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*Published in:*  
 American Journal of Physiology

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*Document Version*  
 Publisher's PDF, also known as Version of record

*Publication date:*  
 1991

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Koopmans, S. J., de Boer, S., Sips, H. C., Radder, J. K., Frölich, M., & Krans, H. M. J. (1991). Whole body and hepatic insulin action in normal, starved, and diabetic rats. *American Journal of Physiology*, 260(6 Pt 1), E825-E832.

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# Whole body and hepatic insulin action in normal, starved, and diabetic rats

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KOOPMANS, S. J., S. F. DE BOER, H. C. M. SIPS, J. K. RADDER, M. FRÖLICH, AND H. M. J. KRANS. *Whole body and hepatic insulin action in normal, starved, and diabetic rats*. *Am. J. Physiol.* 260 (Endocrinol. Metab. 23): E825–E832, 1991.—In normal (N), 3-days starved (S), and streptozotocin-treated (65 mg/kg) 3-days diabetic (D) rats we examined the in vivo dose-response relationship between plasma insulin levels vs. whole body glucose uptake (BGU) and inhibition of hepatic glucose production (HGP) in conscious rats, as determined with the four-step sequential hyperinsulinemic euglycemic clamp technique, combined with [ $^3\text{H}$ ]glucose infusion. Twelve-hour fasting (basal) HGP was  $3.0 \pm 0.2$ ,  $2.1 \pm 0.2$ , and  $5.4 \pm 0.5$  mg/min in N, S, and D rats, respectively. Next, all rats were clamped at matched glycemia (6 mM). Lowering plasma glucose in D rats from  $\pm 20$  to 6.0 mM did not increase plasma norepinephrine, epinephrine, glucagon, and corticosterone levels. For BGU, insulin sensitivity was increased ( $70 \pm 11$   $\mu\text{U}/\text{ml}$ ) in S and unchanged ( $113 \pm 21$   $\mu\text{U}/\text{ml}$ ) in D compared with N rats ( $105 \pm 10$   $\mu\text{U}/\text{ml}$ ). Insulin responsiveness was unchanged ( $12.4 \pm 0.8$  mg/min) in S and decreased ( $8.5 \pm 0.8$  mg/min) in D compared with N rats ( $12.3 \pm 0.7$  mg/min). For HGP, insulin sensitivity was unchanged ( $68 \pm 10$   $\mu\text{U}/\text{ml}$ ) in S and decreased ( $157 \pm 21$   $\mu\text{U}/\text{ml}$ ) in D compared with N rats ( $71 \pm 5$   $\mu\text{U}/\text{ml}$ ). Insulin responsiveness was identical among N, S, and D rats (complete suppression of HGP). In summary, 1) insulin resistance in D rats is caused by hepatic insensitivity and by a reduction in BGU responsiveness. 2) S rats show normal hepatic insulin action, but insulin sensitivity for BGU is increased. Therefore, S and D rats both suffering from a comparable catabolic state (10–15% body wt loss in 3 days) show opposite effects on in vivo insulin action. This indicates that in vivo insulin resistance in D rats is not caused by the catabolic state per se.

norepinephrine; epinephrine; glucagon; corticosterone; glucose uptake; glucose production; clamp; catabolism

**BOTH STARVATION and streptozotocin-induced insulin-deficient diabetes are accompanied by insulinopenia, reduced glucose metabolism, increased lipolysis, and a state of catabolism. In the past decade, a large amount of in vitro studies have examined insulin-sensitive tissues isolated from normal, starved, and diabetic rodents to compare and characterize the cellular alterations in insulin-mediated glucose metabolism. Most studies reveal that adipose tissue from starved and diabetic rodents is resistant to the action of insulin (16, 17, 44). However, data obtained from skeletal muscle are less uniform. It**

seems that muscle becomes insulin resistant when the diabetic state reaches a certain degree of severity and/or lasts for a prolonged time (11, 22, 25, 42). During starvation, insulin action in muscle was reported to be unaltered or even increased (10, 22). From the foregoing, it is concluded that individual tissues can respond in different ways to altered metabolism, and one single tissue might not reflect the in vivo whole body situation. Indeed, the latter has been confirmed in two studies (24, 46).

Recently, several laboratories have adapted the hyperinsulinemic glucose clamp technique to rats for measurement of whole body insulin-mediated glucose uptake in vivo. In vivo insulin resistance was reported to be present in diabetic rats (28, 34), however, others could not detect a defect in insulin action (4, 18). Two studies were performed using starved rats, and one showed a reduction in insulin-mediated glucose disposal (29), whereas the other was unable to detect any in vivo insulin resistance (20).

By analysis of the in vitro and in vivo results, an inconsistent and incomplete picture is obtained. Apart from prevailing glycemia, it has been suggested that time span and severity of the catabolic state during starvation and diabetes and nutritional condition of the rats at the start of starvation and diabetes are important factors that determine the ultimate effect of starvation and diabetes on insulin-mediated metabolism (2, 8, 9, 25). Therefore the importance of a standardized comparative study using normal, starved, and diabetic rats is stressed.

The aim of the present investigation was to determine the in vivo dose-response relationship between plasma insulin levels vs. stimulation of whole body glucose uptake and inhibition of hepatic glucose production in normal, 3-days starved, and 3-days diabetic rats. The dose-response study allows us to discriminate between in vivo insulin sensitivity and responsiveness. This is done to gain a better insight in the mechanism of insulin action (31). In vivo insulin action was determined using the four-step sequential hyperinsulinemic euglycemic clamp technique combined with [ $^3\text{H}$ ]glucose infusion in conscious freely moving rats.

## MATERIALS AND METHODS

*Animals and housing.* Male Wistar rats (300–350 g) with free access to a complete laboratory rat diet (Hope

Farms, 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 12:12 h light-dark cycle (lights on at 0700 h).

**Surgical procedure.** Under complete ether anesthesia, rats were provided with two sterile silicon cannulas [Silastic, medical grade tubing, no. 602-135, inner diameter (ID) 0.020 in., outer diameter (OD) 0.037 in.; no. 602-105, ID 0.012 in., OD 0.025 in.; Dow Corning, Midland, MI]; the large cannula was inserted into the right jugular vein for the infusion of fluids, and the smaller was inserted into the left carotid artery for blood sampling. The tip of the jugular cannula was situated at the entrance of the right atrium and the tip of the carotid cannula at the transition of carotid artery and aortic arch. Surgical manipulations were performed according to the method of Steffens (38), and both cannulas were attached to 90° bent steel tubings that were manufactured from 20-gauge ×1.5 needles (Microlance; Becton Dickinson, Dublin, Ireland). The two steel tubings were fixed on top of the rat skulls by means of four screws (4 × 1 mm) and dental cement (Simplex rapid; Austenal Dental Products, Harrow, UK). The venous catheter was filled with a 0.9% NaCl solution containing 0.2 mg/ml ticarcillin (Ticarpen; Beecham Farma, Amstelveen, The Netherlands), 500 IU/ml heparin (Thromboliquine; Organon Teknika, Oss, The Netherlands), and 0.6 g/ml polyvinylpyrrolidone (PVP, mol wt = 25,000, Merck). The arterial catheter was filled with a 0.9% NaCl solution containing 0.2 mg/ml ticarcillin, 500 IU/ml heparin, and 0.6 g/ml PVP.

After the dental cement had hardened, the venous line was connected to a double-lumen tubing (polyethylene, ID 0.016 in., OD 0.040 in.; Talas, Ommen, The Netherlands) by means of a piece of polyethylene tubing (ID 0.030 in., OD 0.058 in.; Rubber, Hilversum, The Netherlands). The double-lumen tubing was connected to a double-lumen swivel (41), allowing separate fluid infusions (i.e., an insulin and [ $3\text{-}^3\text{H}$ ]glucose solution via one channel and a glucose solution through the other channel) in the conscious undisturbed freely moving rat. Hence, insulin and glucose could be kept separate until reaching the skull of the rat, allowing a low infusion rate (the insulin and [ $3\text{-}^3\text{H}$ ]glucose solution infusion rate was 400  $\mu\text{l/h}$ ; the glucose infusion rate was variable). The venous double-lumen tubing and the swivel were filled with a 0.9% NaCl solution and sealed. The carotid line was connected to a polyethylene tubing (ID 0.030 in., OD 0.058 in., Rubber), filled with the arterial 0.9% NaCl solution with PVP, sealed, and attached aside to the lower rotating part of the swivel. Both venous double-lumen tubing and arterial blood sampling tubing were protected by an outer transparent flexible Tygon tubing (ID 0.16 in., OD 0.28 in.). The rat, permanently attached to the swivel, was placed in a metabolic cage, and the swivel was connected to a contra weight. After surgery, the rats were allowed to recover for at least 1 wk 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 1 wk after surgery the rats reached their preoperative weight

and were used for the clamp studies. In the present form the permanently cannulated rats can be studied for time periods up to 4 wk, and catheter patency could be maintained in  $\pm 80\%$  of the rats during that time period.

**Experimental groups.** Three groups of 23 cannulated animals were studied: *group I*, ad libitum-fed normal rats ( $n = 12$ ); after a period of 5–7 days the animals were restudied as *group II*, 3-days starved rats ( $n = 6$ ) and *group III*, ad libitum-fed streptozotocin-treated (65 mg/kg) 3-days diabetic rats ( $n = 6$ ). In this way, each rat from *groups II* and *III* served as its own control. The starved rats from *group II* had free access to drinking water, and consumption of feces was prevented. The rats in *group III* were made diabetic by means of an intra-arterial injection of 65 mg/kg streptozotocin (Zanosar; Upjohn, Kalamazoo, MI) diluted in 0.01 M citrate buffer (pH 4.5). The streptozotocin solution ( $\pm 300 \mu\text{l}$ ) was injected gradually over a time period of 5 min. Three days after streptozotocin injection, the diabetic rats were clamped. Rats were considered diabetic and suitable for clamp studies when 12-h fasting plasma glucose was between 20 and 30 mM. In vivo insulin action was measured by means of a four-step sequential (dose-response) hyperinsulinemic euglycemic (6 mM) clamp technique.

An additional group of normal ( $n = 6$ ), 3-days diabetic ( $n = 6$ ), and 3-days starved ( $n = 5$ ) rats was introduced and clamped at euglycemia (6 mM) and hyperinsulinemia (1 single insulin infusion step of 16 mU/min) to check the possible “self-exacerbating,” “insulin treatment,” and “glucose feeding” effect of sequential clamps, as previously discussed by Bergman et al. (1) and Doberne et al. (7). The six normal rats used for the one-step clamp study were restudied after 1 wk as the diabetic group after they had been treated with streptozotocin as described. These one-step clamps lasted for 120 min, were performed under comparable conditions as described below for the sequential clamps, and were carried out at the end of the afternoon to match the time of clamping during the day with the maximal insulin infusion step as part of the sequential clamp. Possible effects of circadian rhythm in glucose metabolism were evaded in this way.

**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 was filled 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, UK). 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 [ $3\text{-}^3\text{H}$ ]glucose (Du Pont-NEN, Boston, MA, sp act 13.5 Ci/mmol) and insulin (Actrapid porcine insulin; Novo, Copenhagen, Denmark) at a constant rate (400  $\mu\text{l/h}$ ). The carotid cannula was connected to 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 3 mg/ml citrate to prevent clotting. Citrate was chosen instead to heparin to avoid possible

activation of lipoprotein lipase during the clamp.

Before start of the insulin clamp, a bolus (3  $\mu$ Ci) continuous (0.05  $\mu$ Ci/min) infusion of [ $^3$ -H]glucose was initiated and continued throughout the study. The bolus amount for diabetic rats was 6  $\mu$ Ci. At 70, 80, and 90 min, blood samples were collected to measure plasma glucose ( $n = 3$ ), insulin ( $n = 2$ ), and tritiated glucose specific activity ( $n = 3$ ). Subsequently, a sequential four-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 before each insulin infusion period (2.5, 5, 15, and 80 mU). Blood (40  $\mu$ l) was collected at 5- to 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 (6). During steady state, the final 20 min of each insulin infusion period, blood samples were taken at 10-min intervals for plasma insulin ( $n = 2$ ), plasma glucose ( $n = 3$ ), and tritiated glucose specific activity ( $n = 3$ ). During the first 15 min of each individual insulin infusion step, three samples of 0.7 ml citrated donor blood were transfused to the rat, compensating previous blood loss.

In previous studies, it was shown that hyperglycemia per se will enhance insulin-mediated glucose uptake by a mass action effect (5) and that glucose clearance is dependent of the prevailing blood glucose concentration (18). Therefore, all clamp experiments were carried out at matched glycemia. During the first insulin infusion step, in starved rats, plasma glucose was quickly raised ( $\pm 10$  min) from  $\pm 4.5$  to 6 mM by means of 20% glucose infusion and was gradually lowered in diabetic rats from  $\pm 20$  to 6 mM (in a time period of  $\pm 90$  min) by means of the insulin infusion of 0.5 mU/min. Because of this matched glycemia during the clamp between normal, starved, and diabetic rats, the first insulin infusion step (0.5 mU/min) lasted in fact for 90, 100, and 180 min, respectively. In diabetic rats, plasma samples for corticosterone, glucagon, epinephrine, and norepinephrine were collected before and immediately after lowering plasma glucose from  $\pm 20$  to 6 mM. After these blood samples had been taken, an equal volume of transfusion blood was given. During and after the clamp, urine was collected for the estimation of urinary glucose.

**Chemical determinations.** Blood samples (40  $\mu$ l) 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). Blood samples (150  $\mu$ l) for insulin were transferred to chilled (0°C) heparinized tubes, centrifuged within 5 min, and plasma was removed and stored at  $-80^\circ\text{C}$  until assay. Insulin was measured in duplicate by a specific rat radioimmunoassay (19), rendering identical standard curves for rat and porcine insulin. Intra- and interassay variations of the insulin assay were 6.8 and 8.1%, respectively, with an assay sensitivity of 2  $\mu$ U/ml. Samples for determination of catecholamines (CA) and corticosterone (250  $\mu$ l) were transferred to chilled (0°C) heparinized tubes containing 10  $\mu$ l of a solution of 25 mg/ml disodium EDTA

to prevent CA degradation and were centrifuged immediately. Plasma was removed and stored at  $-80^\circ\text{C}$  until assay. The concentrations of norepinephrine and epinephrine were measured in duplicate in 20  $\mu$ l perchloric acid-deproteinized plasma according to a radioenzymatic catechol-*O*-methyltransferase (COMT) procedure (39). The CAs were converted into their  $^3\text{H}$  methoxy derivatives by incubation with *S*-[methyl- $^3\text{H}$ ]adenosyl-L-methionine (80 Ci/mmol; NEN) in the presence of COMT. Labeled products were isolated by organic extraction and paper chromatography. After elution of labeled products, activity was counted in a liquid scintillation analyzer (Philips; Eindhoven, The Netherlands). The intra- and interassay variabilities were  $<10$  and 15%, respectively. The sensitivity of the assay (amount corresponding to two times the blank) was 1 pg for both norepinephrine and epinephrine. Plasma corticosterone concentrations were determined in duplicate according to a competitive protein-binding method (27). Corticosterone was extracted with dichloromethane from 25- $\mu$ l samples of plasma, and the dry residue was incubated with a corticosteroid-binding globulin tracer solution {0.1% plasma from adrenalectomized female rats containing [1,2- $^3\text{H}$ ] corticosterone (40–50 Ci/mmol) as tracer}. Unbound steroid was removed using dextran-coated charcoal. Standard corticosterone was supplied by Sigma (St. Louis, MO). The intra- and interassay coefficients of variation were  $<10\%$ .

Blood samples (250  $\mu$ l) for glucagon were transferred to chilled (0°C) heparinized tubes, supplemented with 5% Trasylol (10,000 Kallikrein inactivator units/ml; Bayer, Leverkusen, Germany) to prevent hormone degradation and were centrifuged immediately. Plasma was removed and stored at  $-80^\circ\text{C}$  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 [ $^3$ -H]glucose radioactivity (150  $\mu$ l) was deproteinized by barium hydroxide-zinc sulfate (Somogyi procedure). The supernatant was evaporated to dryness at 60°C to eliminate tritiated water and was 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 ( $R_a$ ) is equal to the rate of glucose disappearance ( $R_d$ ), the glucose turnover rate ( $=R_a=R_d$  in mg/min) was calculated by dividing the [ $^3$ -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. Because urinary glucose loss was not present, whole body glucose uptake (BGU) = glucose turnover rate = exogenous glucose infusion rate + rate of HGP. In the basal state the rate

of HGP ( $R_a$ ) is equal to  $R_d$ .

Dose-response curves for *in vivo* insulin action on stimulation of BGU 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 mU/min, resulting in plasma insulin levels of  $\pm 5,000$   $\mu$ U/ml. The index for insulin sensitivity [half-maximal effective dose ( $ED_{50}$ )] was calculated from the individual dose-response curves.

**Data analysis.** Hormone-stimulated responsiveness is defined as the maximal response, and sensitivity is determined by the position of the curve and is defined as the hormone concentration giving  $ED_{50}$ .  $ED_{50}$  was determined by linearizing the ascending or descending part of the dose-response curve, as described previously (19). In short, the biological effect at each hormone concentration is calculated, is expressed as a percentage of the maximal response, and is denoted  $y$ . The logarithm of  $y/(100 - y)$  is plotted against the logarithm values for the hormone concentration. This so-called log-logit plot gives a straight line that can be evaluated by linear regression analysis according to the method of least squares.  $ED_{50}$  is determined by the intercept of the line with the ordinate.

Data are expressed as means  $\pm$  SE. Comparisons between two groups were made using the paired or unpaired Student  $t$  tests, when appropriate. The Bonferroni adjustment was used for multiple comparisons. The criterion of significance was set at  $P < 0.05$ . Statistical analysis and construction of Figs. 1 and 2 and Tables 1-3 were performed on an Apple Macintosh computer.

## RESULTS

**Experimental animals.** Three-days starved and 3-days diabetic rats showed pronounced body weight reduction and low fasting plasma insulin levels compared with normal rats. However, fasting plasma glucose levels and HGP were low in starved rats but high in diabetic rats. When HGP was expressed per gram wet liver weight, HGP was unchanged in starved but increased in diabetic compared with normal rats (Table 1).

**Glycemia-related levels of insulin counterregulatory hormones.** To exclude the possibility that *in vivo* insulin action in diabetic rats was influenced by a rise in insulin counterregulatory hormones related to the induction of a relative hypoglycemia at the start of the clamp, levels of norepinephrine, epinephrine, glucagon, and corticos-

terone were measured. Table 2 shows that, in diabetic rats, the lowering of fasting (basal) hyperglycemia to normoglycemia in a time period of  $\pm 90$  min did not result in a rise of insulin counterregulatory hormones. In fact, plasma glucagon levels decreased by 37%, similar to the decrease observed in normal rats. In the basal state (insulin infusion rate of 0 mU/min), plasma norepinephrine, epinephrine, and glucagon concentrations were similar in diabetic and normal rats. Only basal plasma corticosterone levels were increased in diabetic rats as compared with normal rats. For this reason, we also measured basal plasma corticosterone levels in the six starved rats. These turned out to be  $11.1 \pm 4.7$   $\mu$ g/dl, which is not significantly different from normal and diabetic rats. In normal rats maintaining euglycemia the elevation of plasma insulin from 22.7 to 42.4  $\mu$ U/ml had no effect on the levels of the measured adrenal and sympathetic hormones (Table 2).

***In vivo* dose-response relationship between plasma insulin levels and stimulation of whole body glucose uptake.** Figure 1 depicts the curves for BGU determined by the sequential hyperinsulinemic euglycemic (dose-response) clamp using normal, starved, and diabetic rats. These curves reveal that maximal insulin-stimulated BGU is unchanged ( $12.4 \pm 0.8$  mg/min,  $P < 0.01$ ) in starved but is decreased ( $8.5 \pm 0.8$  mg/min,  $P < 0.05$ ) in diabetic compared with normal rats ( $12.3 \pm 0.7$  mg/min). These values are reached at similar plasma insulin levels of  $5,340 \pm 776$ ,  $4,615 \pm 907$ , and  $5,435 \pm 831$   $\mu$ U/ml, respectively. The curve of the starved rats has shifted to the left ( $P < 0.05$ ), but the relative position of the curve of diabetic rats is unchanged, compared with normal rats. The  $ED_{50}$  values for BGU, which characterize the position of the curves, are given in Table 3. The  $ED_{50}$  values were calculated by linearizing the individual dose-response curves by means of the log-logit method (see MATERIALS AND METHODS). Goodness of fit was excellent, as judged from the correlation coefficients of the regression lines:  $0.98 \pm 0.01$ ,  $0.98 \pm 0.01$ , and  $0.95 \pm 0.02$  in normal, starved, and diabetic rats, respectively.

***In vivo* dose-response relationship between plasma insulin levels and inhibition of hepatic glucose production.** Figure 2 depicts the curves for HGP, determined by the sequential hyperinsulinemic euglycemic (dose-response) clamp using normal, starved, and diabetic rats. From these curves, it is clear that maximal insulin-inhibited HGP is identical among normal ( $-0.5 \pm 0.2$  mg/min), starved ( $-0.3 \pm 0.4$  mg/min), and diabetic ( $-0.4 \pm 0.4$  mg/min) rats. The curve of the diabetic rats has shifted to the right ( $P < 0.05$ ), whereas the relative position of the curve of the starved rats is unchanged compared with

TABLE 1. Characteristics of normal, starved, and diabetic rats at start of 4-step sequential hyperinsulinemic euglycemic clamp

Animals	<i>n</i>	Rat Wt, g	Weight Loss Over 3 Days, %	Fasting Plasma Glucose, mM	Fasting Plasma Insulin, $\mu$ U/ml	Fasting Hepatic Glucose Production, mg/min	Wet Liver Wt, g
Normal	12	347 $\pm$ 4.3	NA	5.9 $\pm$ 0.3	16.9 $\pm$ 2.6	3.0 $\pm$ 0.2	15.4 $\pm$ 0.8
Starved	6	299 $\pm$ 4.8*	14	4.8 $\pm$ 0.4*	7.5 $\pm$ 1.8*	2.1 $\pm$ 0.2*	9.1 $\pm$ 0.4*
Diabetic	6	310 $\pm$ 4.5*	10	22.1 $\pm$ 1.9*	5.7 $\pm$ 2.4*	5.4 $\pm$ 0.5*	14.7 $\pm$ 0.8

Values are means  $\pm$  SE. Wet liver wt was determined in a separate set of experimental rats held under identical conditions. Six normal, 6 starved, and 6 diabetic rats were used. NA, not applicable. \* Significant difference from corresponding normal value.

TABLE 2. Levels of counterregulatory hormones during basal conditions and during first step of sequential clamp in 6 normal and 6 diabetic rats

	Plasma Glucose, mM	Plasma Insulin, $\mu$ U/ml	Plasma Glucagon, pg/ml	Plasma Norepinephrine, pg/ml	Plasma Epinephrine, pg/ml	Plasma Corticosterone, $\mu$ g/dl
Normal rats basal	6.1 $\pm$ 0.3	22.7 $\pm$ 4.2	57.6 $\pm$ 9.3	404 $\pm$ 42	178 $\pm$ 22	9.6 $\pm$ 3.1
Normal rats first step	6.0 $\pm$ 0.2	42.4 $\pm$ 3.4*	45.7 $\pm$ 13.0*	430 $\pm$ 46	163 $\pm$ 26	12.5 $\pm$ 5.2
Diabetic rats basal	22.1 $\pm$ 1.9†	5.7 $\pm$ 2.4†	69.1 $\pm$ 20.3	450 $\pm$ 51	191 $\pm$ 29	17.7 $\pm$ 3.8†
Diabetic rats first step	6.2 $\pm$ 0.3*	33.2 $\pm$ 3.8*	43.6 $\pm$ 4.7*	512 $\pm$ 56	202 $\pm$ 39	21.7 $\pm$ 4.8†

Values are means  $\pm$  SE. Insulin infusion was 0 mU/min during basal conditions and 0.5 mU/min during first step of sequential clamp. In diabetic rats this means before and after reduction of plasma glucose levels from fasting hyperglycemia to euglycemia. \* Significant difference from corresponding basal value. † Significant difference from corresponding normal value.

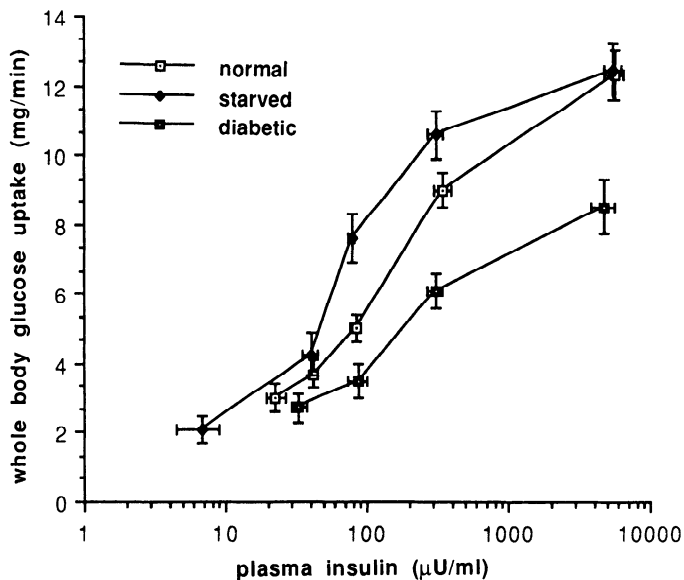


FIG. 1. Dose-response curves for steady-state plasma insulin levels and whole body glucose uptake as determined with sequential hyperinsulinemic euglycemic clamp technique in normal, starved, and streptozotocin-treated diabetic rats. Each point represents mean  $\pm$  SE.

TABLE 3. Sensitivity for insulin-stimulated whole body glucose uptake and insulin-inhibited hepatic glucose production in normal, starved, and diabetic rats

Animals	n	ED <sub>50</sub> , $\mu$ U/ml	
		BGU	HGP
Normal	12	105 $\pm$ 10	71 $\pm$ 5
Starved	6	70 $\pm$ 11*	68 $\pm$ 10
Diabetic	6	113 $\pm$ 21	157 $\pm$ 21†

Values are means  $\pm$  SE. ED<sub>50</sub>, half-maximal effective dose; BGU, whole body glucose uptake; HGP, hepatic glucose production. \* Significant difference from normal value,  $P < 0.005$ . † Significant difference from normal and starved values,  $P < 0.01$ .

normal rats. The ED<sub>50</sub> values for HGP are given in Table 3. The ED<sub>50</sub> values were calculated by linearizing the individual dose-response curves by means of the logit method (see MATERIALS AND METHODS). Goodness of fit was excellent, as judged from the correlation coefficients of the regression lines: 0.95  $\pm$  0.02, 0.97  $\pm$  0.01, and 0.94  $\pm$  0.02 in normal, starved, and diabetic rats, respectively.

*The possible self-exacerbating, glucose feeding, and insulin treatment effect of sequential clamps.* To determine whether antecedent infusion of insulin (and glucose) had altered insulin responsiveness for BGU and HGP (deter-

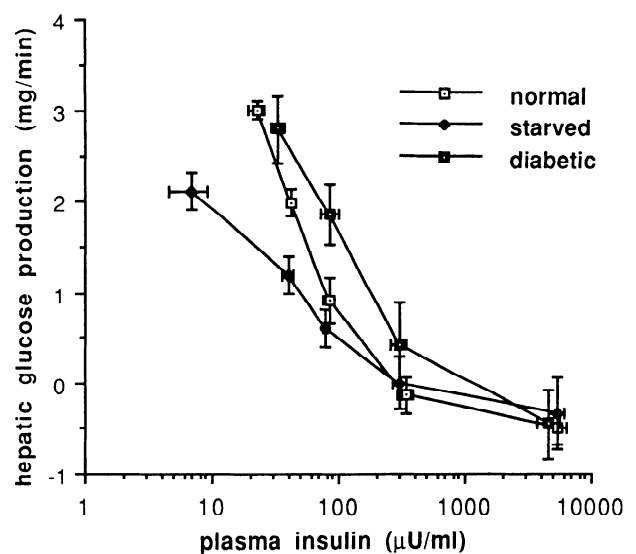


FIG. 2. Dose-response curves for steady-state plasma insulin levels and hepatic glucose production as determined with sequential hyperinsulinemic euglycemic clamp technique in normal, starved, and streptozotocin-treated diabetic rats. Each point represents mean  $\pm$  SE.

mined during the final and maximal insulin infusion step of the sequential clamp), we performed additional one-step clamps at maximal plasma insulin concentrations in normal, starved, and diabetic rats. One-step clamps in normal, starved, and diabetic rats at maximal plasma insulin levels of 6,563  $\pm$  726, 7,268  $\pm$  410, and 5,862  $\pm$  1,015  $\mu$ U/ml resulted in a BGU of 12.4  $\pm$  0.4, 12.3  $\pm$  1.3, and 7.7  $\pm$  0.3 mg/min and in a HGP of 0.2  $\pm$  0.5, -0.1  $\pm$  0.3, and 0.1  $\pm$  0.5 mg/min, respectively. This is similar to the values reached during the sequential clamps in this study. For data, see text describing Figs. 1 and 2.

## DISCUSSION

During insulinopenic diabetes, hyperglycemia is accompanied by a marked catabolic state (19). In the past decade, several experiments have been performed to unravel the effect of catabolism on insulin action (10, 19, 29, 44). Starvation was used as a model to create insulinopenia and catabolism. To determine the role of catabolism on in vivo insulin action in diabetic rats, we studied, apart from diabetic rats, a group of starved rats that showed similar bodyweight reduction.

A second goal of this study was to discriminate between insulin sensitivity (ED<sub>50</sub>) and insulin responsiveness (maximal response) during the clamp. This discrimina-

tion is important because it leads to a better understanding of the mechanism of insulin action. In general, a decrease in insulin responsiveness is due to a defect that is rate limiting for insulin action. A decrease in insulin sensitivity is due to a defect(s) in steps of insulin action that are not rate limiting.

Insulin action was studied with the sequential hyperinsulinemic euglycemic clamp technique in conscious unrestrained rats. This technique was combined with a continuous infusion of [ $^3\text{H}$ ]glucose, allowing calculation of whole body glucose turnover to discriminate between the stimulating effect of insulin on whole body glucose uptake and the inhibiting effect of insulin on hepatic glucose production (1, 29, 34).

In this study, two groups of highly catabolic and insulinopenic animals, i.e., 3-days starved and streptozotocin-treated 3-days diabetic rats, showed opposite effects on *in vivo* insulin action. Starved rats revealed increased insulin sensitivity but normal responsiveness for whole BGU when compared with normal rats. The increased sensitivity is consistent with the findings of Pénicaud et al. (29), but responsiveness was normal in our rats and was reduced in their starved rats. The reason for this discrepancy might be that we used mature and more obese rats (300–350 g), whereas Pénicaud et al. (29) used young and lean rats (150–200 g). Compared with young and lean rats, mature and rather obese rats show a more pronounced protein and fuel metabolism sparing effect during a 3-day period of starvation (8–10). The increased sensitivity is paralleled and might be explained by an increased binding of insulin to muscle, suggesting an increased insulin receptor number and/or affinity, as determined by Le Marchand-Brustel and Freychet (22). Normal insulin responsiveness for BGU indicates that starvation for 3 days using rats of ~350 g causes no insulin resistance at the postbinding level [mechanism discussed by Rizza et al. (31)]. However, in diabetic rats, insulin sensitivity for BGU was unaltered, and insulin responsiveness was decreased. The latter suggests that, in diabetic rats, insulin resistance is located at the postbinding level, as shown before (28). Insulin sensitivity was unaltered, which is in agreement with an *in vivo* study of Nishimura et al. (28) and an *in vitro* study of Goto et al. (11) using soleus muscle. This unaltered sensitivity is observed although the number of insulin receptors and/or affinity of insulin receptors for insulin in muscle is increased (22, 28). This suggests that additional postbinding processes determine the ultimate sensitivity of BGU for insulin.

Summarizing our results, we conclude that the severe catabolic state (body weight loss after a 3-day period of starvation and diabetes was 10–15%) and insulinopenia are not responsible for the diabetes-related insulin resistance. On the contrary, severe catabolism present in starved animals is accompanied with an increase in insulin action.

Although both diabetic and starved animals had similar weight loss, suggesting similar catabolic states, the mechanism of catabolism might be different. There could have been a difference in free fatty acid (FFA) levels before and during the clamp studies. Given the fact that FFA levels can inhibit insulin-mediated glucose uptake

(21) and if FFA levels were higher during the clamp studies in diabetic vs. starved animals, this might have influenced the shape of the dose-response curve. However, this seems unlikely since we have previously shown that adipocytes from both starved and diabetic rats are not resistant to the antilipolytic action of insulin and that lipolysis is identically suppressed by insulin (19). After 7.5 h of clamping, plasma FFA levels are therefore expected to be decreased to a similar extent in starved and diabetic animals. Thus the difference in *in vivo* insulin responsiveness between starved and diabetic rats is probably not caused by a difference in FFA levels.

The striking difference between starved and diabetic rats is the prevailing glycemia. Recently, evidence was presented that hyperglycemia *per se* contributes to the impairment of *in vivo* insulin-mediated glucose disposal in diabetes. Rossetti et al. (34) showed that correction of hyperglycemia with phlorizin normalized *in vivo* insulin action in diabetic rats, and Yki-Järvinen et al. (45) revealed that short-term (24-h) hyperglycemia in type I diabetic patients reduced tissue glucose uptake, as measured with the glucose clamp technique. This and the fact that starved rats with low plasma glucose levels show an increase in *in vivo* BGU suggest that an *in vivo* autoregulation of glucose utilization exists. This is consonant with our previous *in vitro* experiments with preadipocytes (40) and with soleus muscle by others (37), which revealed that glucose regulates its own transmembrane transport and glycolytic flux. In this context, defects in glucose transport have been described in a similar diabetic rat model of insulin resistance (33, 47), suggesting that glucose transport may be the rate-limiting step for BGU. Other defects, such as defects in glycogen synthesis (33), have also been described, indicating that this issue is complicated and needs further investigation. It has been suggested that insulin and glucose delivery (blood flow) to skeletal muscle may be rate limiting for BGU. If blood flow were reduced in diabetic rats, this could give the appearance of a decrease in insulin responsiveness. This possibility cannot be excluded, but this explanation seems unlikely, since James et al. (14) have shown that blood flow is not a major determinant of insulin-mediated glucose uptake in muscle of rats, as studied by means of the hyperinsulinemic euglycemic clamp technique.

The use of conscious rats allowed us to study insulin counterregulatory hormones in the absence of anaesthesia-induced disturbances of the central nervous system. The lowering of plasma glucose from 21.3 to 6.0 mM using an insulin infusion rate of 0.5 mU/min, during the clamp in diabetic rats, did not elicit a response of counterregulatory hormones. We investigated the effect of induction of relative hypoglycemia because Santiago et al. (36) have shown that, during diabetes, there is no absolute plasma glucose concentration threshold for activation of glucose counterregulatory systems. Before and immediately after the decline of glycemia, plasma nor-epinephrine, epinephrine, glucagon, and corticosterone levels behaved in the same way as in normal euglycemic rats during the insulin infusion of 0 and 0.5 mU/min. Thus the measured *in vivo* insulin action in diabetic rats was not influenced by a rise in counterregulatory hor-



mones related to the induction of a relative hypoglycemia. In both normal and diabetic rats the insulin infusion of 0.5 mU/min caused 1) a similar decrease in plasma glucagon levels, suggesting that in 3-days diabetic rats the suppressive effect of insulin on plasma glucagon levels is intact and 2) no effect on plasma norepinephrine, epinephrine, and corticosterone levels. It has been reported that an elevation of plasma insulin levels results in activation of the sympathetic nervous system (35). We cannot support this observation, but the possibility exists that higher insulin levels than used in our study ( $\pm 50$   $\mu$ U/ml) are needed to elicit a response of plasma norepinephrine levels. During the clamp studies of James et al. (13), using high insulin levels, there was no effect on epinephrine levels, which is in agreement with our observations. Corticosterone levels in diabetic rats were elevated compared with normal rats, suggesting that the induction of diabetes promotes the appearance of this adrenal glucocorticoid stress hormone. This raises the question of whether this could explain the *in vivo* hepatic insulin insensitivity and the decrease in insulin responsiveness for BGU in diabetic rats. The fact that we did not observe a significant difference in corticosterone levels between diabetic and starved rats (which show increased insulin action) suggests that an increase in corticosterone concentrations of a magnitude as measured in this study is of minor importance on insulin action. Furthermore, it seems that the effect of glucocorticosteroids on *in vivo* insulin action is primarily directed toward a decrease of insulin sensitivity and not of insulin responsiveness (32).

We have not measured the levels of glucagon, epinephrine, and norepinephrine in starved rats, but, from another study using a comparable experimental set up, we know that 3 days of food deprivation has no effect on resting plasma catecholamine levels (43).

Basal HGP was reduced by 30% in starved compared with normal rats which is similar to the reduction observed by Issad et al. (12). This 30% reduction is paralleled by a similar reduction in wet liver weight. Thus, expressed per gram of liver weight, HGP is identical between normal and starved rats, indicating that there is no defect in liver glucose metabolism. This is supported by our observation that insulin-inhibited HGP is normal in starved rats. In diabetic rats, basal HGP was doubled compared with normal rats (without a change in wet liver wt), indicating that hyperglycemia per se is no longer able to suppress HGP during insulin-deficient diabetes. Müller et al. (26) described that, in the normal physiological situation, hyperglycemia reduces HGP independently of hormonal changes.

Our study reveals that diabetic rats show *in vivo* hepatic insulin insensitivity. This seems to be caused, at least partially, by decreased autophosphorylation of the insulin receptor kinase (15). This indicates that signal transduction per insulin receptor is reduced, a defect that can be overcome by adding surplus insulin, since a maximal biological effect of insulin is normally achieved when only 10% of the total receptor population is occupied by insulin. By occupying more receptors the signal becomes strong enough to reach a maximal effect. This could explain why we do not observe a defect in hepatic

insulin action at maximal plasma insulin concentrations.

The self-exacerbating effect of sequential clamps on the biological action of insulin, as described previously (7), was not observed during our experiments. Stimulation of BGU and inhibition of HGP at maximal insulin concentrations were similar during sequential or one-step clamps in normal, starved, and diabetic rats. This is consistent with the findings of Proietto et al. (30) and Rizza et al. (31) who showed that a precise insulin dose-response curve can be constructed in 1 single day by means of the sequential hyperinsulinemic euglycemic clamp technique. Insulin treatment of diabetic rats and glucose (re)feeding of starved rats, conditions that are present during sequential (long-term) clamping, are known to improve insulin action (16, 17, 42). However, from several studies (3, 23), we know that this improvement does not take place before a time period of 4–6 h. Since our clamp studies take 6–7.5 h, this could explain why we do not observe an effect of long-term clamping on insulin's maximal biological effect in diabetic and starved rats.

In summary, insulin resistance in diabetic rats is caused by hepatic insensitivity and by a reduction of whole body responsiveness, and starved rats show normal hepatic insulin action but increased whole body insulin sensitivity. We conclude that 3-days starved and diabetic rats, both suffering from a comparable catabolic state (10–15% body wt loss), show opposite effects on *in vivo* insulin action. This indicates that *in vivo* insulin resistance in diabetic rats is not caused by the catabolic state per se.

We thank Dr. R. J. Moss for writing the log-logit computer program.

This work was supported by a grant from the Dutch Diabetes Research Foundation.

This work was presented in part at the meeting of the European Association for the study of Diabetes in Paris, France, in September 1988 and has also been presented in abstract form (*Diabetologia* 31: 509A, 1988).

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Received 4 June 1990; accepted in final form 5 February 1991.

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