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Effect of 8 hours of hyperinsulinaemia on haemostatic parameters in healthy man

G. Mauricio-Leguizamo[1], L. Heinemann[1], R. E. Scharf[2] and M. Berger[1]

[1] Department of Nutrition and Metabolism, and
[2] Department of Haematology, Oncology and Clinical Immunology, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, FRG

Summary. The role of variations in plasma insulin concentrations as a factor possibly involved in abnormalities of haemostatic functions, and (or) the development of arterial disease, has been the subject of controversy. This study examines the "in vivo" effect of hyperinsulinaemia on haemostatic parameters in seven healthy men. Two studies were carried out in random order: (a) Hyperinsulinaemia study. Human insulin was infused by a calibrated infusion pump (0.7 mU kg^{-1} min^{-1}, for 8 h) during a euglycaemic glucose clamp, and (b) Control study 0.15 mmol/l NaCl solution was infused over 8 h. Plasma epinephrine and norepinephrine concentrations remained constant throughout the studies. Mean insulin levels during the hyperinsulinaemia study were 46.2 ± 1.6 μU/ml, i.e. approximately eightfold higher than those at baseline, whereas plasma glucose levels remained constant at 4.9 ± 0.1 mmol/l. During the control study, mean insulinaemia was 5.0 ± 0.9 μU/ml, and plasma glucose 5.2 ± 0.1 mmol/l. No statistically significant changes were observed during, or after insulin or 0.15 mmol/l NaCl infusions with regard to platelet parameters, blood coagulation, and coagulation inhibitors. These data suggest that abnormalities of the haemostatic function described during insulin-induced hypoglycaemia or in hyperinsulinaemic patients are not due to a direct action of insulin.

Key words: Haemostasis, hyperinsulinaemia, platelet function.

Arterial disease affecting both large and small calibre vessels is responsible for the major causes of the increased morbidity and mortality in diabetic patients. Considerable efforts have been made to identify the role of the haemostatic system as a factor possibly involved in the pathogenesis of vascular complications in diabetic subjects during hyperglycaemic, normoglycaemic, and hypoglycaemic conditions [1-4]. Multiple abnormalities of haemostatic functions during insulin-induced hypoglycaemia have been described; including increased platelet aggregation, alterations of coagulation factors and abnormal fibrinolytic activity [5-12]. These haemostatic dysfunctions have either been attributed to acute hyperinsulinaemia or the secondary increases of counter regulatory hormone concentrations [5, 7, 11].

On the other hand, several studies have shown that obesity is associated both with alterations of haemostatic functions and hyperinsulinaemia [13]. In addition, it has been shown that patients with hypertension are hyperinsulinaemic, independent of concomitant obesity or antihypertensive medication, and present with haemostatic abnormalities [14, 15]. Recent reports have suggested that hyperinsulinaemia may be a possible risk factor for macrovascular disease in diabetes mellitus, and that hyperinsulinaemia may relate to cardiovascular disease independent of other risk factors [16, 17].

The role of variation in plasma insulin concentration as a factor possibly involved in abnormalities of haemostatic function and (or) the development of arterial disease is unknown. The purpose of the present study was to investigate the "in vivo" effect of hyperinsulinaemia on haemostatic parameters (platelet function, blood coagulation and fibrinolytic activity) in healthy subjects by means of the hyperinsulinaemic-euglycaemic glucose clamp technique.

Subjects and methods

Subjects

Studies were carried out in seven healthy male volunteers ranging in age from 18-32 years with a body mass index of 22.5 ± 1.3 kg/m^2 (mean ± SD), who did not take aspirin or any other medication during the ten days prior to the studies. All subjects were non-smokers, and there was no family history of diabetes mellitus. The subjects were given a detailed oral and written description of the study and their written consent was obtained. The study was carried out according to the principles of the Declaration of Helsinki [18].

Study protocol

We performed two different studies in random order, separated by an interval of at least two weeks: an 8-h infusion of human regular insulin (Hyperinsulinaemia study), or infusion of 0.15 mmol/l NaCl (Control study). All studies were performed at 08.00 hours after an overnight fast, and following a rest period of 30 min after the subjects had arrived in the laboratory.

Hyperinsulinaemia study. The volunteers were, in a resting position, connected to a Biostator (Glucose Controlled Insulin Infusion System, Life Science Instruments, Miles Laboratories Inc, Elkhart, Ind, USA) which has been previously described [19]. Briefly, it consists of the three modules: an on-line glucose analyser, a multichannel infusion system, and a computer-controller module. The glucose analyser allows continuous blood withdrawal, and the infusion system allows precise administration of a glucose solution. A double lumen catheter for continuous blood glucose determination was inserted retrogradely into a forearm vein. The hand was placed in a heated chamber (55° C) for the duration of the study. A second polyethylene catheter was inserted into an antecubital vein for blood sampling. Human insulin (Actrapid HM, Novo Laboratories, Copenhagen, Denmark) in albumin 0.15 mmol/l NaCl dilution (1:20) was infused by a calibrated infusion pump (Harvard Apparatus, South Natick, Mass, USA) at an infusion rate of 0.7 mU kg^{-1} min^{-1}, for 8 h (aimed at elevating plasma insulin levels to 50 μU/ml) into an antecubital vein of the contralateral arm. The euglycaemic glucose clamp was continued for an additional 2 h after the termination of the insulin infusion. The subjects were kept fasting during the experiments.

The basal blood samples for insulin, glucose, epinephrine, norepinephrine, non esterified fatty acids (NEFA), platelet count, β-thromboglobulin (βTG), platelet factor 4 (PF 4), prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, fibrinopeptide A (FPA), factor VIII coagulant activity (F VIII:C), plasminogen, protein C activity, antithrombin III activity (AT III), and α2-antiplasmin (α2-AP) measurements were obtained before the start of the insulin infusion. Seven separate venipunctures in forearm veins were performed at the following times: 1, 2, 4, 6, 8, 9, and 10 h, throughout each study.

Control study. A polyethylene catheter was inserted into an antecubital vein for an 8-h infusion of 0.15 mmol/l NaCl solution by Infusomat (B. Braun, Melsungen, FRG) at an infusion rate of 125 ml/h. The basal blood samples were obtained before the start of the NaCl infusion. Venipunctures for blood samples were performed at the same times as described above for the hyperinsulinaemia study.

Preparation of plasma samples. Blood samples were withdrawn through a 21 gauge siliconized "butterfly" needle (Abbott, Sligo, Ireland). Immediately after venipuncture, the tourniquet was released, and blood was withdrawn into a 5 ml polypropylene syringe (containing the respective and adequate amount of anti-coagulant solution), and transferred immediately to pre-cooled tubes placed in melting ice. For the plasma βTG and PF 4 assays 2.7 ml blood was collected into a mixture of 0.1 ml Na_2EDTA 0.27 mol/l, 0.1 ml theophylline 30 mmol/l, 0.1 ml PGE_1 100 μmol/l and 30 μl indomethacin 800 μmol/l. For the FPA assay 4.5 ml blood was collected into 0.5 ml of a mixture of heparin 1000 U/ml and aprotinin 1000 U/ml in a buffer containing NaCl 0.13 mol/l. For the determination of coagulation, fibrinolytic and inhibitory parameters nine volumes of blood were collected into one volume of trisodium citrate 0.11 mol/l [20]. Syringes for blood collection were used in the following chronological order: (a) βTG and PF4, (b) FPA, (c) citrated blood, (d) glucose and chemistry assays, and (e) insulin and catecholamines. Platelet rich plasma (PRP) and platelet poor plasma (PPP) were obtained by centrifugation at 150 *g* for 15 min or at 1500 *g* for 30 min at 4° C, respectively. Citrated blood was centrifuged at 2000 *g* for 30 min at room temperature. The plasma was carefully separated and stored frozen at −20° C until the day of assay. Each determination was made in duplicate, the mean values being taken as the final result. Platelet count, PT, aPTT, and F VIII:C were determined immediately after blood processing.

Analytical methods

Plasma and intraplatelet levels of βTG and PF4 were determined by radioimmunoassay kits from the Radiochemical Centre (Amersham Buchler, Braunschweig, FRG) and Abbott Laboratories (Wiesbaden-Delkenheim, FRG) respectively; details of the methods have been previously described [20, 21]. For determination of intraplatelet βTG and PF4 content platelets were lysed by adding 20 μl of 10% volume/volume Triton X-100 to 180 μl of PRP. Blood platelet counts were performed in PRP using a Thrombocounter (Coulter Electronics, Hialeah, Fla., USA). PT and aPT were assayed by one-stage technique using an automated clot detection system and reagents purchased from Boehringer Mannheim (Mannheim, FRG). Plasma fibrinogen was measured according to Clauss [22]. F VIII:C was assayed by one-stage technique based on the normalization of the clotting time of specific factor deficient plasmas [23]. FPA was measured by radioimmunoassay using the RIA-Quant FPA kits (Mallinckrodt Diagnostica, Dietzenbach, FRG) [24]. Protein C activity was determined photometrically by the chromogenic method using BCP 300 (pGlu-Pro-Arg-MNA [2-Methoxinitroaniline]) as substrate (Behringwerke AG, Marburg, FRG) [25]. AT III was evaluated by an antithrombin (heparin cofactor) assay (KabiVitrum AB, Stockholm, Sweden) [26]. Plasminogen was measured using an excess of streptokinase as described by Gram and Jespersen [27] (KabiVitrum AB, Stockholm, Sweden). α_2-AP was assayed by a chromogenic method (KabiVitrum AB, Stockholm, Sweden) [28]. Plasma glucose was monitored by the glucose oxidase method (Glucose Analyzer, Beckman In-

Table 1. Plasma concentrations of free insulin, glucose, non esterified fatty acid (NEFA) and catecholamines at baseline, during and after the hyperinsulinaemia (A) and control study (B) in seven healthy subjects

		Insulin or 0.15 mmol/l NaCl infusion							
	Time (h)	0	1	2	4	6	8	9	10
Free insulin (μU/ml)	A	6.1± 4.3	45.5± 7.0[a, b]	50.9± 14.7[a, b]	47.6± 10.6[a, b]	46.3± 5.6[a, b]	43.4± 8.7[a, b]	7.6± 3.6	5.3± 1.3
	B	5.2± 1.9	5.5± 1.7	6.0± 1.6	5.3± 1.5	3.9± 1.8	3.9± 1.2	3.7± 2.0	4.8± 1.7
Glucose mmol/l	A	4.9± 0.3	4.7± 0.3	4.9± 0.0	4.9± 0.0	4.9± 0.0	4.9± 0.1	5.0± 0.5	5.3± 0.2
	B	5.2± 0.1	5.2± 0.1	5.0± 0.1	4.9± 0.1	4.9± 0.1	4.9± 0.1	4.8± 0.0	4.8± 0.1
Plasma NEFA μmol/l)	A	1013 ±118	888 ±170[a, b]	841 ±158[a, b]	810 ±153[a, b]	743 ±143[a, b]	708 ±138[a, b]	728 ±149[a, b]	763 ±172[a, b]
	B	1032 ±176	977 ±161	962 ±167	930 ±176	962 ±151	1007 ±136	1006 ±151	1073 ±206
Epinephrine (pg/ml)	A	43 ± 14	41 ± 9	42 ± 13	39 ± 13	45 ± 25	38 ± 11	41 ± 12	43 ± 10
	B	52 ± 17	38 ± 10	37 ± 11	40 ± 12	39 ± 11	47 ± 15	47 ± 11	53 ± 11
Norepinephrine (pg/ml)	A	250 ± 73	195 ± 75	181 ± 74	176 ± 48	185 ± 67	194 ± 48	215 ± 65	204 ± 47
	B	283 ±102	254 ±103	165 ± 78	192 ±128	186 ±122	193 ± 91	165 ± 26	212 ± 33

[a] $p < 0.01$ vs baseline at 0 h; [b] $p < 0.01$ vs respective value during the control study (B)

Table 2. Effect of insulin (A) and 0.15 mmol/l NaCl infusion (B) on platelet parameters in seven healthy subjects

		Insulin or 0.15 mmol/l NaCl infusion							
	Time (h)	0	1	2	4	6	8	9	10
Platelet count ($x10^9$/l)	A	337 ±71	325 ±117	288 ±86	265 ±76	348 ±138	296 ±105	364 ±97	330 ±68
	B	288 ±44	287 ± 43	281 ±61	285 ±32	273 ± 45	276 ± 68	320 ±42	300 ±57
βTG (ng/ml)	A	25.3± 5.1	27.3± 8.3	28.8± 6.4	31.3± 9.0	33.1± 8.9	30.4± 7.9	33.3± 7.3	28.1± 8.6
	B	24.1± 7.6	20.8± 9.0	18.4± 6.9	18.4± 5.2	17.1± 7.7	16.8± 5.5	17.1± 8.3	18.4± 6.9
βTG ($\mu g/10^9$ pla[a])	A	45.5± 5.6	37.1± 7.5	42.2±10.6	42.4±11.7	34.8± 8.0	42.0± 10.5	33.1± 7.5	42.5± 9.7
	B	43.1±10.5	42.5± 10.3	44.1± 8.7	40.6± 6.6	42.6± 12.3	44.5± 7.0	42.0± 9.0	39.0±11.6
PF4 (ng/ml)	A	5.5± 1.7	5.1± 1.9	4.5± 1.9	5.1± 1.6	4.7± 1.7	4.7± 2.0	5.1± 1.9	3.8± 2.1
	B	4.8± 1.9	3.7± 2.4	2.7± 1.9	3.4± 1.9	3.0± 2.1	2.8± 1.8	3.0± 2.1	3.1± 2.1
PF4 ($\mu g/10^9$ pla[a])	A	14.0± 3.7	13.2± 3.0	14.7± 3.7	13.8± 3.7	12.2± 3.9	14.7± 2.8	13.0± 3.5	14.1± 2.9
	B	12.8± 3.3	14.0± 3.5	14.5± 4.5	14.8± 3.9	14.6± 4.8	14.1± 2.3	13.5± 3.9	13.8± 3.4

[a] Platelet βTG = β-Thromboglobulin, PF4 = platelet factor 4

Table 3. Effect of insulin (A) and 0.15 mmol/l NaCl infusion (B) on blood coagulation, fibrinolytic and inhibitory parameters in seven healthy subjects

		Insulin or 0.15 mmol/l NaCl infusion							
	Time (h)	0	1	2	4	6	8	9	10
PT (s)	A	13.3± 1.3	12.5± 1.0	12.7± 1.1	13.3± 1.5	12.8± 0.9	12.9± 0.6	13.0± 0.7	13.3± 1.1
	B	12.2± 0.6	12.5± 0.6	12.1± 0.5	12.4± 0.7	12.5± 0.5	12.3± 0.5	12.8± 0.6	12.5± 0.5
aPTT (s)	A	36.9± 3.4	36.8± 4.2	36.6± 3.0	38.9± 4.7	37.8± 3.3	37.5± 2.7	37.6± 2.1	36.9± 2.5
	B	39.3± 4.2	39.0± 3.9	40.5± 4.7	40.2± 4.1	39.4± 4.8	38.4± 4.8	38.1± 4.4	37.6± 4.3
Fibrinogen (mg%)	A	217 ±75	197 ±57	199 ±63	194 ±55	216 ±109	208 ±68	191 ±44	200 ±51
	B	219 ±47	234 ±47	231 ±46	233 ±38	245 ± 31	239 ±36	237 ±46	237 ±47
FPA (ng/ml)	A	1.6± 0.3	1.4± 0.3	1.4± 0.3	1.7± 0.5	1.8± 0.7	1.6± 0.5	1.5± 0.4	1.6± 0.2
	B	2.2± 0.6	1.3± 0.5	1.4± 0.3	1.5± 0.7	1.1± 0.2	1.5± 0.5	1.8± 0.6	1.7± 0.7
F VIII:C (s)	A	61.2± 6.3	67.2± 8.8	64.6± 8.6	66.1± 7.5	66.3± 8.4	64.7± 8.3	65.9± 6.4	66.9± 4.1
	B	65.5± 8.0	66.4± 9.3	65.6±10.0	68.0±10.0	67.2± 9.9	65.1± 7.7	64.9± 7.8	64.7± 5.3
Plasminogen (%)	A	90.7±10.3	84.2±12.1	85.7± 6.1	85.2± 7.8	85.2± 7.2	84.2± 5.4	85.0± 8.2	86.8± 8.1
	B	99.6±19.4	95.7±17.7	94.0± 8.5	91.4±13.7	87.3± 8.9	85.9± 9.0	89.5±12.2	92.2±15.7
Protein C (%)	A	82.0±19.5	78.5±17.0	76.0±19.5	77.3±14.9	76.3± 15.0	76.3±13.4	73.5±16.7	78.5±13.5
	B	80.0± 9.5	75.0±11.0	80.5±14.1	76.8±11.9	77.8± 11.3	78.5±12.1	79.5±11.3	81.8±14.0
AT III (%)	A	98.2± 9.8	93.1± 7.5	92.7± 8.3	92.7±12.6	91.2± 10.2	85.7±13.4	87.7± 8.8	88.7± 5.6
	B	93.8± 4.8	100.2± 9.4	91.3± 6.7	92.7± 6.8	90.5± 9.7	88.3± 9.4	93.4± 9.4	94.9± 9.4
α2-AP (%)	A	102.1± 7.0	93.5± 6.1	98.5± 8.7	96.6± 8.9	95.1± 9.5	94.0± 7.2	97.7±12.8	101.6±11.2
	B	102.5± 4.8	100.0± 7.7	105.2±12.3	100.0± 7.5	98.1± 6.5	94.3± 4.9	98.2± 4.4	98.5± 2.9

PT = prothrombin time, aPTT = activated partial thromboplastin time, FPA = fibrinopeptide A, F VIII:C = factor VIII coagulant activity, AT III = antithrombin III activity, α_2-AP = α_2-antiplasmin

struments, Fullerton, Calif., USA). Insulin was measured with a commercially available double antibody radioimmunoassay (Phadebas Co., Uppsala, Sweden). The inter-assay coefficient of variation of the insulin assay was 10.3% at 4 μU/ml and 5.1% at values greataer 30 μU/ml. The intra-assay coefficient of variation ranged between 2.1 and 3.1% for low and high insulin concentrations. The polyethylene glycol extraction for plasma free insulin was performed immediately after blood withdrawal, i.e. after centrifugation of 3 ml blood, 1.0 ml plasma and 1.0 ml of added 30% polyethylene glycol (mol 6000) were thoroughly mixed. After centrifugation the supernatant was stored at −20°C. Plasma catecholamine and NEFA concentrations were assayed as described earlier by Kemmer et al. [29].

Statistical analysis

All data are expressed as mean ± SD. Their statistical significance was evaluated by two-tailed paried Student's *t*-test.

Results

Circulating levels of free insulin, glucose, NEFA and catecholamines before, during and after insulin or 0.15 mmol/l NaCl infusion are presented in Table 1.

Plasma insulin. The fasting levels of free insulin were similar in both studies: 6.1 ± 4.3 μU/ml (hyperinsulinaemia study), and 5.2 ± 1.9 μU/ml (control study). The insulin infusion during the hyperinsulinaemia study induced an increase of plasma insulin concentrations from basal values to mean steady-state levels of 46.2 ± 1.6 μU/ml, i.e. approximately eightfold higher than those at baseline. After the end of the insulin infusion, insulin levels returned toward the basal values. In the control study, plasma insulin concentrations re-

mained constant during the 0.15 mmol/l NaCl infusion at 5.0 ± 0.9 μU/ml.

Plasma glucose. In the hyperinsulinaemia study, mean plasma glucose levels were 4.9 ± 0.3 mmol/l before induction of 8-h hyperinsulinaemia and remained at 4.9 ± 0.1 mmol/l during the steady-state period of insulin infusion. The mean basal plasma glucose concentrations in the control study were 5.2 ± 0.1 mmol/l and did not change throughout the studies. No significant differences were observed between the two studies. The total amount of glucose infused during the hyperinsulinaemia study was 366 ± 41 g.

Plasma free fatty acids. The fasting NEFA concentrations were not different in both studies. During the 8-h insulin infusion period, plasma NEFA levels decreased progressively from a basal concentration of 1013 ± 118 μmol/l to 708 ± 138 μmol/l. In contrast, during 0.15 mmol/l NaCl infusion, plasma NEFA levels remained constant throughout the studies.

Plasma catecholamines. No changes were seen between hyperinsulinaemia and control studies with regard to plasma epinephrine or norepinephrine concentrations.

Haemostatic parameters. The results for platelet parameters are shown in Table 2. No significant changes in platelet count or mean plasma levels of platelet specific proteins were observed throughout hyperinsulinaemia and control studies. The same was true for intraplatelet concentrations of alpha-agranular constituents that remained unchanged, both in the hyperinsulinaemia study and the control study. The plasma parameters of blood coagulation, fibrinolytic and inhibitory activity are shown in Table 3. No differences were observed in PT, aPTT, fibrinogen, FPA, F VIII:C, plasminogen, protein C, AT III, or α_2-AP between the hyperinsulinaemia and the control studies.

Discussion

In this study the effects of plasma insulin on various factors involved in normal haemostatic function were investigated during hyperinsulinaemic-euglycaemic clamp conditions compared to a normoinsulinaemic-euglycaemic control study. The expected decrease in plasma NEFA levels during the infusion of exogenous insulin demonstrated the biological effectiveness of the obtained insulin levels (≈ 50 μU/ml), whilst blood glucose was clamped at 5.0 mmol/l [30].

Varying concentrations in plasma insulin levels that may be involved in abnormalities of haemostatic functions have been the subject of controversy. Recently, it has been suggested that insulin has direct effects on the endothelial cell and erythrocyte functions [31]. In the present study haemostatic parameters of seven healthy subjects were evaluated before, during and after an 8-h period of sustained hyperinsulinaemia. No significant changes between hyperinsulinaemia and control studies were observed with regard to platelet parameters, blood coagulation, and coagulation inhibitors. Trovati et al. described that insulin-induced hypoglycaemia influences various platelet functions [5]. It has been suggested that epinephrine may be responsible for the hypoglycaemia-induced increase of platelet aggregation and release of platelet specific proteins [5, 32]. Likewise, Corrall et al. noted that the rise in F VIII:C following hypoglycaemia is mediated via an adrenergic mechanism [7]. Based on our hyperinsulinaemia study where the haemostatic parameters and plasma catecholamine concentrations remained constant in all subjects, it is suggested that haemostatic changes occurring in response to insulin-induced hypoglycaemia in normal and diabetic subjects result from the secretion of counter regulatory hormones, which are part of the physiological response to hypoglycaemia, but not due to any direct action of insulin. Another area of controversy concerns the association of hyperinsulinaemia with fibrinolytic alterations as a factor possibly related to the development of macrovascular disease in diabetes mellitus and coronary heart disease in hyperinsulinaemic patients. With respect to the fibrinolytic system, no conclusions can be drawn from this study since we did not evaluate euglobulin clot lysis time, tissue-type plasminogen activator, or plasminogen activator inhibitors. In this study, 8 h of sustained hyperinsulinaemia in normoglycaemic subjects did not cause any changes of the haemostatic parameters evaluated. These data suggest that abnormalities of haemostatic functions described in hyperinsulinaemic patients are not due to hyperinsulinaemia per se. However, possible effects of more prolonged hyperinsulinaemia when associated with other atherosclerosis risk factors (such as hypertension or lipoprotein abnormalities) with regard to the development of atherogenesis and (or) thrombosis cannot be excluded on the basis of this study.

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Prof. M. Berger
Abteilung für Stoffwechsel und Ernährung
Medizinische Klinik der Heinrich-Heine-Universität
Moorenstraße 5
D-4000 Düsseldorf 1
FRG