Cortisol response to individualised graded insulin infusions: a reproducible biomarker for CNS compounds inhibiting HPA activation

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- The insulin tolerance test (ITT) is commonly used to induce cortisol release in response to physiological stress. This methodology can be employed in studies evaluating the hypothalamic-pituitary-adrenocortical axis.

WHAT THIS STUDY ADDS

- An exploration of the threshold for cortisol release in response to blood glucose lowering, indicating that the induction of hypoglycaemia with significant symptoms is not required for a highly reproducible maximal cortisol response.

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AIM

To determine the potential of cortisol secretion, in response to a physiological stressor, as a biomarker for centrally active compounds targeting the hypothalamic-pituitary-adrenocortical (HPA) axis.

METHODS

Cortisol response to hypoglycaemia was measured in 26 healthy males in two stages: firstly to derive an algorithm for individualized, graded insulin infusion rates to achieve defined hypoglycaemic targets over 3 h and secondly to determine the inter- and intra-subject variability of cortisol response to hypoglycaemia over two identical periods by measuring the maximum (t_{max}), time to maximum (C_{max}) response and cortisol area under the response curve (AUC).

RESULTS

Hypoglycaemia induced a consistent cortisol response starting at approximately 1 h, corresponding to blood glucose concentrations of approximately 3.3 mmol L⁻¹, and peaking approximately 3 h after the start of infusion. The inter- and intra-subject coefficients of variation (CVs) of cortisol response were approximately 19 and 19% (AUC), 15 and 19% (C_{max}) and 10 and 14% (t_{max}), respectively. The intra-subject CVs for the ratio of maximum cortisol response to baseline concentration and rate of initial cortisol response between study days were more variable (32.8% and 59.0%, respectively). The blood glucose-cortisol response model derived from the study was predictive of the individual observed cortisol responses, and estimated a blood glucose EC₅₀ associated with onset of the cortisol response of 3.3 mmol L⁻¹.

CONCLUSIONS

Gradual hypoglycaemia is an effective, reproducible and well-tolerated method of stimulating a cortisol response and may therefore be useful in assessing the neuroendocrine response to HPA axis inhibitors, such as corticotropin-releasing hormone-1 (CRH-1) antagonists.

Introduction

Hyperactivity of the hypothalamic-pituitary-adrenocortical (HPA) axis has been implicated in several neuropsychiatric diseases including anxiety and depression [1, 2]. Elevated cortisol has been shown to be associated with mood disorders in several behavioural models of stress, such as the Trier Social Stress Test (TSST), in humans [3]. Furthermore, pharmacological challenges acting on several neurotransmitter systems implicated in depression/anxiety, such as 5-hydroxytryptamine (5-HT) and corticotropin-releasing hormone (CRH) have been shown to attenuate stress-induced ACTH/corticosterone release in animal models of stress. For example, both the CRH-1 antagonists R121919 and NBI-34041 attenuated stress-induced ACTH/corticosterone release in rats. However, in clinical studies, neither R121919 nor NBI-34041 attenuated CRH-induced ACTH and cortisol release in patients with depression and volunteers, respectively. In contrast, NBI-34041 attenuated stress-induced cortisol release using the Trier Social Stress Test (TSST) [4–6]. Given the lack of reliable methods to assess acutely central target engagement of drugs in human subjects, the aim of this study was to explore novel methods to measure stress induced release of neurohormones that could be acutely modulated by drugs. A common model of induced stress resulting in cortisol release is the insulin tolerance test (ITT). The ITT is a standard diagnostic test to evaluate integrity of the HPA axis in patients [7]. Previous studies have examined the variability of the hormonal counter-regulatory response to the ITT in healthy volunteers. For example, the cortisol response to an ITT is more reproducible in healthy volunteers than the growth hormone response [8, 9].

In the standard ITT, hypoglycaemia is achieved by administration of an intravenous (i.v.) bolus of insulin and clinical hypoglycaemia is attained at a somewhat variable rate [9]. In order to investigate drug effects on the neuroendocrine response to hypoglycaemia, a more predictable and reproducible endocrine response to hypoglycaemia with less adverse clinical symptomatology is desired, which may be achieved by gradual induction of hypoglycaemia. Earlier studies investigating the counter-regulatory response to an i.v. infusion of insulin have shown that a predictable cortisol response was achieved using a low insulin infusion rate of $0.35 \text{ mU kg}^{-1} \text{ min}^{-1}$ to produce hypoglycaemia gradually over a 180 min period [10, 11]. Moreover, such a controlled approach could yield other potential neuroendocrine response measures, such as time to onset, and the rate of initial response, which is not possible using a standard ITT. These parameters may provide additional, potentially more discrete, measures other than the maximal response of cortisol. Whilst there are data available on the reproducibility of the standard ITT [9], similar data are not available on cortisol responses to a more gradual hypoglycaemia.

The aim of this study was to determine in healthy volunteers the extent of variability in the cortisol response to the HPA axis perturbed by hypoglycaemia as a physiological stressor, to aid in the development of study techniques to evaluate the potential of this biomarker method as a marker of drug target engagement.

Several studies investigating the counter-regulatory response to an i.v. infusion of insulin have demonstrated a threshold cortisol response above the 39.6 mg dl^{-1} (2.2 mmol l^{-1}) glucose threshold [11, 12]. The blood glucose threshold for an insulin infusion-induced cortisol response was also assessed in this study.

Methods

This was an open label study in 26 healthy subjects. For inclusion into the study, subjects were required to be overtly healthy males, as determined by medical history and physical examination, between the ages of 21 and 55 years inclusive, with a screening body mass index (BMI) of 18.5 and 29.9 kg m^{-2} and a fasting blood glucose $<6.1 \text{ mmol l}^{-1}$ at screening. Subjects were excluded if they had known allergies or sensitivity to insulin; had used systemic glucocorticoids within 3 months of study entry and topical or inhaled glucocorticoids within 7 days of study entry; and/or if they had a first-degree family history of diabetes.

This study was approved by the Domain Specific Ethics Review Board, National Healthcare Group, Singapore and written informed consent was obtained. All study procedures were carried out in accordance with the Declaration of Helsinki.

A pilot study was carried out to determine the optimal conditions for inducing a controlled stepped hypoglycaemic response to an insulin infusion. Subjects were divided into a fixed infusion rate group and a variable infusion rate group. In each group, two pairs of subjects were assessed at the same time. The results of each assessment were used to evaluate the hypoglycaemic response caused by the insulin infusion regimen and determine modifications for future groups, if warranted, to achieve the desired hourly target blood glucose concentrations of 3.5 , 2.8 and 2.2 mmol l^{-1} at 1 , 2 and 3 h , respectively [11].

All subjects were fasted for 4 h following consumption of a standardized meal. Initially, a group of four subjects (group 1) was administered insulin at a fixed infusion rate of $0.7 \text{ mU kg}^{-1} \text{ min}^{-1}$ over 3 h . Blood glucose concentrations were monitored at intervals of 2.5 – 15 min from 30 min prior to the start of the infusion until their return to baseline. The insulin infusions were terminated once the blood glucose reached a concentration of 2.2 mmol l^{-1} or the subject exhibited clinical symptoms of hypoglycaemia that were intolerable. Subjects were administered i.v. dextrose ($20\% \text{ v/v}$) as required.

The insulin infusion rate for a subsequent group of four subjects (group 2) was adjusted at 1 and 2 h from onset, if their glucose concentration was not within 0.2 mmol l⁻¹ of intended targets (based on those reported by Fanelli *et al.*) of 3.5 and 2.8 mmol l⁻¹, respectively [11]. The insulin infusions were terminated as described above.

The data from the pilot study were used to develop an algorithm for determining the initial rate of insulin infusion for each subject in the main study. The inter- and intra-subject variability of the cortisol response to hypoglycaemia was assessed in a second cohort of 18 subjects (groups of 6) on two separate study days (day 1 and day 2), 7 days apart.

Procedures and precautions were instigated to minimize the influence of external factors on neuroendocrine levels by standardizing diet and environmental factors. Subjects were admitted to the Clinical Research Unit (CRU) the night before procedures and were fasted from approximately midnight. A standardized light breakfast was provided at about 0800 h on dosing days. Environmental conditions such as light intensity, room temperature and sound level, were kept constant between study periods. To minimize the effect of diurnal hormone rhythms, in particular the morning decline in cortisol concentrations, the initiation of the insulin infusion and collection of samples for hormone concentrations were performed at approximately the same time, mid-day, for each study period for an individual subject.

The pilot study indicated that subject response to the insulin infusion would be highly variable. To allow for this, changes in the insulin infusion rate were permitted at 15–30 min intervals, with minimum and maximum rates of 0.2 and 2.0 mU kg⁻¹ min⁻¹. The insulin infusions were terminated as described previously. Cortisol concentrations were measured over a 5 h period during each infusion, from approximately 30 min prior to the start, then at regular intervals (0.25–1 h) until 2 h following cessation of the infusion.

Blood glucose concentrations were measured as mg dl⁻¹ (Yellow Springs Instrumentation, Ohio, USA), and converted to mmol l⁻¹ by the conversion factor of 0.0555.

Serum cortisol samples were analyzed using a chemiluminescent competitive immunoassay (Cobras e411, Roche Diagnostics, Mannheim, Germany) by the National University Hospital, Singapore. The dynamic range of this assay was 0.5–1750 nmol l⁻¹ with a lower limit of 0.5 nmol l⁻¹ and an inter-assay accuracy (% CV) during validation of 2.7% to 5.5% and an intra-assay accuracy (% CV) of 0.9 to 2.4%. Samples above the limit of quantification were diluted and re-analyzed to yield results within the calibrated range.

Throughout both parts of the study, safety data in the form of clinical examinations, vital signs, clinical laboratory tests, and a record of adverse events were collected. A follow-up visit was completed for each subject approximately 10 days following receipt of the last insulin dose.

Pharmacodynamic analyses

Using the results of the pilot insulin administration study, an algorithm was developed to assist in setting initial insulin infusion rates. Observed blood glucose data in the pilot study were analyzed using S-Plus for Windows (version 6.2, Insightful Corp.).

Noncompartmental analysis was conducted using the software program WinNonlin (Enterprise, Version 5.0.1, Pharsight, Cary, NC) to determine AUC(0, t_{last}), C_{max} , and t_{max} for serum cortisol for each individual on each study day. AUC(0, t_{last}) was calculated for the total sampling period by the linear logarithmic/trapezoidal method, wherein the linear trapezoidal method was employed up to t_{max} and the logarithmic trapezoidal method was used from t_{max} through the last quantifiable sample time (t_{last}).

Mean serum cortisol concentrations at each sampling time were determined for each treatment for graphical evaluation only. Means were calculated for those time points at which at least two-thirds of the subjects had data concentrations above the limit of quantification and had samples drawn within 10% proximity to the scheduled sampling time.

In addition to non-compartmental analysis, the relationship between blood glucose and serum cortisol concentrations was evaluated using a nonlinear mixed-effects model with the NONMEM program (Level 1.1, Globomax, Ellicott City, MD, USA, version VI). Cortisol pharmacokinetics were assumed to follow a one-compartment model [13]. Over the course of the experiment, it was assumed that baseline cortisol concentrations would remain relatively unchanged. Decreases in glucose concentrations were linked to increases in the rate of cortisol input according to the following equation:

$$\frac{dCort}{dt} = R_{baseline\ release} + \left(1 - \frac{C_{gluc}^{\gamma}}{EC_{50}^{\gamma} + C_{gluc}^{\gamma}}\right) \times R_{hypoglycaemia\ release} - Cort \times k_{cortisol} \quad (1)$$

In Equation 1, Cort represents the cortisol concentration at any particular time, $k_{cortisol}$ is the elimination rate constant for cortisol, and $R_{baseline\ release}$ is the baseline rate of cortisol release into the plasma. $R_{hypoglycaemia\ release}$ is the maximum rate of cortisol release in response to hypoglycaemia. C_{gluc} is the blood glucose concentration, and EC_{50} represents the blood glucose concentration corresponding with the half-maximal rate of hypoglycaemia induced cortisol release. Gamma (γ) is the Hill coefficient. For initial modelling, the Hill coefficient was set to a very high number such that at glucose concentrations above the EC_{50} the rate of hypoglycaemia-induced cortisol release was effectively 0, while below the EC_{50} this rate was at its maximum. Because there is no hypoglycaemia-induced cortisol release at baseline, $R_{baseline\ release}$ can be estimated as $Cort \times k_{cortisol}$ using initial cortisol concentrations. All measurable serum cortisol and blood glucose concentrations were included in the analysis. Blood glucose measurements were imputed if a serum cortisol concentration did

not have a corresponding blood glucose concentration. Because of the relatively frequent measurement of blood glucose, it was assumed that the missing blood glucose measurements could be reliably estimated by linear interpolation. The first-order conditional estimation method with interaction (FOCEI) was used as the estimation method throughout the analysis.

Statistical analysis

Derived pharmacokinetic parameters such as $C_{\max}$ and $AUC(0, t_{\text{last}})$ had skewed data distributions and variances which increased with the means. Therefore it was not appropriate to apply the usual variance homogeneity and normality assumptions. The usual normal assumption also does not account for the non-negative $C_{\max}$ and $AUC(0, t_{\text{last}})$ values. These parameters were log-transformed to allow a more symmetrical distribution and the variances to be independent of the mean. The analysis was carried out using SAS (version 9.1.3).

Log-transformed $C_{\max}$ and $AUC(0, t_{\text{last}})$ were analyzed with a mixed effects model to estimate inter- and intra-subject (between each study day) variability in the presence of hypoglycaemia in healthy male subjects. Study day was included in the model as a fixed effect to test for differences between days 1 and 2 and to describe the specific differences that exist. Subject was included in the model as a random effect so that conclusions regarding the differences between study days could be generalized to the population.

The variance component covariance structure was chosen for these analyses.

Additional outcome measures were explored in the analysis, namely $C_{\max} : C_{\text{baseline}}$ ratio, slope of cortisol rise and time to maximum cortisol rise ($t_{\max}$). Each outcome measures' inter- and intra- subject CV% were also analyzed.

The $C_{\max} : C_{\text{baseline}}$ ratio gives a quantifiable number for observing the range (or rise) of the maximum concentration from the minimum concentration.

$C_{\max} : C_{\text{baseline}}$ ratios (derived for each subject at each period) were compared to detect between-study differences in the range or rise in cortisol concentration. Since the distribution of this ratio is not normally distributed and the comparisons were paired by period, the non-parametric Friedman's-test [17] was used to compare the ratios between period 1 and 2.

A mixed model was fitted with slope of cortisol rise (or onset time) as the dependent variable, period as the fixed variable and subject as the random variable. The slope of cortisol rise was normally distributed. For $t_{\max}$, since the data were not normally distributed, the Kruskal-Wallis test was used to detect differences between periods.

Results

A total of 26 healthy male subjects aged 22 to 53 years participated in this study. Eight healthy male subjects were

Figure 1

Individual blood glucose vs. time profiles of subjects administered insulin at fixed infusion rate of $0.7 \text{ mU kg}^{-1} \text{ min}^{-1}$ (Group 1, A) and variable infusion rate (Group 2, B). Note: Dotted vertical lines demarcate the target time-points of 1, 2 and 3 h to achieve hypoglycaemic concentrations of 3.5, 2.8 and 2.2 mmol l^{-1} , respectively. Infusion rates for subject 104 was initially $1.0 \text{ mU kg}^{-1} \text{ min}^{-1}$, before adjustment at 1 h to $0.7 \text{ mU kg}^{-1} \text{ min}^{-1}$ and $1.0 \text{ mU kg}^{-1} \text{ min}^{-1}$ at 2 h. Respective infusion rates scheme for subjects 105, 106, and 302 were (0.7, 0.7, 1.0) (1.0, 1.5, 2.0) and (0.2, 0.7, 1.5) $\text{mU kg}^{-1} \text{ min}^{-1}$. Glucose concentration 2.2 mmol l^{-1} (—); Glucose concentration 2.8 mmol l^{-1} (---); Glucose concentration 3.5 mmol l^{-1} (—); Glucose concentration 4.5 mmol l^{-1} (---). Subject 104 (—); Subject 105 (---); Subject 106 (—); Subject 302 (---).

enrolled in the pilot study, with a mean age of 31 years (range 22–44 years), mean ($\pm$ SD) BMI of $23.1 (\pm 0.59) \text{ kg m}^{-2}$ and mean weight of $69.3 (\pm 5.1) \text{ kg}$. In the cortisol assessment study, 18 healthy male subjects were enrolled, with a mean age of 34 years (range 22–53 years), mean ($\pm$ SD) BMI of $23.7 (\pm 0.9) \text{ kg m}^{-2}$ and mean weight of $70.6 (\pm 5.5) \text{ kg}$.

Figure 1 illustrates the effect of the insulin infusion rates on each group of subjects in the pilot study. In group

1, the target blood glucose concentrations of 3.5, 2.8 and 2.2 mmol l⁻¹ at 1, 2 and 3 h, respectively, were not reached by all subjects and a wide variability in individual subject response was observed. The introduction of adjustments in the insulin infusion rates at 1 and 2 h in group 2 improved the ability to reach target glucose concentrations.

Of the eight subjects in the pilot study, three (38%) experienced symptoms of hypoglycaemia during the insulin infusion period, but none reported any intolerable symptoms requiring cessation of the infusion.

An algorithm was developed based upon the observed glucose concentrations from the pilot study, to predict the initial insulin infusion rate for use in the cortisol assessment study. The algorithm was developed after making the empirical observation that the rate of change in glucose concentrations appeared to be relatively linear during insulin infusion, at least until counter-regulatory effects were observed, which appeared to occur at glucose concentrations below 3.5 mmol l⁻¹. A linear model was developed relating the initial rate of change in glucose

concentrations to insulin infusion rates. This model was utilized to develop an algorithm for setting initial insulin infusion rates, based on the goal of reaching blood glucose concentrations of 3.5 mmol l⁻¹ over the course of 60 min:

$$\text{Rate} = \frac{3.5 - C_{\text{baseline}}}{-60 \cdot 0.0375} \quad (2)$$

where 0.0375 is the estimated parameter relating insulin infusion rates to the initial rate of glucose decline. Rate is the insulin infusion rate in mU kg⁻¹ min⁻¹ and C_{baseline} is the mean baseline glucose concentration taken over 30 min prior to the start of insulin infusion, in mmol l⁻¹.

A total of 16 out of 18 subjects completed both study days in the cortisol assessment study. Figure 2 illustrates the glucose and cortisol mean concentration vs. time profiles of both study days.

Of these 18 subjects, 13 (72%) experienced symptoms of hypoglycaemia of whom three had intolerable symptoms requiring cessation of the insulin infusion. With the incorporation of adjustments to the insulin infusion every 15–30 min based on the individual subject's response, it was possible to reach the pre-defined target glucose concentrations more consistently with each subject. The inter- and intra-subject variability of hypoglycaemia between days 1 and 2 is shown in Table 1. The slope of decline in glucose concentration between study days was more variable than the time to onset of hypoglycemia (intra-subject CV% of 59.0% and 23.6%, respectively).

The onset of the cortisol surge was observed approximately 1 h after the start of the insulin infusion. This appeared to correspond with the time blood glucose concentrations reached approximately 3.3 mmol l⁻¹. The cortisol response peaked after approximately 3 h of insulin infusion and gradually returned to baseline approximately 2 h following cessation of the insulin infusion.

The inter- and intra-subject variability of cortisol response between days 1 and 2 is shown in Table 1. The ratio of maximum cortisol response to baseline concentration ($C_{\text{max}}:C_{\text{baseline}}$) and rate of initial cortisol response (slope of cortisol rise) between study days were the most variable parameters observed, with intra-subject CV% of

Figure 2

Mean serum cortisol and blood glucose concentration–time profiles following individualized, graded insulin infusion ($\pm 95\%$ SEM). Glucose – Day 1 ($\blacktriangle$); Glucose – Day 2 ($\triangle$); Cortisol – Day 1 ($\bullet$); Cortisol – Day 2 ($\circ$)

Table 1

Inter- and intra-subject variability of pharmacodynamic parameters

Parameter (units)	Inter-subject CV% (95% CI)	Intra-subject CV% (95% CI)
Cortisol		
AUC(0, t_{last}) (nmol l ⁻¹ h)	19 (12, 40)	19 (14, 30)
Ratio of $C_{\text{max}}:C_{\text{baseline}}$	18 (9, 258)	33 (24, 52)
Slope of cortisol rise (nmol l ⁻¹ h ⁻¹)	27 (12, 6.59×10^2)	59 (43, 100)
t_{max} (h)	10 (6, 32)	14 (10, 21)
C_{max} (nmol l ⁻¹)	15 (9, 40)	19 (14, 29)
Glucose		
t_{onset} of glucose decline (h)	16 (9, 65)	24 (17, 37)
Slope of glucose decline (mg dl ⁻¹ h ⁻¹)	27 (12, 6.59×10^2)	59 (43, 100)

Table 2

Pharmacodynamic parameters for cortisol response

Parameter (units)	Period	LS geometric means (%CV)	Geometric means ratio (90% CI)	P value
AUC(0, t_{last}) (nmol l ⁻¹ h)	1	2198.1 (21.6)	1.06 (0.95, 1.19)	0.376
	2	2069.1 (33.3)		
C _{max} (nmol l ⁻¹)	1	740.50 (18.8)	1.03 (0.92, 1.15)	0.678
	2	720.61 (29.4)		

Table 3

Model parameters for hypoglycaemia-induced cortisol response

Parameter (units)	Central value (%SEE)	Total variability (%SEE)
Cortisol baseline (nmol l ⁻¹)	178 (6.29)	37.3% (24.8)
EC ₅₀ (mmol l ⁻¹)	3.3 (2.22)	10.8% (14.7)
R _{hypoglycaemia response} (nmol l min ⁻¹)	9.23 (6.12)	NE
k _{cortisol} (min ⁻¹)	0.0123 (8.86)	46.7% (30.7)
Gamma	30 (Fixed)	NE
Residual variability (% CV)	20.4 (11.8)	

Abbreviations: k_{cortisol}, the elimination constant for cortisol; NE, Not estimated; EC₅₀, blood glucose concentration corresponding with the half-maximal rate of hypoglycaemia-induced cortisol release; Gamma, the model Hill coefficient for glucose; R_{hypoglycaemia response}, is the maximum rate of cortisol release in response to hypoglycaemia; SEE, Standard error of the estimate. Total variability refers to the combination of inter- and intra-subject variability.

32.8% and 59.0%, respectively. Maximum response, time to maximum response and cortisol area under the response curve showed the least variability. AUC and C_{max} were comparable for both study days (Table 2). The median and range of t_{max} (h) was observed to be 2.72 (1.82 to 3.08) and 2.58 (1.58 to 3.08) for days 1 and 2, respectively. Overall, the cortisol response was generally consistent between days 1 and 2 and there were no significant differences in any of the cortisol parameters investigated ($P \geq 0.05$).

A nonlinear mixed effects model was used to examine the relationship between blood glucose and serum cortisol concentrations (equation 1). The final model parameters are summarized in Table 3. The results of the modelling effort found an EC₅₀ of 3.3 mmol l⁻¹. It was observed that this concentration corresponded closely with glucose concentrations at the onset of the cortisol surge. In Figure 2, a reference line is plotted to represent the EC₅₀. The Hill coefficient (gamma term) was fixed to a value of 30 while developing initial estimates for each parameter. Following the final fitting of the model parameters, a sensitivity analysis was performed for the gamma term, re-fitting the model fixing gamma to values between 4.5 and 70. It was determined that values of gamma between 18 and 60 produced similar minimum objective function values and caused no significant changes in fitted model values, supporting the use of a value of 30 for the gamma term. For each model parameter, a variability term was included in the model, if supported by the data. Attempts to model both intra- and inter-subject variability

for the cortisol baseline, EC₅₀, and k_{cortisol} terms were made using exponential and additive error models, but were not successful. The total (intra- and inter-subject) variability was characterized using an exponential error model. Figure 3 shows the observed cortisol concentrations and the cortisol time course predicted by the model for selected subjects, chosen to represent the best and worst model fits observed. The model's predicted cortisol time course closely fitted the data from each individual subject.

Safety analysis

Although not considered adverse events (AEs) as they were an expected outcome of the insulin infusion, 16 subjects experienced clinical symptoms of hypoglycaemia, which included fatigue, sweating, hunger, drowsiness and dizziness. In the cortisol assessment study, of the 11 subjects with symptoms of hypoglycaemia on the first study day and who subsequently returned for repeat infusion, only one did not have recurrent symptoms.

Out of 26 subjects who received study treatments, 19 subjects reported a total of 56 AEs. AEs associated with study procedures were the most common (70%), with bruising/swelling due to cannulation/venepuncture (30%) and allergic reaction to ECG electrodes (29%) being the most frequently reported.

Figure 3

Representative samples of observed serum cortisol concentrations in individualized, graded insulin infusion analysis (open circles) and predicted serum concentrations (solid line) from various study days and study periods. Graphs were judged to show the best (top row) and worst (bottom row) model fits seen throughout the course of the study

Discussion

This study has demonstrated that a gradual induction of hypoglycaemia can be reliably and reproducibly initiated by means of a variable insulin infusion. Earlier studies utilized a fixed insulin regime to induce gradual hypoglycaemia [13]. Such fixed regimes were not found to be effective in this study, a difference which may be accounted for by variations in study design and factors indicative of individual insulin sensitivity such as length of fasting, baseline glucose, body mass composition and ethnicity [14]. The insulin infusion methodology used in the present study enabled target glucose concentrations to be reached in many of the subjects, and was reproducible when employed on a separate occasion. Although the final target of 2.2 mmol l^{-1} was only attained in approximately 12% of subjects due to apparent counter-regulatory responses, this did not appear to impact on the cortisol response of the subjects and thus the overall aim of the study, as described below.

Such a gradual induction of hypoglycaemia reliably produced a cortisol response with an onset corresponding with the time blood glucose concentrations reached approximately 3.3 mmol l^{-1} . This is in general agreement with Fanelli *et al.*, where insulin was administered as an i.v. infusion at a concentration of $0.35, 1$ and $2 \text{ mU kg}^{-1} \text{ min}^{-1}$ over approximately 6 h with concomitant stepped hypoglycaemia over the period of study [11]. In the present

study the 3.3 mmol l^{-1} threshold was confirmed using a nonlinear mixed-effects model from which a mean EC_{50} for the hypoglycaemia induced cortisol surge was 3.3 mmol l^{-1} . This model also showed that the maximal cortisol response was associated with the length of time a subject was below the EC_{50} for the hypoglycaemia induced cortisol surge. Hence, attaining clinical or biochemical hypoglycaemia ($<2.2 \text{ mmol l}^{-1}$) which is the aim of a standard ITT, with its attendant risks, is not necessary for inducing a cortisol response.

The gradual hypoglycaemia protocol was also shown to be well-tolerated. Of the 19 subjects who underwent the protocol, only three did not complete the full 180 min infusion period because of intolerable hypoglycaemic symptoms whilst a further two had infusions stopped because target hypoglycaemia was reached. By comparison, in a study of the reproducibility of the standard ITT, all subjects had a minimum blood sugar of less than 1.9 mmol l^{-1} and five of the 16 subjects had a minimum blood sugar of less than 1.0 mmol l^{-1} [8].

The cortisol response produced by the gradual hypoglycaemia protocol could be assessed by several parameters, some of which were more reproducible than others. The more reproducible measures were t_{max} (time to peak cortisol) and C_{max} (peak cortisol) (Table 1). Other measures such as AUC, ratio of $C_{\text{max}} : C_{\text{baseline}}$ and slope of the cortisol surge proved to be less reproducible. By comparison, earlier work using the standard ITT showed good

reproducibility for peak cortisol but other parameters could not be calculated given the nature of the standard ITT [8]. The greater reproducibility of $C_{\max}$ compared with the ratio of $C_{\max} : C_{\text{baseline}}$ would suggest that the maximal cortisol response is similar for each subject regardless of their baseline cortisol concentrations being more variable. Further work involving patient populations will be required before this novel method of inducing a cortisol response to hypoglycaemia can be validated for clinical use as an alternative to the standard ITT.

The selection of reproducible outcome measures is vital for evaluating the neuroendocrine response to compounds that act on the HPA axis, such as CRH-1 antagonists. Moreover, the ability of the gradual hypoglycaemia protocol to provide several measures of the neuroendocrine response is potentially useful as these measures may be differentially affected by CRH-1 antagonists. Whilst a contribution by systemic CRH-1 receptors to the observed cortisol response cannot be excluded, it is unlikely to be substantial as the stress response is mediated via central mechanisms.

The only published evaluations of the clinical efficacy of CRH-1 antagonists utilized the neuroendocrine response to CRH [15] and psychosocial stress [6]. Neither R121919 nor NBI-34041 impaired cortisol response to exogenous CRH whilst NBI-34041 was shown to attenuate the cortisol response in the TSST. However, data on the reproducibility of this test are not available and may not be possible due to the nature of the test which involves a public speaking task and an unexpected mental arithmetic task. It may also be anticipated that there may be considerable inter- and intra-individual variation in emotional response to such a task whereas a neuroendocrine response to hypoglycaemia is much less likely to be so susceptible.

Furthermore, the use of the modified ITT with an individualized, graded insulin infusion to stimulate the HPA axis is a more physiologically relevant approach than those methods that involve pharmacological provocation with exogenous CRH [16].

Demonstrating an effect of a CRH-1 antagonist on a reliable and validated test of hypoglycaemia induced cortisol response would provide evidence of target engagement and hence inform future drug development. Moreover, such a test may also serve as a surrogate endpoint of target engagement in early phase clinical trials.

In conclusion, gradual hypoglycaemia is an effective, physiologically-relevant and safe means of reproducibly producing a cortisol response and may therefore be useful as a biomarker method in assessing the neuroendocrine response to HPA axis inhibitors.

Competing interests

BAW, CNL, SLL, STW and SDW are employees of Eli Lilly & Company. LM, GKO and GBC are former employees of Eli

Lilly & Company. CLHC was a Lilly-NUS/Singapore Institute for Clinical Sciences Joint Fellow in Investigational Medicine. BAW owns stock in Eli Lilly & Company. GBC has consulted for and is a shareholder of Eli Lilly & Company.

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