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Counterregulatory Hormone Responses During Graded Hyperinsulinemic Euglycemia in Conscious Rats

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**Department of Endocrinology and Metabolic Diseases, University Hospital, Building 1, C4R82, Rijnsburgerweg 10, 2333 AA Leiden, The Netherlands †Netherlands Institute for Drugs and Doping Research, Department of Psychopharmacology, University of Utrecht, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands*

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KOOPMANS, S. J., S. F. DE BOER, J. K. RADDER, M. FRÖLICH AND H. M. J. KRANS. *Counterregulatory hormone responses during graded hyperinsulinemic euglycemia in conscious rats.* PHYSIOL BEHAV **54**(6) 1141–1148, 1993.—It has been suggested that hyperinsulinemia per se may affect the levels of some counterregulatory hormones in the absence of hypoglycemia. We studied the effect of graded hyperinsulinemia and concomitant increased glucose metabolism on the levels of counterregulatory hormones by means of the 5-step sequential hyperinsulinemic euglycemic clamp technique, combined with [3-^{3}H]-glucose infusion, in conscious rats. Insulin infusion rates (IIR) of 0, 0.5, 1, 3, and 16 mU/min, resulted in steady-state plasma insulin levels (mean ± SEM) of 24 ± 4, 44 ± 3, 98 ± 8, 418 ± 48, and 6626 ± 361 μU/ml, peripheral glucose uptake (PGU) of 3.1 ± 0.2, 3.6 ± 0.3, 5.4 ± 0.3, 9.2 ± 0.4, and 12.4 ± 0.2 mg/min and hepatic glucose production (HGP) of 3.1 ± 0.2, 2.4 ± 0.4, 0.8 ± 0.3, −0.1 ± 0.2, and −0.5 ± 0.3 mg/min, respectively. Plasma glucagon levels were half maximally suppressed between IIRs of 0.5 and 1 mU/min and maximally suppressed at 3 mU/min. The suppression exactly paralleled the inhibition of HGP ($r = 0.87 \pm 0.04$, $p < 0.02$) but not the stimulation of PGU ($r = -0.66 \pm 0.12$, p = NS). This suggests that the inhibition of HGP by insulin is at least partially mediated by a simultaneous suppression of plasma glucagon levels. The adrenal hormones corticosterone and epinephrine were not influenced during the clamp. Moreover, the circadian rhythm of corticosterone seemed unaffected. Plasma norepinephrine levels were increased (±50%, $p < 0.05$) at IIRs of 1 mU/min and higher, suggesting an insulin-induced stimulation of the sympathetic nervous system when peripheral plasma insulin levels exceed 98 ± 8 μU/ml. In conclusion, measurement of in vivo insulin action by means of the euglycemic clamp induces dose-dependent changes in the levels of glucagon and norepinephrine but not in epinephrine and corticosterone. This has to be taken into account because it is, in fact, the interaction between insulin and these counterregulatory hormones that determines the ultimate action of insulin on glucose metabolism in vivo.

Euglycemic clamp Peripheral glucose uptake Hepatic glucose production Insulin Glucagon Norepinephrine Epinephrine Corticosterone Sodium Potassium Calcium Plasma protein

HYPOGLYCEMIA stimulates the release of insulin-counter-regulatory hormones (7,36). In most studies, hypoglycemia was induced by the administration of insulin (19,44,47). It has been suggested that hyperinsulinemia per se might also affect the levels of certain counterregulatory hormones. In humans and dogs, some investigators have shown that hyperinsulinemia, in the absence of hypoglycemia, elevates the levels of plasma norepinephrine, indicating that insulin elicits a stimulatory effect on the sympathetic nervous system (13,20,27,31). Others could not confirm this (10,40,41) or the data were controversial (9). Plasma epinephrine levels seem not to change under conditions of moderate hyperinsulinemia (8,10,13,14,20,40,41). The suppressive effect of insulin on plasma glucagon, irrespective of ambient glucose levels, has been described (8,24,37), although a recent study (4) could not detect any effect of chronic hyperinsulinemia on plasma glucagon levels in rats. Finally, to our knowledge, no data are available concerning plasma corticosterone responses during hyperinsulinemic euglycemia.

At present, no dose–response study has been performed to determine the effect of a broad range of plasma insulin concentrations, from basal physiological to pharmacological levels, on the plasma levels of the four mentioned counterregulatory hormones in the same study. Knowledge of this relationship would give us an indication about the sensitivity and responsiveness (maximal response) of counterregulatory hormones for hyper-insulinemia in rats. This is of particular interest because it is

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well known that a change in the levels of norepinephrine, epinephrine, glucagon, and corticosterone have a great impact on glucose metabolism and it is, in fact, the interaction between insulin and counterregulatory hormones that determines the ultimate effect of hyperinsulinemia on glucose metabolism (23).

In the present study we investigated the effect of graded hyperinsulinemia and concomitant increased glucose metabolism on the levels of plasma norepinephrine, epinephrine, glucagon, and corticosterone by means of the 5-step sequential hyperinsulinemic euglycemic clamp technique, combined with 3-^{3}H-glucose infusion, in unanaesthetized, unrestrained rats.

The use of the hyperinsulinemic euglycemic clamp technique allowed us to induce hyperinsulinemia without changing plasma glucose levels. Furthermore, the clamp technique was combined with a continuous infusion of [3-^{3}H]-glucose for measurement of whole body glucose turnover. Now, a discrimination could be made between hepatic glucose production and peripheral tissue glucose uptake (33). In this way we were able to correlate changes in glucose production and glucose uptake with possible changes in the levels of counterregulatory hormones. Finally, the use of conscious rats allowed us to study insulin-counterregulatory hormones in the absence of anaesthesia-induced disturbances of the central nervous system. When counterregulatory hormones are studied it is important to perform the investigations in a nonstressed animal model, because these hormones are known to be sensitive to stressful stimuli (1,29). In addition, insulin-mediated glucose production and glucose uptake are affected by anaesthesia (29).

METHOD

Animals and Housing

Fifteen male Wistar rats (±400 g) with free access to complete laboratory rat chow (Hope Farms b.v. Woerden, The Netherlands) and water were individually housed in Plexiglas metabolic cages (21 × 22 × 26 cm) at a constant temperature (23°C) and a fixed 12-h light/12-h dark cycle (lights on at 0700 h). Rats were acquired and used in compliance with the Dutch law and institutional regulations.

Surgical Procedure

Under complete ether anaesthesia, rats were provided with two sterile silicon cannulae, one into the right jugular vein for the infusion of fluids and the other into the left carotid artery for blood sampling, as described before (17). Surgical manipulations were performed according to the method of Steffens (38). After surgery, the rat was attached to a swivel (42) and was placed in a metabolic cage. The rats were allowed to recover for at least 1 week before the clamp experiments were performed. During this period the arterial catheter was aspirated every other day to maintain catheter patency and to adapt the rats to the clamp and blood-sampling procedure. Within a week after surgery the rats reached their preoperative weight and were used for the clamp studies.

Euglycemic Clamp Studies

Food was withdrawn, if applicable, 12 h before the clamp was performed. One hour before clamping the venous and arterial cannulae were aspirated, the venous line was filled with a 0.9% NaCl solution, and the arterial line with a 0.9% NaCl solution containing 5 IU/ml heparin. The double lumen swivel, allowing separate fluid infusions, was connected to two peristaltic pumps (Watson Marlow 202U/AA, Falmouth, England). One venous line was used for the infusion of a 20% glucose solution at a variable rate and the other line was used for a combined infusion of [3-^{3}H]-glucose (Du Pont-NEN, Boston, MA, spec. act. 13.5 Ci/mmol), insulin (Actrapid porcine insulin, Novo, Copenhagen, Denmark), and 1% rat plasma in insulin dilution medium (Novo, Copenhagen, Denmark) at a constant rate (400 μl/h). The carotid cannula was connected to a blood-sampling tubing which allows frequent sampling of arterial blood and repletion of blood loss by means of fresh prewarmed whole blood obtained from littermates. The transfusion blood contained 50 IU/ml heparin to prevent clotting.

Before starting the insulin tracer clamp in seven rats, a bolus (3 μCi) continuous (0.05 μCi/min) infusion of [3-^{3}H]-glucose was initiated and continued throughout the study. At t = 70, 80, and 90 min blood samples were collected to measure plasma glucose (n = 3), tritiated glucose specific activity (n = 3), insulin (n = 2), and glucagon (n = 1). Subsequently a sequential 4-step hyperinsulinemic euglycemic glucose clamp was started in periods of 90 min at increasing insulin infusion rates of 0.5, 1, 3, and 16 mU/min. A bolus of insulin was given prior to each insulin infusion period (2.5, 5, 15, and 80 mU). Blood (40 μl) was collected at 5–10-min intervals for fast determination of plasma glucose, so the variable 20% glucose infusion could be adjusted to reach and maintain plasma glucose at 6 mM according to the negative feedback principle (30,33). During steady state, the final 20 min of each insulin infusion period, blood samples were taken at 10-min intervals for plasma glucose (n = 3), tritiated glucose specific activity (n = 3), insulin (n = 2), and glucagon (n = 1). During the first 15 min of each individual insulin infusion step three samples of 0.7 ml heparinized donor blood were transfused to the rat, compensating previous blood loss.

In a separate set of similar clamp experiments in eight rats, plasma samples for determination of corticosterone, epinephrine, norepinephrine, calcium, sodium, potassium, and total protein were collected. During these clamps, no [3-^{3}H]-glucose was infused and plasma glucagon was not determined. Thus, the data were collected in two separate sets of experiments. This was done to avoid excessive blood transfusion during these long-term clamps.

Six control studies (SHAM) were performed mimicking the 5-step clamp, however, without insulin and glucose infusion. During the 7.5 h lasting 5-step sequential clamp the amount of fluid infused was approximately 0.5, 1, 2.5, 4, and 6 ml at insulin infusion rates of 0, 0.5, 1, 3, and 16 mU/min, respectively. During the control infusion experiments 14 ml of insulin dilution medium (Novo, Copenhagen, Denmark) was infused and 8 ml donor blood was transfused over the same time period and at the end of the experiment blood samples were collected for determination of plasma glucagon, epinephrine, norepinephrine, and corticosterone. The control experiment was carried out to see whether fluid infusion and blood transfusion per se would lead to changes in counterregulatory hormones.

Chemical Determinations

Blood samples (40 μl) for glucose determinations were collected in heparinized tubes and immediately centrifuged in a microcentrifuge (Beckman Instruments, Palo Alto, CA). Plasma glucose was measured by the glucose oxidase method (Glucose Analyzer II, Beckman Instruments, Palo Alto, CA). Blood samples (150 μl) for insulin were transferred to chilled (0°C) heparinized tubes, centrifuged within 5 min, plasma was removed, and stored at −80°C until assay. Insulin was measured in duplicate by a specific rat radioimmunoassay (16), rendering identical standard curves for rat and porcine insulin. Intra- and interassay

TABLE 1

LEVELS, RANGE, AND COEFFICIENT OF VARIATION IN PLASMA GLUCOSE LEVELS DURING EACH INSULIN INFUSION STEP OF THE SEQUENTIAL HYPERINSULINEMIC EUGLYCEMIC CLAMP IN EIGHT CONSCIOUS RATS

	Insulin Infusion Rate (mU/min)				
	0	0.5	1	3	16
Plasma glucose levels (m*M*)	6.0 ± 0.2	6.2 ± 0.1	6.1 ± 0.2	6.0 ± 0.1	5.8 ± 0.1
Range in plasma glucose levels (m*M*)	5.2–6.9	5.1–7.2	4.8–7.0	4.7–6.8	4.9–6.6
CV in plasma glucose levels (%)	4.3 ± 0.4	6.5 ± 0.7	6.8 ± 0.9	7.4 ± 1.1	6.3 ± 0.5

variations of the insulin assay were 6.8% and 8.1%, respectively, with an assay sensitivity of 2 μU/ml. Plasma calcium, sodium, potassium, and total protein concentrations were determined in 20 μl aliquots. Calcium was measured by means of atomic absorption spectrophotometry (AAS), sodium, and potassium by means of an IL-943 flame photometer (Instrumentation Laboratory, Inc., Lexington, MA) and total protein by means of the bicinchoninic acid method described by Smith et al. (34). Samples for determination of catecholamines (CA) and corticosterone (CS) (250 μl) were transferred to chilled (0°C) heparinized tubes, containing 10 μl of a solution of 25 mg/ml disodium EDTA to prevent CA degradation and centrifuged immediately. Plasma was removed and stored at −80°C until assay. The concentrations of norepinephrine and epinephrine were measured in duplicate in 20 μl perchloric acid-deproteinized plasma according to a radioenzymatic COMT-procedure (12), as described before (17). The intra- and interassay variabilities were less than 10% and 15%, respectively. Plasma CS concentrations were determined in duplicate according to a competitive protein-binding method (25), as described before (17). The intra- and interassay coefficients of variation were less than 10%.

Blood samples (250 μl) for glucagon were transferred to chilled (0°C) heparinized tubes, supplemented with 5% trasylol (10.000 KIE/ml, Bayer, Leverkusen, Germany) to prevent hormone degradation and centrifuged immediately. Plasma was removed and stored at −80°C until assay. Plasma glucagon concentrations were determined in duplicate by a radioimmunoassay (Daiichi, Tokyo, Japan). The limit of detection was 15 pg/ml, with an interassay coefficient of variation of 8.6%. Plasma for [3-^{3}H]-glucose radioactivity (150 μl) was deproteinized by barium hydroxide-zinc sulfate (Somogyi procedure), the supernatant was evaporated to dryness at 60°C to eliminate tritiated water, and counted for 10 min in a beta scintillation counter (LKB, Woerden, The Netherlands).

Calculations

The rate of exogenous infused glucose to maintain euglycemia during the steady-state period (final 20 min) of each individual insulin infusion step was used for the assessment of insulin action. All calculations were carried out in this period when the total amount of glucose taken up by all tissues of the body is equal to the input of glucose into the body. During this steady state, when the rate of glucose appearance (Ra) is equal to the rate of glucose disappearance (Rd), the glucose turnover rate (=Ra=Rd in mg/min) was calculated by dividing the [3-^{3}H]-glucose infusion rate (dpm/min) by the steady-state value of glucose specific activity (dpm/mg). Under these conditions, the glucose turnover rate is equal to the sum of the rates of exogenous infused glucose and of hepatic glucose production (HGP). From this equation the rate of HGP can be calculated. Peripheral glucose uptake (PGU) = glucose turnover rate = exogenous glucose infusion rate + rate of HGP. In the basal state, the rate of HGP (Ra) is equal to Rd.

Dose-response curves for in vivo insulin action on stimulation of PGU and suppression of HGP were constructed by plotting the steady-state plasma insulin levels vs. the corresponding rates of glucose uptake and production, respectively.

Maximum effect of insulin (insulin responsiveness) was assumed to exert at the insulin infusion rate of 16 mE/min resulting in plasma insulin levels of ± 5000 μU/ml.

Data Analysis

Data are expressed as mean ± SEM. Statistical analysis was performed by means of the Fisher PLSD test, after ANOVA for repeated measurements, within-subject design. The criterion of significance was set at $p < 0.05$. Statistical analysis and construction of graphs and tables were performed on an Apple Macintosh computer.

RESULTS

The clamp technique was used to induce hyperinsulinemia without changing glucose levels. This was done to study the effect of hyperinsulinemia per se, i.e., in the absence of hypoglycemia, on the levels of counterregulatory hormones. Plasma glucose levels, the range in plasma glucose levels, and the coefficient of variation (CV%) of plasma glucose levels during the 5-step hyperinsulinemic clamp are presented in Table 1. It is clear that hyperinsulinemia was applied in rats without a major change in glycemia.

Insulin-mediated glucose metabolism, as measured with the 5-step sequential hyperinsulinemic euglycemic clamp technique, combined with [3-^{3}H]-glucose infusion, is depicted in Fig. 1. The dose-response relationship between steady-state plasma insulin levels vs. inhibition of hepatic glucose production and stimulation of peripheral tissue glucose uptake reveals that glucose production is more sensitive than glucose uptake to the action of insulin.

Figure 2A shows the steady-state levels of plasma insulin and glucagon reached during the 5-step clamp. It is clear that the five consecutive insulin infusion rates have led to significantly different insulin levels. Plasma glucagon levels are half maximally suppressed between insulin infusion rates of 0.5 and 1 mU/min and maximally suppressed at 3 mU/min [one-way ANOVA for glucagon, $F(4, 24) = 4.39$, $p < 0.01$]. The control infusion/blood sampling experiment (SHAM) had no effect on plasma glucagon levels.

The inhibition of hepatic glucose production by insulin (Fig. 1) parallels the suppression of glucagon levels (Fig. 2A) during the 5-step clamp. Therefore, the correlations between plasma glucagon levels and a) hepatic glucose production and b) pe-

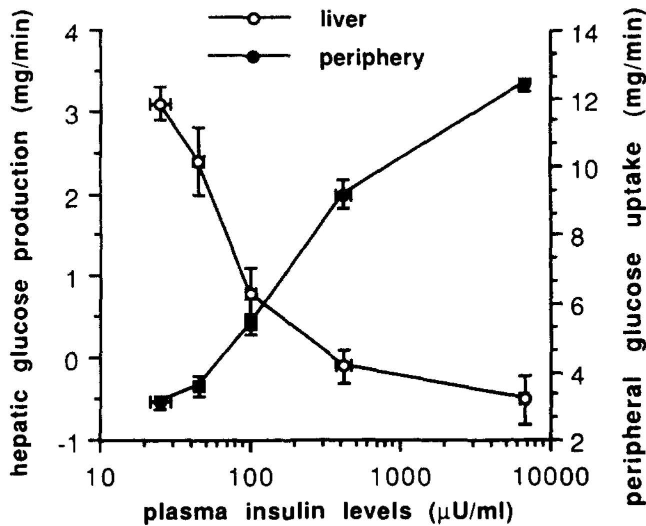


FIG. 1. Dose–response relationship between steady-state plasma insulin levels and inhibition of hepatic glucose production and stimulation of peripheral tissue glucose uptake (n = 7 rats). Data are presented as means ± SEM.

ripheral tissue glucose uptake were determined in each individual rat (Fig. 3). Between glucose production and glucagon levels a significant ($p < 0.02$) correlation ($r = 0.87 \pm 0.04$) is observed. The correlation between glucose uptake and glucagon levels is not significant with a correlation coefficient of $r = -0.66 \pm 0.12$.

Figure 2B depicts the steady-state levels of norepinephrine and epinephrine, measured during the 5-step clamp. At insulin infusion rates of 1, 3, and 16 mU/min a ±50% increase in norepinephrine levels is observed [one-way ANOVA for norepinephrine, $F(4, 28) = 3.99$, $p < 0.01$] that is not present during the control experiment (SHAM). Only at the pharmacological insulin infusion rate of 16 mU/min a small but significant increase in epinephrine levels can be seen [one-way ANOVA for epinephrine, $F(4, 28) = 2.77$, $p < 0.05$]. However, this increase is also present during the SHAM experiment, suggesting that hyperinsulinemia has no effect on epinephrine levels.

Figure 2C shows the steady-state levels of corticosterone during the 5-step clamp. At insulin infusion rates of 3 and 16 mU/min a significant increase in corticosterone levels is present [one-way ANOVA for corticosterone, $F(4, 24) = 2.98$, $p < 0.05$]. However, the SHAM-experiment reveals a similar increase. Furthermore, when the normal circadian rhythm of corticosterone is taken into account (Fig. 2C), it is clear that hyperinsulinemia and concomitant increased glucose metabolism have no effect on corticosterone levels. Moreover, the circadian rhythm seems not to be affected during the 5-step clamp.

In addition, we studied the effect of graded hyperinsulinemia and concomitant increased glucose metabolism on the plasma concentrations of several electrolytes by means of the 5-step clamp (Table 2). No significant change in calcium levels and a very small but significant decrease in sodium levels was observed [one-way ANOVA for sodium, $F(4, 20) = 5.14$, $p < 0.01$]. In contrast, potassium levels showed a significant decrease of ±20% during the 5-step clamp [one-way ANOVA for potassium, $F(4, 20) = 28.67$, $p < 0.0001$].

Furthermore, to investigate whether long-term clamping with concomitant infusion of fluids and transfusion of blood would result in a dilution problem, which could then, in turn, affect the levels of the measured hormones and electrolytes, we determined the concentration of total protein in plasma during the 5-step clamp (Table 2). No significant change in plasma total protein content was observed.

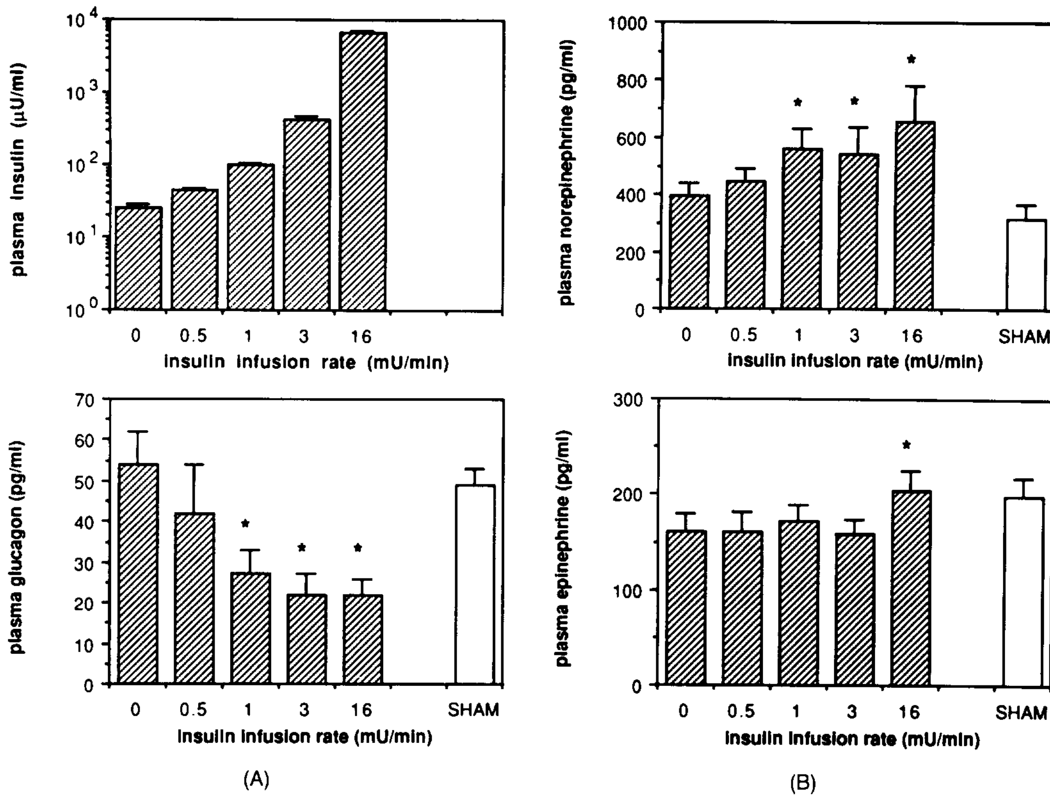
DISCUSSION

The use of conscious rats allowed us to study insulin-counterregulatory hormones in the absence of anaesthesia-induced disturbances of the central nervous system. When counterregulatory hormones are studied it is important to perform the investigations in a nonstressed animal model, because these hormones are known to be sensitive to stressful stimuli (1,29). The techniques used in this study for high frequency blood sampling and transfusion of donor blood in awake rats have been shown not influence the secretion of stress hormones like prolactin and corticosterone. In addition, blood composition has been shown to remain normal and no metabolic alterations could be detected (39,40,43). These observations are in concordance with our data that show no major changes in the levels of the stress hormones epinephrine and corticosterone during hyperinsulinemic euglycemic clamping. Also, we did not detect any changes in total protein content in plasma during the clamp.

During the 5-step sequential hyperinsulinemic euglycemic clamp, plasma glucose levels were continuously kept at the basal level of 6 mM (Table 1). Therefore, the decrease in glucagon levels and the increase in norepinephrine levels observed in this study were not indirectly caused by a change in glycemia but by an effect of hyperinsulinemia per se.

There was a significant ($p < 0.02$) correlation between plasma glucagon levels and hepatic glucose production ($r = 0.87 \pm 0.04$), whereas no significant correlation existed between plasma glucagon and peripheral tissue glucose uptake ($r = -0.66 \pm 0.12$). Although this does not prove causation, it suggests that the inhibition of hepatic glucose production by insulin is, at least partially mediated by a simultaneous suppression of glucagon levels. There is good evidence that this is, in fact, the case because several authors (5,26,36) have shown that glucagon is a more important regulator of glucose production than of glucose uptake. In this context, inhibition of hepatic glucose production is more sensitive to the action of insulin than stimulation of peripheral tissue glucose uptake (Fig. 1), which is in accordance with previous observations (33). This might be explained by the simultaneous suppressive effect of hyperinsulinemia on glucagon levels that are known to have a great impact on glucose production (5,26,36).

A discrepancy in the behavior of the catecholamines norepinephrine and epinephrine is present during the 5-step clamp. Whereas hyperinsulinemia and concomitant increased glucose metabolism elevate norepinephrine levels by ±50%, no effect is observed on epinephrine levels, suggesting that the increase in norepinephrine levels is not from adrenal output. Although the adrenal medulla secretes, besides epinephrine, norepinephrine as well (11), it is now believed that plasma norepinephrine originates from leakage from peripheral sympathetic nerve endings into the blood stream. Our data suggest, therefore, that hyperinsulinemia and concomitant increased glucose metabolism have no effect on the adrenal medulla but have a stimulatory effect on the sympathetic nervous system. Indeed, there is now direct evidence from microelectrode nerve recordings that euglycemic hyperinsulinemia increases muscle nerve sympathetic activity (2). This stimulation might be triggered by insulin binding to insulin receptors that are present in the brain (21). Recently it has been shown that peripheral circulating insulin can penetrate in the cerebrospinal fluid and, thus, can easily reach the central nervous system (39). Furthermore, insulin transport through the



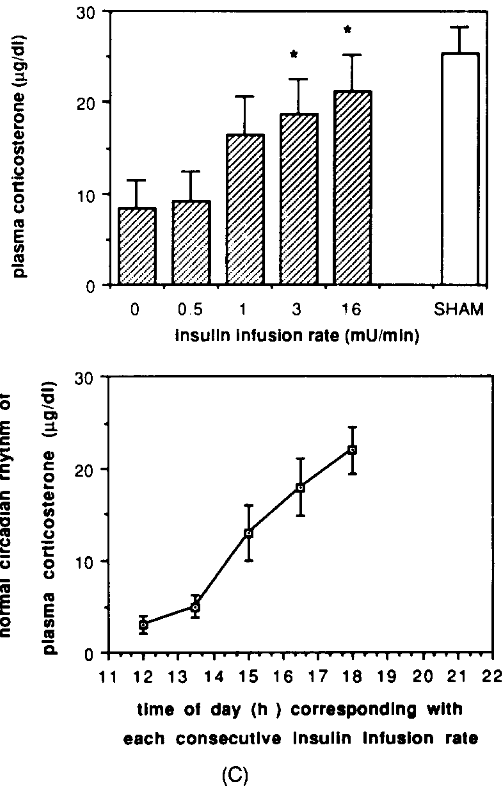


FIG. 2. (A–C) Steady state plasma insulin, glucagon, norepinephrine, epinephrine, and corticosterone levels measured during the 5-step sequential hyperinsulinemic euglycemic clamp technique. *Significant difference from basal insulin infusion rate (0 mU/min), $p < 0.05$ by Fisher PLSD test, after ANOVA for repeated measurements, within-subject design (n = 6–8 rats). Data are presented as means ± SEM. SHAM = control infusion/blood transfusion experiment.

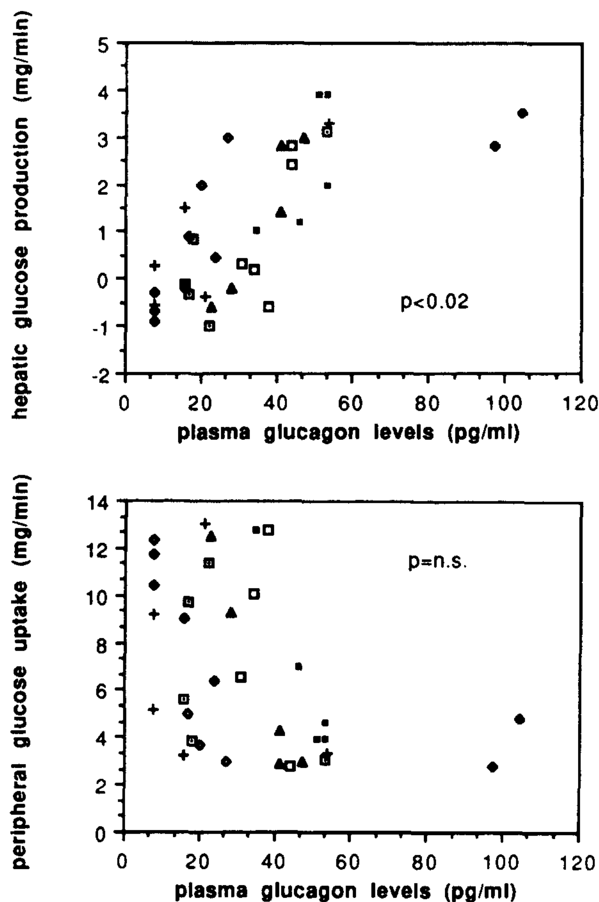


FIG. 3. Correlation between steady-state plasma glucagon levels and inhibition of hepatic glucose production and stimulation of peripheral tissue glucose uptake during the 5-step sequential hyperinsulinemic euglycemic clamp technique. Each symbol represents an individual rat and the mean correlation was calculated from each individual rat (n = 7 rats).

blood–brain barrier by means of a combined endocytosis-exocytosis system is demonstrated (28).

The physiological role of increased sympathetic activity during hyperinsulinemia remains speculative, but it might be that norepinephrine acts as a local modulator of glucose metabolism in specific innervated tissues (skeletal muscle), in the role of a neurotransmitter, and in the entire body, in the role of a peripheral circulating hormone modulating, for instance, lipolysis in adipose tissue (6,22,35). Because norepinephrine leaks from nerve endings into the bloodstream it is expected that locally (i.e., in innervated muscle) high concentrations of norepinephrine are present when, as in our study, in the blood stream a 50% increase is measured.

Finally, postganglionic norepinephrine-containing nerve endings have mainly been found in the vascular walls of peripheral tissues (i.e., skeletal muscle) (11,15). This indicates that the sympathetic nervous system (norepinephrine) could play an important role in modulating blood flow and, thus, the supply of metabolic substrates for specific peripheral tissues during hyperinsulinemia. Because insulin has direct vasodilating effects (18,20) and norepinephrine exerts vasoconstricting effects (32), the hyperinsulinemia-induced local rise in tissue norepinephrine concentrations and the general rise in plasma norepinephrine levels could thereby act in concert to (locally) modulate blood flow.

In contrast to norepinephrine, epinephrine, and glucagon, there is a significant circadian rhythm in corticosterone levels during the daytime (2) that has to be taken into account when studying this hormone. Hyperinsulinemia and concomitant increased glucose metabolism have no effect on corticosterone, and even seem not to influence its circadian rhythm. However, under basal conditions (0 mU/min insulin infusion rate) plasma corticosterone levels are close to 8 μg/dl, while under noninfusion conditions the levels are ±twofold lower. This indicates that the stress-hormone corticosterone is very sensitive to the clamp procedure and that probably the saline infusion per se elicits a rise in the level of this adrenal cortex hormone.

In addition to the hormone measurements, we studied the effect of graded hyperinsulinemia and concomitant increased glucose metabolism on plasma concentrations of several electrolytes by means of the 5-step clamp (Table 2). No dramatic changes in the levels of calcium, sodium, and potassium were observed. Plasma calcium and sodium concentrations remained fairly constant during the 5-step clamp. In contrast, a 20% decrease was measured in plasma potassium levels. This phenomenon has been described before and seems to be caused by the insulin-stimulating effect on potassium uptake into skeletal muscle by activation of the Na^+, K^+ pump (30).

To investigate whether long-term clamping with concomitant infusion of fluids and transfusion of blood would result in a dilution problem, which could then, in turn, affect the levels of the measured hormones and electrolytes, we determined the concentration of total protein in plasma during the 5-step clamp (Table 2). It was calculated that during the 7.5 h lasting 5-step

TABLE 2

LEVELS DURING THE STEADY-STATE PERIODS OF EACH INSULIN INFUSION STEP OF THE SEQUENTIAL HYPERINSULINEMIC EUGLYCEMIC CLAMP IN SIX CONSCIOUS RATS

	Insulin Infusion Rate (mU/min)				
	0	0.5	1	3	16
Calcium (mmol/l)	2.39 ± 0.06	2.40 ± 0.05	2.36 ± 0.05	2.35 ± 0.07	2.33 ± 0.07
Sodium (mmol/l)	146.8 ± 0.7	146.1 ± 0.9	145.4 ± 0.9	144.5 ± 0.9*	143.6 ± 0.4*
Potassium (mmol/l)	4.22 ± 0.05	4.11 ± 0.07	3.86 ± 0.03*	3.72 ± 0.02*	3.53 ± 0.09*
Protein (mg/ml)	37.7 ± 2.8	39.6 ± 2.8	39.9 ± 2.1	38.8 ± 2.2	39.8 ± 2.7

* Significant difference from basal insulin infusion rate (0 mU/min), $p < 0.05$ by Fisher PLSD test, after ANOVA for repeated measurements, within-subject design.

sequential clamp the amount of fluid infused was approximately 0.5, 1, 2.5, 4, and 6 ml at insulin infusion rates of 0, 0.5, 1, 3, and 16 mU/min, respectively. Furthermore ±2 ml of donor blood was used per individual insulin infusion step for repletion of blood loss during the clamp. These manipulations did not lead to a significant change in plasma total protein content during the 5-step clamp.

In conclusion, this study evaluates the changes in the levels of counterregulatory hormones and electrolytes during the 5-step sequential hyperinsulinemic euglycemic clamp technique. Insight in the levels of all these parameters is important because it leads to a better understanding of this dynamic in vivo model used for measurement of glucose metabolism, because it is actually the interaction between insulin and counterregulatory hormones that will determine the ultimate effect of hyperinsulinemic, euglycemic clamping on hepatic glucose production, and peripheral tissue glucose uptake (23).

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