIS on day 2

	Triglycerides (mmol/l)				NEFA (mol/l)			
	SAL	IH	GLU	GLU-IH	SAL	IH	GLU	GLU-IH
Day 1								
08:00 hours	1.4±0.2	1.6±0.1	1.5±0.3	1.4±0.2	0.58±0.12	0.48±0.06	0.54±0.09	0.43±0.09
12:00 hours	2.2±0.2	3.2±0.5	3.2±0.4	1.6±0.2	0.45±0.16	1.11±0.19	0.20±0.10	0.74±0.11
16:00 hours	2.0±0.1	5.2±0.7	4.3±0.5	2.2±0.3	0.49±0.16	1.40±0.28	0.13±0.02	0.94±0.20
Day 2								
8:00 hours	1.71±0.09	4.73±1.01	1.65±0.25	1.86±0.22	0.50±0.07	1.12±0.13	0.10±0.03	0.70±0.07
p-value[a]	NS	0.01	NS	NS	NS	<0.01	<0.01	<0.04
p-value[b]	NA	0.01	NS	NS	NA	<0.01	NS	<0.01

Data are presented as means ± SEM. *p*-value[a] refers to the comparison of the 08:00 hours value on day 2 with the 08:00 value on day 1 in that particular study (statistics by ANOVA); *p*-value[b] refers to comparison of the 08:00 hours value on day 2 in IH, GLU, and GLU-IH with the 08:00 value on day 2 in SAL (statistics by ANOVA), Please note that the 12:00 and 16:00 hours samples on day 1 were not fasting samples. GSIS, glucose-stimulated insulin secretion; SAL, saline infusion study; IH, intralipid and heparin infusion study; GLU, glucose infusion study; GLU-IH, intralipid and heparin and glucose infusion study

creased (7.5±0.3 mmol/l). The addition of intralipid and heparin to the glucose infusion (GLU-IH) raised plasma glucose even further to 9.7±0.6 mmol/l. There was no difference in glucose concentrations between the IH and SAL studies. Plasma insulin concentrations followed the same pattern as plasma glucose concentrations.

There were also no differences in pre-clamp plasma C-peptide concentrations between the SAL and IH studies (0.93±0.22 pmol/l in SAL; 0.85±0.18 pmol/l in IH; *p*=NS). In the GLU study plasma C-peptide concentrations increased about twofold (2.12±0.21 pmol/l, *p*<0.01 vs SAL). This value increased further with the combined heparin, intralipid and glucose infusion (3.31±0.44 pmol/l in GLU-IH, *p*<0.01 vs SAL and GLU).

Fasting plasma triglyceride and NEFA concentrations at 0 and 24 h of the four infusion protocols prior to study of GSIS on day 2 (Table 2). Plasma NEFA concentrations increased in the IH and GLU-IH studies, but were suppressed in the GLU study. As anticipated, plasma NEFA concentrations were highest in the IH study, intermediate in the GLU-IH infusion study, and lowest in the GLU study. Plasma triglyceride concentration was also highest in the IH study and similar in the other three studies.

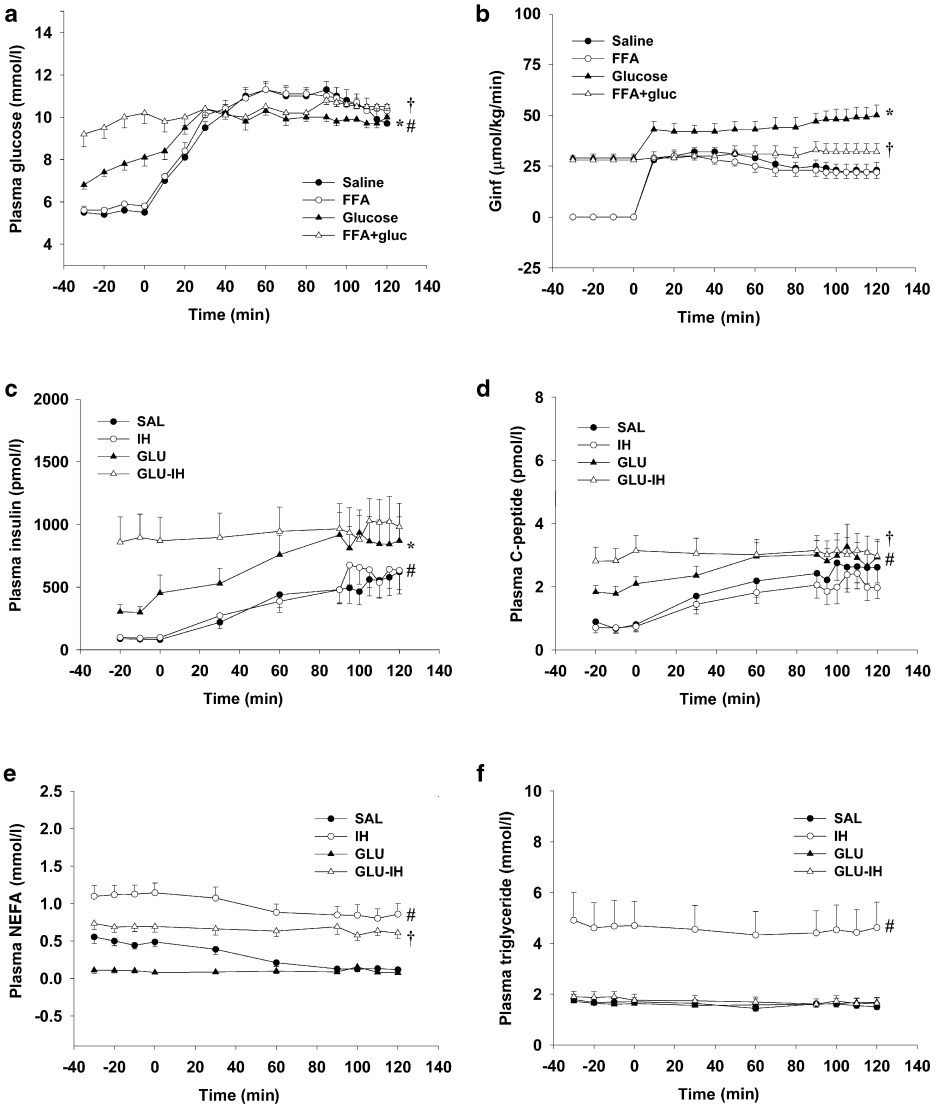

Fig. 1a–f. Plasma glucose concentration, glucose infusion rate, plasma insulin, C-peptide, NEFA and triglyceride concentrations during the GSIS study on day two. Plasma glucose concentration (**a**), glucose infusion rate (Ginf) (**b**), plasma insulin concentration (**c**), plasma C-peptide concentration (**d**), plasma NEFA concentration (**e**) and plasma triglyceride concentration (**f**) during hyperglycaemic clamp studies are shown for saline control studies (SAL, closed circles) and NEFA infusion (IH, open circles), glucose infusion (GLU, closed triangles) and NEFA+glucose infusion studies (GLU-IH, open triangles). Statistics are by ANOVA for the last 30 min of the clamp. *p* values: (**a**) *GLU vs SAL, IH and GLU-IH, $p<0.01$; (**b**) *GLU vs SAL, IH and GLU-IH, $p<0.05$; †GLU-IH vs SAL, IH and GLU, $p<0.05$; (**c**) ‡IH vs GLU and GLU-IH, $p<0.01$; *p*=NS vs SAL; *GLU vs SAL and IH, $p<0.01$; *p*=NS vsGLU-IH; (**d**) ‡IH, $p<0.01$ vs SAL; $p<0.01$ vs GLU and GLU-IH; †GLU-IH, $p<0.05$ vs SAL and $p<0.01$ vs IH; *p*=NS vs GLU; (**e**) ‡IH vs SAL, GLU and GLU-IH, $p<0.01$; †GLU-IH vs SAL, GLU and IH, $p<0.01$; (**f**) ‡IH vs SAL, GLU and GLU-IH, $p<0.01$. GSIS, glucose-stimulated insulin secretion; Ginf, glucose infusion rate

Plasma glucose concentration, glucose infusion rate, and plasma insulin, C-peptide, NEFA and triglyceride concentrations during the GSIS study (Table 3, Fig. 1). This section deals with results from the last 30 min of the hyperglycaemic clamp. Plasma glucose was lower in the GLU study than in the three other studies ($p<0.01$ vs SAL, IH, GLU-IH) (Table 3, Fig. 1a). There was no difference in clamp glucose concentrations between the SAL, IH and GLU-IH studies.

Although clamp glucose concentrations were lower in the GLU group, the glucose infusion rate was highest in that group ($p<0.01$ vs SAL, IH, and GLU-IH) (Table 3, Fig. 1b). The glucose infusion rate was intermediate in the GLU-IH study ($p<0.05$ vs SAL, IH, GLU) and lowest in the IH and the SAL studies (*p*=NS for SAL vs IH).

Plasma insulin concentrations (Table 3, Fig. 1c) were higher in the GLU and GLU-IH studies during the hyperglycaemic clamp (*p*=NS between the two studies and $p<0.01$ vs SAL and IH). In the SAL and IH studies plasma insulin concentrations were similar.

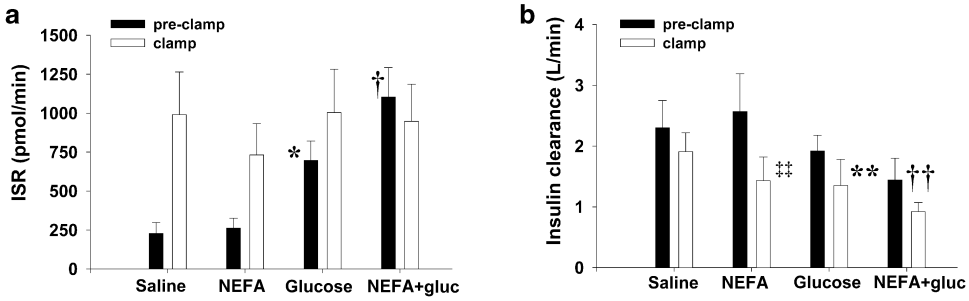

Fig. 2a, b. ISR and insulin clearance prior to and during the GSIS. ISR (**a**) and insulin clearance (**b**) during the hyperglycaemic clamp are shown for saline control studies (SAL) and NEFA infusion (IH), glucose infusion (GLU) and NEFA and glucose infusion studies (GLU-IH). Statistics are by ANOVA. *p* values are: (**a**) *pre-clamp GLU, $p<0.01$ vs pre-clamp SAL, IH and GLU-IH; †pre-clamp GLU-IH, $p<0.01$ vs pre-clamp SAL, IH and GLU; (**b**) ‡‡clamp IH vs clamp SAL, $p<0.01$; **clamp GLU vs clamp SAL, $p<0.05$; ††clamp GLU-IH vs clamp SAL, $p<0.01$. ISR, insulin secretion rate; GSIS, glucose-stimulated insulin secretion; NEFA+ gluc, NEFA and glucose infusion

Table 3. Outcome variables between the 4 infusion protocols during the last 30 minutes of the hyperglycaemic clamp study

	SAL	GLU	IH	GLU-IH
Glucose (mmol/l)	10.5±0.4	9.9±0.2	10.6±0.3	10.6±0.2
Ginf (μmol·kg^{-1}·min^{-1})	22.0±3.1	48.0±4.7	23.0±3.4	31.9±4.3
Insulin (pmol/l)	637±145	1037±183	732±142	1148±193
C-peptide (pmol/l)	2.97±0.55	3.39±0.50	2.38±0.51	3.45±0.56
ISR (pmol/min)	990±274	1003±279	731±201	948±237
Insulin clearance (l/min)	1.91±0.31	1.35±0.43	1.43±0.39	0.92±0.15
NEFA (mmol/l)	0.15±0.06	0.10±0.04	0.81±0.16	0.53±0.07
Triglyceride (mmol/l)	1.50±0.11	1.79±0.23	5.11±1.05	1.65±0.21
S_I (dl/kg.min per μU/ml)	2.86±0.63×10^{-4}	3.82±0.95×10^{-4}	2.50±0.65×10^{-4}	2.37±0.63×10^{-4}
DI (l^2·kg^{-1}·min^{-2})	4.09±0.92×10^{-3}	6.11±1.62×10^{-3}	2.80±0.81×10^{-3}	2.95±0.48×10^{-3}

Data are presented as means ± SEM. Please see Methods for definitions and calculation of the variables. See Results for statistically significant differences between parameters. Abbreviations: SAL, saline infusion study; IH, intralipid and heparin infusion study; GLU, glucose infusion study; GLU-IH, intralipid and heparin and glucose infusion study; Ginf, glucose infusion rate; ISR, insulin secretion rate; S_I, insulin sensitivity index; DI, disposition index

Plasma C-peptide concentrations (Table 3, Fig. 1d) were lower in the IH study than in the other three studies ($p<0.01$) and higher in the GLU-IH study than in the SAL study ($p<0.05$ vs SAL, p=NS vs GLU). There was no difference in plasma C-peptide concentrations between the GLU and SAL studies.

During the hyperglycaemic clamp, plasma NEFA concentrations (Table 3, Fig. 1e) remained highest in the IH study ($p<0.01$ vs SAL, GLU and GLU-IH), intermediate in the GLU-IH study ($p<0.01$ vs SAL, IH and GLU) and lowest in the GLU and SAL studies (p=NS GLU vs SAL). Plasma triglyceride concentrations (Table 3, Fig. 1f) were also highest in the IH study ($p<0.01$ vs SAL, GLU and GLU-IH) and were comparable in the three remaining groups.

ISR and insulin clearance on day 2 prior to and during the hyperglycaemic clamp study. There were no differences in pre-clamp ISR (Fig. 2a) between the SAL and IH studies (227±71 pmol/min in SAL; 262±64 pmol/min in IH, p=NS). As with the C-peptide data, there was a threefold increase in pre-clamp ISR in the GLU study (697±124 pmol/min, $p<0.01$ vs SAL, IH and GLU-IH) and a fivefold increase in pre-clamp ISR in the GLU-IH study (1103±189 pmol/min, $p<0.01$ vs SAL, GLU and IH). During the last 30 min of the 10 mmol/l hyperglycaemic clamp, there were no differences in ISR between the four groups (Table 3, Fig. 2a).

With regard to insulin clearance, there were no differences between pre-clamp values for the four groups (2.30±0.45 l/min in SAL; 2.57±0.62 l/min in IH; 1.92±0.26 l/min in GLU; 1.44±0.36 l/min in GLU-IH, p=NS for SAL vs IH vs GLU vs GLU-IH) (Fig. 2b). Insulin clearance during the clamp was lower in the IH, GLU and GLU-IH studies than in the SAL study ($p<0.01$ vs SAL). There was no difference in insulin clearance during the clamp between the IH, GLU and GLU-IH groups (Table 3, Fig. 2b).

Insulin sensitivity index and disposition index. The S_I was calculated for the pre-clamp period for GLU and GLU-IH (not illustrated). The S_I could not be calculated pre-clamp for SAL and IH as the glucose infusion rate was zero for those two studies pre-clamp, and a

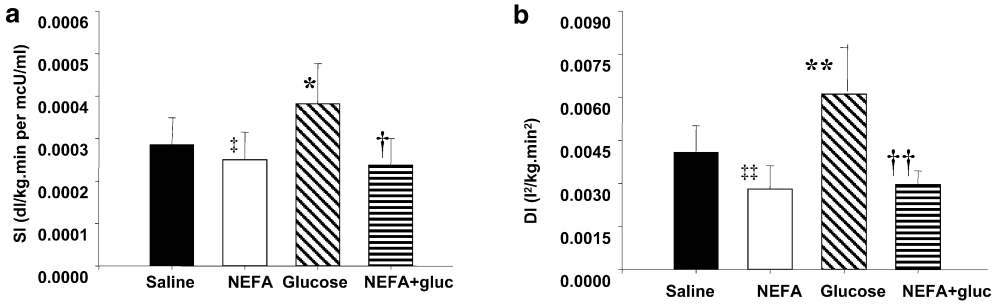

Fig. 3a, b. Insulin sensitivity index (**a**) and disposition index (**b**) during the hyperglycaemic clamp studies are shown for saline control studies (SAL), and NEFA infusion (IH), glucose infusion (GLU) and NEFA and glucose infusion (GLU-IH) studies. Statistics are by ANOVA. *p* values are: (**a**) ‡IH vs SAL, $p=0.09$; *GLU vs SAL or IH or GLU-IH, $p<0.01$; †GLU-IH, $p<0.05$ vs SAL; (**b**) ‡‡IH vs SAL, $p<0.05$; **GLU vs SAL or IH or GLU-IH, $p<0.01$; ††GLU-IH, $p=0.09$ vs SAL. SI, insulin sensitivity index; DI, disposition index; NEFA+gluc, NEFA and glucose infusion

glucose infusion rate value is needed to calculate S_I. In the GLU-IH study the S_I was 43% lower ($4.08+1.44\times10^{-4}$ dl/kg·min per μU/ml) than in the GLU study ($7.18+1.42\times10^{-4}$), but this difference was not significant. With regard to S_I calculated during the hyperglycaemic clamp, it increased by approximately 20% in the GLU study compared with the saline control study ($p<0.01$ vs SAL) (Table 3, Fig. 3a). The S_I tended to be lower in the IH group than in the SAL group ($p=0.09$ vs SAL). The addition of NEFA to glucose impaired the glucose-induced increase in S_I ($p<0.01$ vs GLU and $p<0.05$ vs SAL).

As with S_I data, there was also an approximately 50% increase in DI in the GLU study ($p<0.01$ vs SAL) (Table 3, Fig. 3b). The DI was lower in the IH group than in the SAL group ($p<0.05$ vs SAL). The addition of NEFA to glucose (GLU-IH study) abolished the increase in the DI ($p<0.01$ vs GLU and $p=0.09$ vs SAL).

Discussion

Our study showed that a 24-h intravenous infusion of glucose, which resulted in moderate hyperglycaemia, was associated with an increase in pancreatic insulin secretion, S_I and DI. Our findings, which are consistent with previous studies, do not support the idea of a beta cell glucotoxic effect of moderate hyperglycaemia for 24 h induced by an intravenous glucose infusion in non-diabetic humans. We did, however, confirm previous in vivo observations in humans that a prolonged NEFA increase reduces DI [23]. The combination of an increase in plasma NEFA and moderate hyperglycaemia completely abolished the increase in S_I and DI seen with hyperglycaemia alone. These results show that an increase of plasma NEFA for 24 h impairs the stimulatory effect of short-term modest hyperglycaemia on beta-cell function and insulin sensitivity in obese humans.

Although a twofold increase in plasma NEFA alone had no significant effect on plasma glucose concentrations, adding intralipid and heparin to the glucose infusion raised plasma glucose concentrations above those of the glucose infusion alone. This is further evidence that the addition of increased NEFA concentrations impaired insulin sensitivity, since absolute insulin secretion was not lower in GLU-IH than in GLU, even though relative insulin secretion (i.e. DI) was diminished. Previous studies have shown that increases of plasma glucose to above 9 mmol/l for 68 h resulted in decreased GSIS in healthy humans, whereas a more modest increase of up to 7.5 mmol/l increased GSIS [16]. Therefore we cannot exclude the possibility that the aggravated hyperglycaemia induced by NEFA accounts for the differences between the GLU-IH and GLU groups. However, when we analysed our data using ANCOVA to take into account the different glucose concentrations between the two groups, our results did not change, suggesting that the impairments of SI and DI in the GLU-IH group are not due to the higher glucose.

With regard to the increases in S_I, ISR and DI observed in our study in connection with 24 h of moderate hyperglycaemia, we note that mild hyperglycaemia potentiated insulin secretion in previous studies [17, 18, 19], while mild hyperinsulinaemia enhanced glucose utilisation [19]. Our results are consistent with one of these studies, in which a 24-h infusion of glucose in non-obese male subjects led to an increase in insulin secretion and insulin sensitivity [19]. Moreover, in the present study, a prolonged increase of plasma glucose alone (GLU) resulted in a similar reduction of insulin clearance compared to the two studies in which NEFA concentrations were increased (i.e. the IH and GLU-IH studies). We have previously shown that an increase of plasma NEFA in humans leads to reduced insulin clearance [27, 29]. The reduction in insulin clearance with prolonged hyperglycaemia as seen in the present study could be due to prolonged hyperinsulinaemia and consequent down-regulation of insulin receptors. The precise mechanism, however, remains unclear.

Interestingly, plasma NEFA concentrations were very low on the morning of GSIS testing in the 24-h

glucose experiment. Such a reduction in morning plasma NEFA concentrations using treatment with acipimox, an analogue of nicotinic acid, has been shown to increase insulin sensitivity and GSIS [35, 36]. It is possible therefore that the suppression of NEFA during the 24-h hyperglycaemic study contributed to the increase in S_I and DI. However, statistical adjustment for the morning plasma NEFA concentrations or the total area under the NEFA curve during the hyperglycaemic clamp did not eliminate the difference between the glucose infusion study and the other experimental protocols with regard to the S_I and DI results. Other mechanisms therefore must be involved in mediating the insulin-sensitising effect of 24 h of moderate hyperglycaemia.

The trend seen in the IH study towards an absolute reduction of ISR and S_I, while DI was significantly reduced, is consistent with our previous study, in which we examined the acute (1.5 h) and chronic (48 h) effects of a twofold increase of plasma NEFA in lean healthy males [29]. In that study, although there was no change in DI at 1.5 h, there was a reduction in S_I and DI at 48 h. S_I and DI, however, were measured during a 10 and 20 mmol/l hyperglycaemic clamp, in contrast to the present study, where S_I and DI were measured during a 10 mmol/l hyperglycaemic clamp only. In our previous study [29] DI tended to be reduced during the 10 mmol/l clamp, whereas it was only significantly reduced during the 20 mmol/l clamp. Our present results in obese individuals, where DI was also reduced at 10 mmol/l, confirm our previous findings that a prolonged increase of NEFA in obese individuals induces increased susceptibility to the impairment of beta-cell function [27].

The findings of the present study suggest that the impairment of GSIS with a prolonged increase of plasma NEFA is present at hyperglycaemia as well as at euglycaemia**.** Other studies have shown that glucose and NEFA regulate the expression of beta-cell genes encoding key regulatory enzymes in the glycolytic [37, 38, 39] and fat oxidation pathways [40]. In INS-1 cells, glucose causes a sustained increase in lipogenic genes, an almost total inhibition of fat oxidation, and a pronounced increase in lipid esterification and triglyceride deposition [41]. The authors of that study have proposed the 'glucolipotoxic' hypothesis and suggested that beta-cell abnormalities become particularly apparent when glucose and fatty acids are high and the NEFA esterification pathways are enhanced [25]. Recently, other teams carried out several in vitro studies which support the glucolipotoxic hypothesis. They found that (i) prolonged in vitro exposure of isolated islets to fatty acids decreased insulin gene expression only in the presence of high glucose concentrations [42]; (ii) prolonged culture of islets with palmitate resulted in the accumulation of intracellular triglycerides only in the presence of high glucose [43]; and (iii) normalisation of plasma glucose with phlorizin in Zucker diabetic fatty rats prevented the accumulation of triglycerides and decreased insulin mRNA levels in islets, whereas normalisation of plasma lipid concentrations with bezafibrate was without effect [44]. In the present study, the reduction in DI between the GLU-IH and GLU experiments was threefold greater than between the IH and SAL experiments. However, this was not statistically significant and absolute DI was similar in GLU-IH and IH alone. Our study, therefore, does not support the theory of glucolipotoxicity, perhaps because glucotoxicity itself was not evident at this level of hyperglycaemia for this duration, but rather the opposite effect, i.e. glucose-enhanced insulin sensitivity and beta-cell function. As mentioned above, the severity and duration of hyperglycaemia are likely to be critical determinants of glucotoxicity. We cannot, therefore, exclude the possibility that with longer duration or severity of hyperglycaemia glucolipotoxicity does occur in vivo.

With regard to our study design, there were some limitations. Firstly, there were differences in the energy load provided by the various treatment protocols (7.2 MJ vs 12 MJ vs 19.3 MJ over 24 h for IH, GLU and GLU-IH respectively). Although we observed qualitative and quantitative differences in Si and DI between the treatments, there was no obvious relationship with the different calorie loads. However, we cannot completely rule out that it could have affected insulin sensitivity and/or GSIS. Secondly, there were also differences in volumes of total fluid infused in the different experimental protocols, although these differences are unlikely to have significantly affected the primary variables of interest. Finally, the plasma glucose concentrations were lower in the GLU study than in the other three studies during the hyperglycaemic clamp, due to technically imperfect matching. This slight undershooting of plasma glucose was taken into account in the calculation of S_I, but could have resulted in an underestimation of ISR and DI in the GLU study.

In conclusion, we showed that 24 h of moderate hyperglycaemia alone was associated with an increase in plasma insulin concentration, GSIS, S_I and DI. The combination of an increase in plasma NEFA and moderate hyperglycaemia completely abolished the increase in S_I and DI which was seen with hyperglycaemia alone. Since the combination of NEFA and glucose did not suppress S_I or DI below concentrations seen in the NEFA study alone, we found no evidence of true glucolipotoxicity. It remains unclear whether more chronic or more marked combined increases of glucose and NEFA would absolutely impair pancreatic beta-cell function, thereby hastening and aggravating the beta-cell failure that is characteristic of the development of Type 2 diabetes.

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