Counterregulatory Hormone Responses and Symptoms During Hypoglycaemia Induced by Porcine, Human Regular Insulin, and Lys(B28), Pro(B29) Human Insulin Analogue (Insulin Lispro) in Healthy Male Volunteers

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Lys(B28)Pro(B29) human insulin analogue (Lispro) is a newly developed monomeric insulin analogue with a rapid onset and short duration of action. The aim of the study was to compare the thresholds for the counterregulatory responses during a stepwise euglycaemic/hypoglycaemic clamp for insulin lispro (LP), human (H), and porcine (P) insulin in a randomized order in 12 healthy male volunteers (age 22.4 ± 1.7 years, BMI 21.9 ± 1.7 kg m^{-2}). A euglycaemic period of 2 h was followed by three hypoglycaemic levels of 60 min each: from 150–210 min at 3.5 mmol l^{-1}, 240–300 min at 3.0 mmol l^{-1}, and 330–390 min at 2.5 mmol l^{-1}. Plasma insulin levels during the 50 mU kg^{-1} h^{-1} infusions and blood glucose levels were not significantly different. The glucose requirements (mean ± SD) during the last part of the euglycaemic period (90–120 min) tended to be higher during LP compared to those during H and P; 2239 ± 702 and 1929 ± 769, 1957 ± 725 mg kg^{-1}, $P = 0.067$, respectively. The thresholds (blood glucose level at which a sustained elevation of the counterregulatory hormones as compared to the mean at normoglycaemia level 4.0 mmol l^{-1} , occurs) for the various hormones were very similar during LP, H, and P insulin infusions and occurred at 253.8 ± 56.7, 256.3 ± 55.3 and 257.5 ± 70.0 min for adrenaline; 241.4 ± 80.3, 260.5 ± 82.5 and 225.0 ± 75.9 min for noradrenaline; 307.5 ± 65.5, 304.1 ± 74.1 and 322.5 ± 40.4 min for cortisol; 263.8 ± 50.3, 255.0 ± 63.6 and 249.6 ± 50.9 min for growth hormone; 236.3 ± 78.2, 200.0 ± 73.1 and 226.3 ± 65.5 for pancreatic polypeptide. The autonomic and neuroglycopenic symptoms were elicited at 240 and 300 min, respectively. In conclusion, our data indicate a tendency to a higher biological activity of approximately 10 % for Lispro insulin. During a stepwise euglycaemic/hypoglycaemic clamp, the counterregulatory hormone responses to insulin lispro, human insulin, and porcine insulin were similar.

KEY WORDS **Insulin lispro Human insulin Porcine insulin Hypoglycaemia Counterregulation Insulin analogue Hypoglycaemic symptoms**

Introduction

Maintenance of strict glycaemic control is the main objective of therapy in patients with diabetes mellitus since it reduces the occurrence of microvascular complications.[1,2] In most patients, this can only be achieved with intensified insulin therapy, which is accompanied by an increased risk of severe hypoglycaemia.[1,3–5] The latter is associated with a prolonged duration of diabetes,[6–8] impaired glucose counterregulation, and frequent episodes of mild hypoglycaemia.[9–12] An appropriate autonomic warning system for incipient hypoglycaemia before neuroglycopaenia develops enables the patient to ingest carbohydrates, avoiding severe hypoglycaemia. Whether this awareness may be affected by insulin species *per se* remains controversial.[13–22]

Recently a newly developed short acting insulin analogue, Lys (B28), Pro (B29) human insulin analogue (insulin lispro) has been introduced. Insulin lispro only differs from human insulin at positions 28 and 29 of the B-chain in which the natural amino acid sequence is inverted, resulting in an insulin molecule with a greatly reduced capacity for self-association.[23] Because of these modifications, insulin lispro exhibits monomeric behaviour in solution and shows a fast pharmacodynamic action compared with other soluble insulin preparations.[24–26] However, for a safe therapeutic application the magnitude of the counterregulatory hormone response, and the

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 CCC 0742–3071/97/030248–10$17.50
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Accepted 17 November 1996
DIABETIC MEDICINE, 1997; **14**: 248–257

nature of symptoms during hypoglycaemia, should at least be similar to those induced by human insulin.

The present investigation was designed to compare the counterregulatory hormonal responses and subjective symptoms during controlled hypoglycaemia induced by infusions of insulin lispro, human and pork insulin. To exclude potential patient-related confounders such as previous glycaemic control, duration of disease or presence of autonomic neuropathy, we used the stepwise euglycaemic-hypoglycaemic clamp method in healthy male subjects.

Subjects, Materials, and Methods

Subjects

Twelve healthy, normal weight, male subjects (age 22.6 ± 1.7 years, body mass index 21.9 ± 1.7 kg m^{-2}) were recruited for the study. The subjects had never received insulin before, were not suffering from any disease, and had no family history of diabetes mellitus. All subjects were instructed to maintain their usual dietary and physical behaviour during the study period but refrained from alcohol and strenuous exercise for 24 h before each study day. Subjects gave written informed consent prior to the study. The protocol had been approved by the local ethical committee and the study was conducted in accordance with the Declaration of Helsinki.

Materials and Methods

All 12 subjects were studied on three occasions separated by at least 2 weeks but not more than 4 weeks. After a 10–12 h overnight fast, subjects were admitted to the metabolic ward at 0800 h. Two intravenous cannulae were inserted. One cannula, for infusion of insulin and 20 % glucose was inserted into an antecubital vein. The second cannula was placed retrogradely into a dorsal hand vein, for sampling arterialized venous blood. The hand was placed in a plexiglas box heated with air to 55–60°C to arterialize venous blood.[27,28] All subjects rested for at least 30 min after inserting the cannulae before baseline sampling was obtained. The subjects received on three occasions in double blinded, randomized (Latin square design) order either insulin lispro (Lys(B28)Pro(B29)-Human Insulin Analog (rDNA); Eli Lilly and Company, Indianapolis, USA), Humulin® R (insulin human injection, USP (recombinant DNA origin); Eli Lilly and Company, Indianapolis, USA), or Regular Iletin® II (insulin injection, USP, purified pork; Eli Lilly and Company, Indianapolis, USA). Since the receptor binding characteristics and the potency of the insulin species were assumed to be equal, equimolar amounts of insulin were given.[29,30] Forty-two IU of insulin (0.42 ml) were diluted in 53.6 ml saline (NaCl 0.9 %) to which 6 ml albumen was added (20 %).

Baseline measurements for hormones, blood pressure, and heart rate were taken and a symptom questionnaire was administered at –30, –15, and 0 min. At time t = 0, intravenous infusion of insulin were started (50 mU kg^{-1} h^{-1}) to run for 390 min. Blood glucose was maintained at levels of 4.0 mmol l^{-1} for 120 min by a variable glucose infusion (Imed 928, Abingdon Oxon, UK). The blood glucose was then allowed to decline gradually over 30 min from 4.0 to 3.5 mmol l^{-1} and maintained at that level for another 60 min. This sequence of steps, i.e. gradual decline in blood glucose of 0.5 mmol l^{-1} over 30 min and stabilization for 60 min, was repeated twice until the lowest level of blood glucose (2.5 mmol l^{-1}) was achieved. The blood glucose level was monitored every 2.5 min for the first hour and every 5 min thereafter. Samples for determination of plasma insulin levels were taken every 15 min in the 0–120 min period, and every 30 min thereafter. C-peptide was determined at baseline and every 30 min. Additional blood samples for later measurement of catecholamines, glucagon, growth hormone, cortisol, and pancreatic polypeptide were taken at 90 min and every 15 min thereafter. Blood pressure and heart rate were measured every 15 min. Every 30 min, the subjects were asked to complete a symptom questionnaire. Symptoms related to hypoglycaemia were ranked on a linear analogue scale from 0 = none to 6 = very severe. Throughout the experiments the subjects remained supine or semi-recumbent.

Analytical Methods

During the clamps, blood glucose was measured at the bedside using a glucose oxidase method on a Yellow Springs glucose analyzer (YSI 2300 STAT PLUS, Yellow Spring Instruments, Yellow Springs, Ohio, USA). Plasma catecholamines were measured using high-pressure liquid chromatography (HPLC) with electrochemical detection.[31] Cortisol (Coat-A-Count, DPC, Los Angeles, USA), plasma glucagon (Serono Diagnostics, Milano, Italy), growth hormone (Sorin Biomedica, HGHK-2, Saluggia, Italy), pancreatic polypeptide (Inhouse developed radioimmunoassay (RIA), using antibodies raised in rabbits, labelled pancreatic polypeptide (Novo, Copenhagen, Denmark), SAC Cell (Wellcome, Dartford, UK) as precipitating second antibody), C-peptide ((Novo Nordisk, antiserum M 1221, Copenhagen, Denmark) using SAC Cell (Wellcome, Dartford, UK) as precipitating second antibody), and plasma insulin (Coat-A-Count, DPC, Los Angeles, USA) using RIA. Intra-assay variation was not greater than 10 % for any assay. Blood pressure and heart rate were automatically measured (Colin, Hayashi Komaki City, Japan).

The symptom questionnaire was analysed using the symptom allocation as described by Hepburn.[32]

Calculations and Statistical Methods

Data are given as means ± standard deviation (SD). The differences among the three treatments were analysed

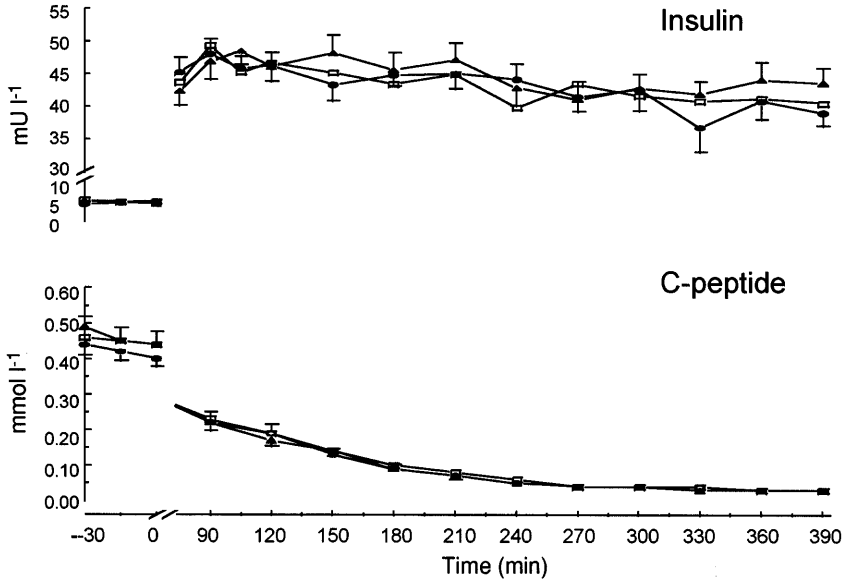

Figure 1. Insulin and C-peptide levels (mean ± SE) for human insulin analogue (LP), porcine insulin, and human regular insulin during the euglycaemic-hypoglycaemic clamp test. Lispro (-●-), human (-□-), and porcine (-▲-) insulin

using an analysis of variance contrasting regular human insulin treatment against both other treatments and insulin lispro treatment against regular pork insulin treatment. Thresholds were defined to coincide with a sustained elevation of the various counterregulatory hormones above the upper 95 % confidence limit observed for that parameter during euglycaemia. The symptom scores were analysed using a repeated difference contrast test to obtain the timepoint at which a significant rise of symptoms occurred. A commercially available software package (SPSS, Microsoft, Redmond, WA) was used for statistical analysis.

Results

Blood Glucose and Plasma Insulin Concentration

Plasma insulin increased in the three studies to comparable values of approximately 44 mU l^{-1} during the 50 mU kg^{-1} insulin infusion 43.5 ± 8.7, 43.2 ± 8.5, and 44.9 ± 8.6 mU l^{-1} for insulin lispro, human insulin, and pork insulin, respectively (Figure 1). Blood glucose concentrations in the hypoglycaemic studies decreased from fasting basal values of 4.5 ± 0.2 mmol l^{-1} to the target

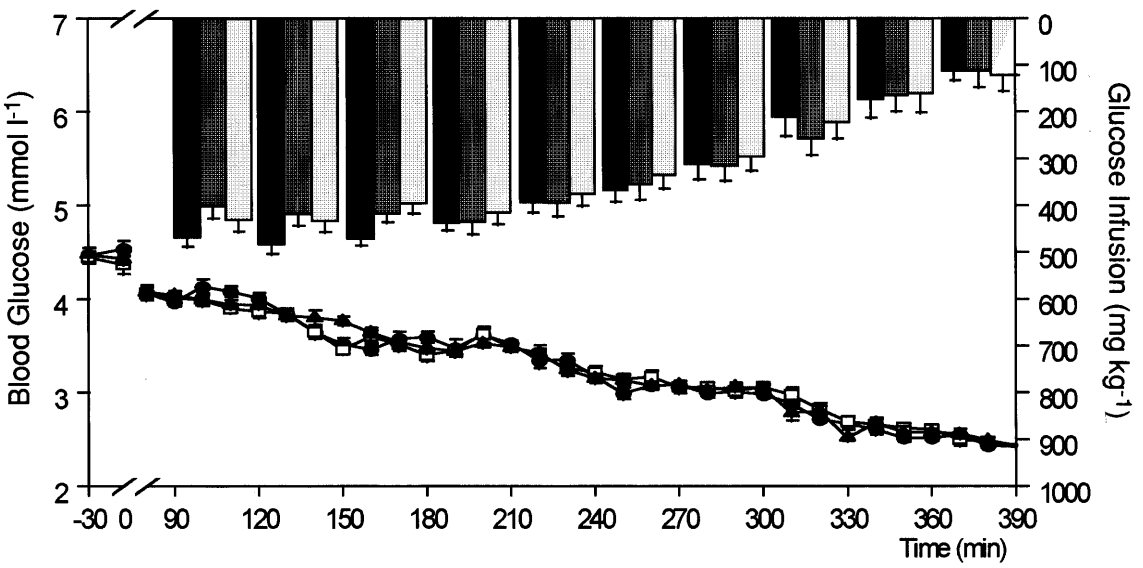

Figure 2. Blood glucose and glucose requirements (mean ± SE) for the various preparations during the euglycaemic-hypoglycaemic clamp test. (For key to shading see caption to Figure 3.)

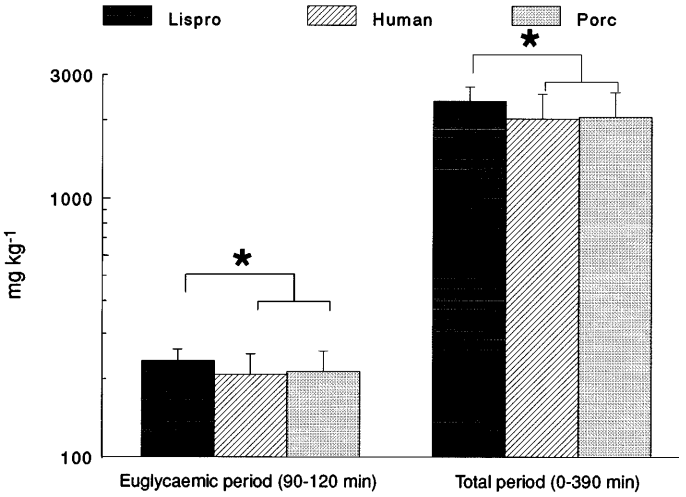

Figure 3. Total glucose requirements during the euglycaemic and the total clamp period; *$p = 0.06$ (LP analogue vs human and porcine insulin)

clamp values of 4.0 ± 0.19, 3.5 ± 0.2, 3.0 ± 0.18, and 2.5 ± 0.18 mmol l^{-1} during the first (90 to 120 min), second (150 to 210 min), third (240 to 300 min), and fourth plateaus (330 to 390 min), respectively (Figure 2). There were no differences for the blood glucose levels between the three treatments ($p > 0.1$).

Glucose Requirements

The glucose infusion rates (uncorrected for changes in measured blood glucose) during the glucose clamps tended to be higher for insulin lispro compared to human and pork insulin: 2239 ± 702 versus 1929 ± 769 versus 1957 ± 725 mg kg^{-1} (F[1,11] = 4.3; $p = 0.06$), respectively (Figure 3). This difference was also apparent when calculated for the euglycaemic clamp phase only (90 to 120 min): 236 ± 25 versus 207 ± 43 and 213 ± 42 mg kg^{-1}.

Assessment of Thresholds for Activation of Counterregulation and Initiation of Symptoms

A significant rise over baseline levels of all counterregulatory hormones, except cortisol, was observed at earlier time points than for the occurrence of symptoms. The time and blood glucose thresholds for secretion of growth hormone, adrenaline, noradrenaline, and pancreatic polypeptide were not significantly different for insulin lispro, human, and pork insulin infusions (Table 1).

Plasma Counterregulatory Hormones

Except for glucagon, the plasma levels of all counterregulatory hormones were raised significantly during the hypoglycaemic stimulus (Figure 4). No significant differences were found in the counterregulatory hormonal levels during the clamps for the three insulin preparations. The response of glucagon appeared blunted because of the assay method used. When we used another method, there was a small but significant rise. However, the amount of available spare serum was limited, so we did not include these data in our analysis.

Blood Pressure and Heart Rate

The blood pressure and heart rate are depicted in Figure 5. Analysis of the curves showed that the systolic blood pressure and the heart rate did not change during the euglycaemic-hypoglycaemic clamps. Only the diastolic blood pressure declined during the tests from initial values of 63.7 ± 7.2, 64.9 ± 6.2, and 62.9 ± 5.9 mmHg to 59.1 ± 4.7, 58.5 ± 5.4 and 58.2 ± 6.7 mmHg ($p<0.001$) for lispro, human and porcine insulin infusions. This decline in diastolic blood pressure was not different between the three treatments (F[1,11] = 28.43; $p>0.05$).

Table 1. Thresholds of counterregulatory hormones for the various insulin species: time to threshold (min) and glucose level (mmol l^{-1})

	Epinephrine		Norepinephrine		Growth hormone	
	Time to threshold	Glucose level	Time to threshold	Glucose level	Time to threshold	Glucose level
Lispro	253.8 ± 56.7	3.1 ± 0.35	241.4 ± 80.3	3.1 ± 0.49	263.8 ± 50.3	3.1 ± 0.42
Human	256.3 ± 55.3	3.0 ± 0.30	260.5 ± 82.5	3.1 ± 0.52	255.0 ± 63.6	3.2 ± 0.37
Porc	257.5 ± 70.0	3.1 ± 0.41	225.0 ± 75.9	3.2 ± 0.46	249.5 ± 50.9	3.1 ± 0.39
	Cortisol		Pancreatic polypeptide			
	Time to threshold	Glucose level	Time to threshold	Glucose level		
Lispro	307.5 ± 65.5	2.8 ± 0.43	236.3 ± 78.2	3.1 ± 0.41		
Human	304.1 ± 74.1	2.9 ± 0.43	200.0 ± 73.1	3.4 ± 0.48		
Porc	322.5 ± 40.4	2.7 ± 0.29	226.3 ± 65.5	3.3 ± 0.35		

Results expressed as mean ± SD.

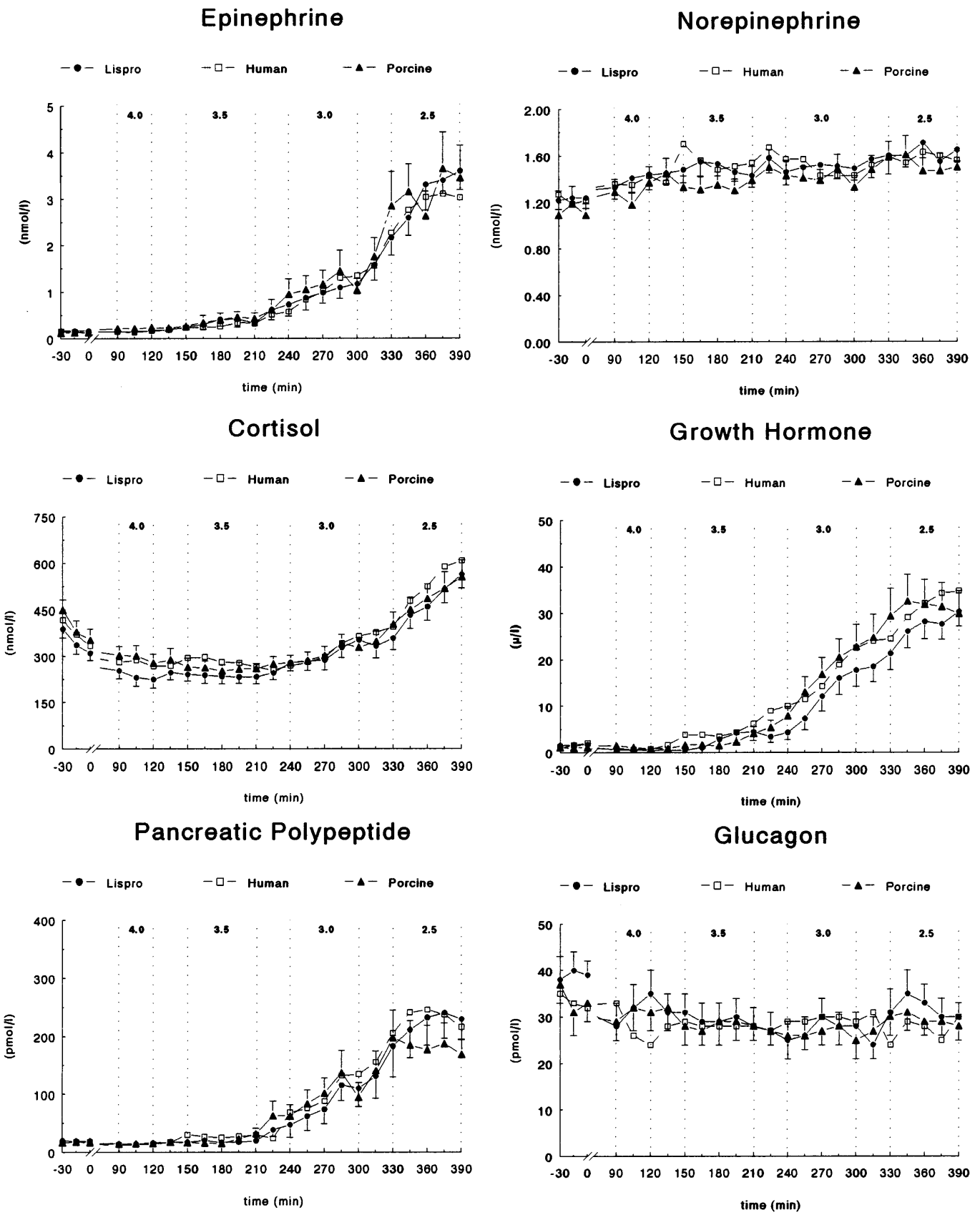

Figure 4. Hormone concentrations (mean ± SE) during the euglycaemic-hypoglycaemic clamp test. The various clamp plateaus are indicated by the dotted lines. Lispro (-●-), human (-□-), and porcine (-▲-) insulin

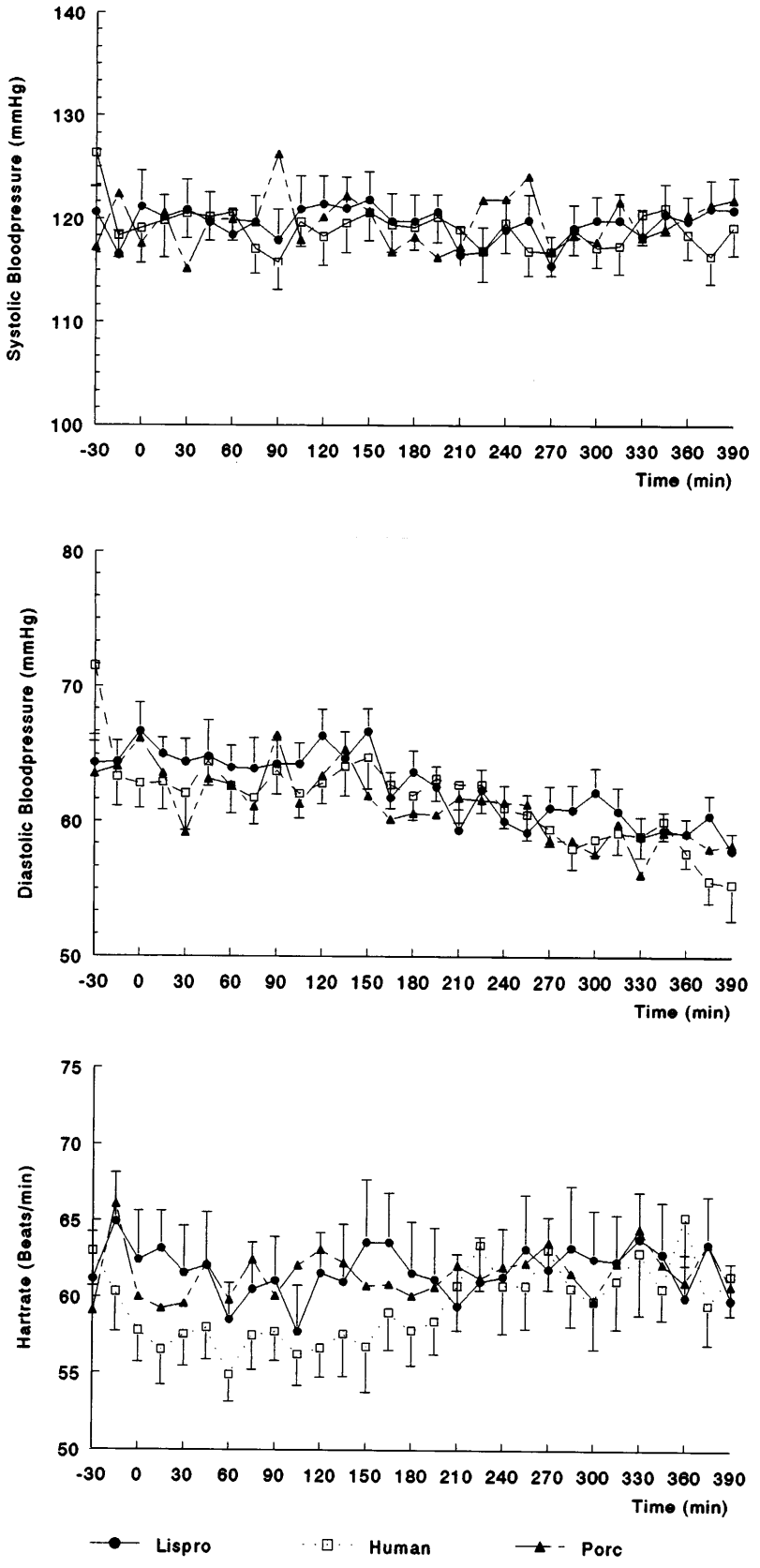

Figure 5. blood pressure and heart rate (mean ± SE) during the euglycaemic-hypoglycaemic clamp test with different species of insulin infused. Lispro (-●-), human (-□-), and porcine (-▲-) insulin

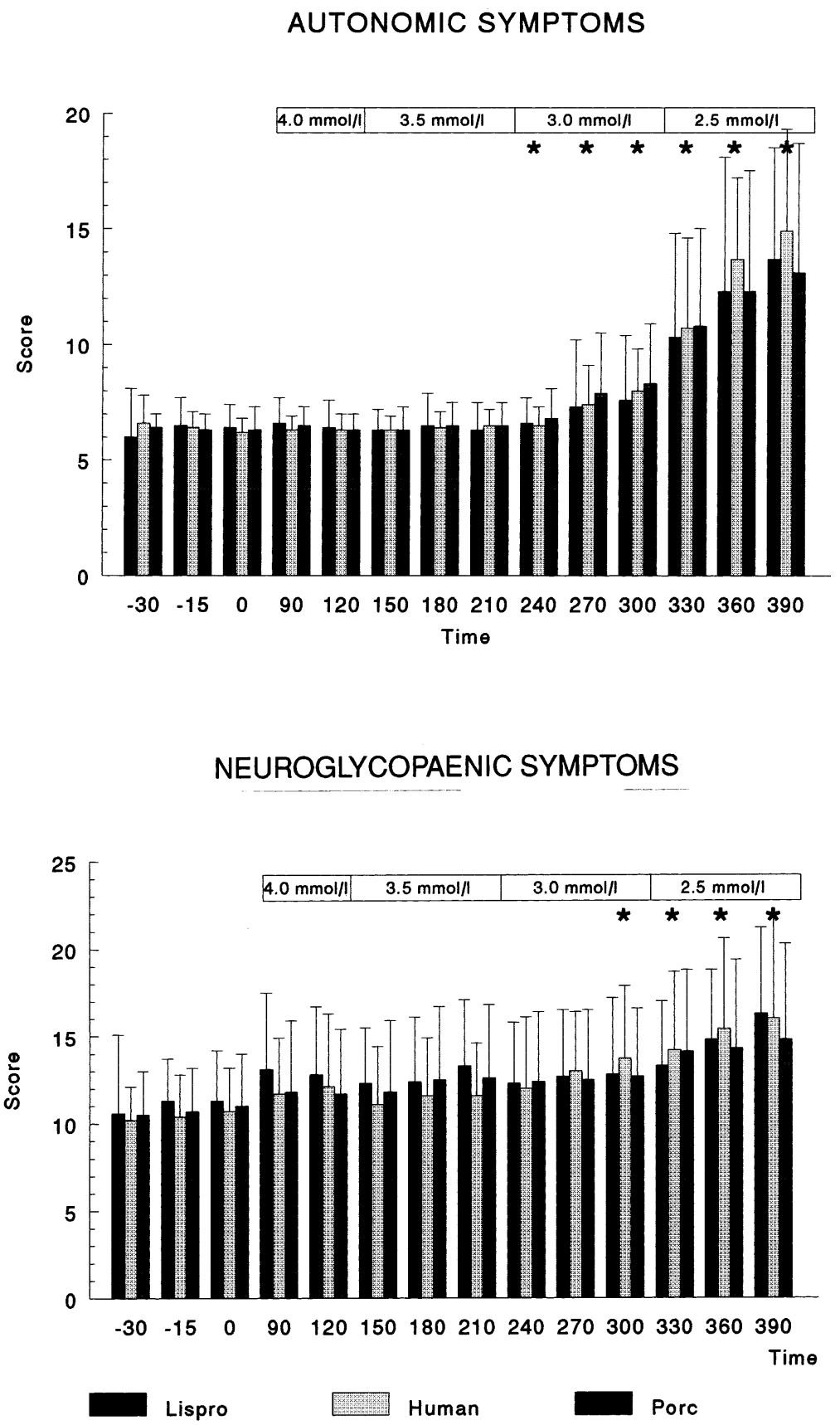

Figure 6. Symptom scores (mean ± SE) at various timepoints (min) during a stepwise insulin induced hypoglycaemia; *significant difference ($p<0.05$) with the previous score

Symptom Scores

The autonomic and neuroglycopenic symptom scores are given in Figure 6. The threshold for autonomic symptoms was attained at an earlier timepoint than the neuroglycopenic symptom threshold (240 min, plasma glucose 3.3 mmol l^{-1} versus 300 min, plasma glucose 3.0 mmol l^{-1}), but the results were not different for the treatments.

Discussion

This study demonstrates that the counterregulatory hormonal responses and symptoms during a standardized stepwise decline of the blood glucose levels, induced by insulin lispro, are comparable to those elicited by human and porcine insulin infusions. Earlier reports suggested differences between human and porcine insulin resulting in an increase in the incidence of severe hypoglycaemia due to loss of awareness.[15,16,33] Previous studies observed subnormal adrenaline and noradrenaline responses to hypoglycaemia induced by human insulin in nondiabetic subjects.[13,14] Also, a transiently weaker auditory evoked potential response (P300) was described.[34] However, other well-controlled studies could not provide evidence in favour of species differences.[21,35] These discrepancies are probably due to the variety of the methods used to assess counterregulatory hormone responses and symptoms. The present study was designed considering these potential pitfalls.

First, until recently disagreement existed about the proper classification of symptoms in autonomic and neuroglycopenic symptoms. Towler *et al*. used pharmacological blockade to confirm the pathogenic mechanisms of the symptom groups,[36] whereas Hepburn *et al*. developed an effective questionnaire for assessing the intensity of symptoms. Correct classification of autonomic and neuroglycopenic symptoms was confirmed by factor analysis.[32] In our study, no differences in intensity or distribution of symptoms could be observed for the three insulins. Furthermore, the thresholds for autonomic and neuroglycopenic symptoms in this comparative study are remarkably similar to those reported by others using the stepwise hypoglycaemic clamp.[37,38]

Secondly, the method used to induce hypoglycaemia has an important impact on the counterregulatory response. In our study, we used the stepwise euglycaemic-hypoglycaemic clamp technique with plateaus of 60 min in order to allow time for a specific level to elicit a response. The importance of maintaining the blood glucose level at a certain plateau is confirmed by the variability in response times for the counterregulatory hormone responses and trigger points for the symptom scores. This is illustrated by the rise in the neuroglycopenic symptom score at the end of the 60 min at the 3.0 mmol l^{-1} plateau. Studies applying a continuous decline are bound to find lower trigger levels due to the required response time between achieving the glucose trigger level and eliciting a response.[39,40]

Thirdly, insulin *per se* may influence the magnitude of the counterregulatory response and therefore has to be considered.[41–43] In this study, we used an infusion of 50 mU kg^{-1} h^{-1}, which attained steady plasma insulin levels in the physiologic range. Comparable insulin levels with the three insulin preparations were achieved, also illustrating the fact that when given i.v. insulin lispro displays the same kinetics as regular human and regular pork insulin.

The euglycaemic-hypoglycaemic clamp uses a glucose infusion to 'clamp' the blood glucose at desired levels providing a measurement of insulin action. Our study suggests that insulin lispro is slightly more bioactive than regular human and porcine insulin although the data analysed are raw glucose infusion rates. A comparably small potency difference was observed by other investigators.[23] However, results from other *in vivo* and *in vitro* studies showed equipotency of insulin lispro and human regular insulin.[24,26,29,44]

Acute hypoglycaemia causes an increase in heart rate, a rise in systolic blood pressure, and a modest decline in diastolic blood pressure. We observed only the last physiological response. A previous study which applied a controlled and stepwise fall in blood glucose, as in our study, found only transient increases in heart rate.[45] The slight decline in diastolic blood pressure, which was equal for the three treatments, was related to the autonomic symptoms. This suggests involvement of adrenergic mechanisms, but the effect of the resting position of the subjects during the clamp cannot be ruled out.

In conclusion, using these methods and avoiding possible confounding effects associated with insulin-dependent diabetes mellitus, gender and age, by using normal volunteers, we have demonstrated that insulin lispro *per se* elicits the same hypoglycaemic counterregulatory and symptom response as human and porcine insulins. As such there is no reason to anticipate that hypoglycaemia unawareness will be a problem with insulin lispro when administered in the treatment of diabetes. The different action profile of insulin lispro may cause changes in hypoglycaemic experience in daily life for patients and clearly this warrants further investigation.

Acknowledgements

We gratefully thank A. v. Iperen, M. Aarsen, and Gera Strijker for their skilful technical assistance and the staff of the endocrine laboratory for the various measurements. We are indebted to Eli Lilly and Company for financial support.

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