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Alterations in alanine metabolism in diabetic dogs during short-term treatment with an artificial B cell

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Summary. The flux rates of plasma glucose and alanine were studied isotopically (6-^{3}H-glucose and U-^{14}C-alanine simultaneously) in resting chronically diabetic dogs during short-term treatment with an artificial B cell where the insulin was infused into a peripheral vein. Despite perfect blood glucose control and normal glucose flux rates, the concentration and rates of appearance and disappearance of alanine were significantly elevated in the diabetic animals before, during and after an exogenous glucose load. The incorporation of the carbon moiety of alanine into circulating glucose was also increased, but diminished to a near-normal extent when exogenous glucose was given. The plasma clearance rates for alanine in the diabetic dogs were normal throughout the study. It is concluded that normal blood glucose control in diabetes does not necessarily mean normalization of the entire metabolic network. On the basis of peripheral hyperinsulinaemia alanine formation from glucose and branched chain amino acids is elevated in muscle. This may explain increased flux of alanine despite normal blood glucose control.

Key words: Artificial B cell, dog, experimental diabetes, alanine metabolism, gluconeogenesis, tracer study.

Artificial B cell-mediated blood glucose control provides normal glucose flux rates both under basal conditions and after a glucose load in diabetes [1, 2]. If, however, during short-term application, insulin is administered via the peripheral route, several other metabolic parameters remain pathological. Thus, circulating glucose precursor concentrations are usually elevated [3-6]. In an apparent contrast to these findings, rates of glucose carbon re-circulation were found to be diminished under these conditions both in diabetic patients [6] and dogs [1].

The present study deals with alanine metabolism in chronically diabetic dogs during normal glucose regulation by means of feedback-controlled peripheral venous insulin administration. Endogenous alanine is produced in skeletal muscle on the basis of carbon moieties from glucose and amino acids and of the amino groups resulting from protein catabolism; alanine serves predominantly as a substrate for hepatic gluconeogenesis (glucose-alanine cycle: 7). In untreated diabetes, decreased plasma alanine concentrations have been observed in combination with both a reduced production rate [8] and increased gluconeogenesis [8-12]. The long-term use of a peripheral venous insulin pump, however, leads to elevated plasma alanine concentrations [3]. In a preliminary study of insulin-treated diabetic dogs, we observed hyperalaninaemia even if insulin therapy was not optimal; an intravenous bolus injection of a blood glucose lowering insulin dose did not affect the plasma alanine values in these animals or in healthy controls (unpublished data). Therefore the question arises whether the flux rates of alanine are altered under the influence of insulin and glucose in diabetes.

Since there is an apparent relation between concentrations and metabolic rates of alanine in vivo [8, 13], it was the aim of this study to investigate the effect of perfect blood glucose control on simultaneously measured flux rates of glucose (6-^{3}H-glucose) and alanine (U-^{14}C-alanine) under basal conditions and during an intravenous glucose load.

Materials and methods

Animals

Six non-diabetic (mean ± SEM, weight: 28 ± 2 kg, age: 31 ± 5 months) and five diabetic Alsatian dogs of both sexes (34 ± 2 kg, 44 ± 5 months; duration of diabetes 10 ± 2 months, insulin requirement in daily therapy employing three subcutaneous injections of regular porcine monocomponent insulin 1.55 ± 0.06 IU/kg Actrapid MC, Novo, Copenhagen, Denmark) were studied. One of the diabetic animals was investigated in the control group before it was rendered diabetic.

Diabetes was produced by subtotal pancreatectomy with intrasurgical infusion of a small dose of streptozotocin [14]. The animals were canine C-peptide-negative and had no anti-insulin antibodies. Their long-term metabolic situation was characterized by an average glycaemia over the day of 10.4 ± 0.8 and a maximum amplitude of glycaemic excursions of 19.6 ± 2.2 mmol/l as taken from seven estimates around the clock (control animals: 5.2 ± 0.1 and 1.0 ± 0.1 mmol/l respectively).

Experimental design

The turnover rates of glucose and of alanine were estimated isotopically before, during and after an exogenous load of unlabelled glucose in the non-medicated animals [1]. During these tests the diabetic animals were treated intravenously with glucose-controlled insulin infusions (GCII) to restore fasting normoglycaemia and to keep the glycaemic responses as close to normal as possible [15].

Immediately before the experiments (14 h after the last meal and – in the case of the diabetic dogs – the last subcutaneous insulin injection), the animals were prepared with three indwelling catheters without anaesthesia, one in the jugular vein (Cavafix RMT, Braun, Melsungen, FRG) for blood sampling from the upper caval vein; one in the cephalic vein for glucose monitoring during GCII (Polyethylene Cannula, VEB Kombinat MLW, Medizinplaste Lichtenberg, GDR); and another in the pelvic lower caval vein via a saphenous vein (Teflon Portex Intravenous Tubing, Hythe, Kent, UK) to administer infusions. These consisted of a constant background infusion of saline (0.154 mmol/l, 0.5 ml/min) which was supplemented with a solution of tracer (0.1 ml/min), unlabelled glucose according to protocol (1.0 ml/min), and – during the GCII – insulin ($0.1\ \mathrm{ml \cdot kg^{-1} \cdot min^{-1}}$, pulses of variable duration according to glycaemia).

At 05.00 h on the day of the experiment, the diabetic animals were injected intravenously with an individually adapted dose of the insulin formulation as described above (average 9.0 ± 1.2 IU/animal). From 07.00 h, the GCII were applied throughout the experiment: the average total insulin requirement over 10 h was 16.3 ± 2.7 IU/animal, basal dose $0.51 \pm 0.10\ \mathrm{mU \cdot kg^{-1} \cdot min^{-1}}$, dose during exogenous glucose load 0.32 ± 0.12 IU/kg within 2 h.

The specific activities of circulating substrates were analyzed at intervals of 15 min from 90 min after the beginning of tracer infusion. The intravenous load of unlabelled glucose was given from 210 to 330 min after the beginning of tracer infusion. During the experiments, the hematocrit declined to the same extent in the diabetic animals (beginning: 0.45 ± 0.01, end: 0.42 ± 0.02) as in the controls (0.44 ± 0.01 and 0.40 ± 0.01 respectively).

Infusates

L-U-^{14}C-alanine (7.4 mBq, sp. act. 6.327 GBq/mmol) and 37.0 MBq D-6-^{3}H-glucose (sp. act. 1.369 GBq/mmol), both from Amersham International (Amersham, Bucks, UK), were purified by ion exchange chromatography the day before the experiment and made up with saline (0.154 mmol/l) to 50.0 ml under sterile conditions. Ten percent of this total dose was applied as an initial bolus injection; the remainder was infused continuously during the experiment. The calculated constant dose was 14.8 KBq/min for ^{14}C-alanine and 74 KBq/min for ^{3}H-glucose; the applied dose was analyzed in each experiment [1]. Unlabelled glucose was dissolved in water to allow the same dose ($56.67\ \mathrm{\mu mol \cdot kg^{-1} \cdot min^{-1}}$) and the same infusion rate in each experiment (average concentration 337 mg/ml). Insulin (same formulation as above) was diluted with saline (0.154 mmol/l) and 10% sterile homologous serum (v/v) to give a concentration of 240 mU/ml.

Chemical analyses

Sampling and storage of specimens and the determination of ^{3}H-glucose specific activity have been described elsewhere [1]. Plasma glucose for glucose monitoring was determined by a glucose oxidase/PO_2 technique (Beckman analyzer, Fullerton, California, USA); immunoreactive insulin activity was analyzed according to a back titration/alcohol precipitation principle [16], and immunoreactive glucagon was estimated by a double antibody technique [17]. The guineapig anti-glucagon antiserum No. 43 (Zentralinstitut für Diabetes "Gerhardt Katsch", Karlsburg, GDR) and the WHO pancreatic glucagon calibration standard No. 69/194 were used for this assay which exhibited a 5% cross-reactivity with porcine entero-glucagon-like immunoreactivity. The concentrations of glucose [18], lactate [19], β-hydroxybutyrate [20] and alanine [21] were measured spectrophotometrically (Photometer Eppendorf 1101 M, Netheler + Hinz GmbH, Hamburg, FRG) from 2 ml of plasma. After deproteination (2 ml 0.5 mol/l $HClO_4$), alanine was separated from the extract by chromatography on a 5-ml column (Quick-Sep, Panchem, Kleinwallstadt, FRG) by a cation exchange resin (Dowex 50 WX8, 200–400 mesh, Serva, Heidelberg, FRG) and was re-eluted with 1.78 mol/l ammonia [22]. Since no appreciable carbon incorporation into other amino acids has been reported under comparable conditions [23], no attempt was made to separate them from this fraction. To obtain glucose and lactate specific activities, the chromatographed acid extracts were neutralized (5 mol/l K_2CO_3) and re-chromatographed (anion exchange resin Dowex 1X8, 200–400 mesh, Serva) [24].

For liquid scintillation counting, all chromatographed samples were lyophylized and dissolved in distilled water. All scintillation counting was performed over 5 or 10 min in Bray's solution [25] on a liquid scintillation spectrometer (Tricarb, Model 2650, Packard, Vienna, Austria).

Data analysis

Recovery of the analytical substrates after this procedure was 92 ± 1% (glucose, $n = 25$), 75 ± 2% (lactate, $n = 28$), and 86 ± 3% (alanine, $n = 23$). On the basis of previous reports [26, 27] Steele's one-compartment model as adapted to non-steady state conditions [28] was employed to calculate the turnover rates of glucose and alanine. On the basis of our own unpublished data, the apparent distribution spaces employed in all computations were 200 ml/kg for glucose and 176 ml/kg for alanine with no difference between normal dogs and normoglycaemic diabetic dogs. The formation of glucose from the alanine carbon moiety was calculated [29] as

$$\text{percentage glucose from alanine} = \frac{\text{sp. act. } ^{14}\text{C glucose}}{\text{sp. act. } ^{14}\text{C alanine}} \times 0.5 \times 100$$

where the factor 0.5 results from the two alanine molecules that are theoretically necessary to yield one glucose molecule. These calculations were not performed for the intervals when unlabelled exogenous glucose was applied, because the specific activities of ^{14}C in circulating glucose is artificially diluted by the test load. To compensate for under-estimation of glucose formation from alanine due to random metabolic exchange of carbon atoms in the hepatic oxaloacetate pool, the glucose formation data were multiplied by 2.2, as established by Hetenyi [30]. The results are expressed as mean ± SEM, and Student's t-test for non-paired data ($p < 0.05$) was used where appropriate.

Results

Glucose metabolism

The diabetic animals had an initial plasma glucose concentration of 22.3 ± 3.8 mmol/l which was restored to normal within 3 h after the beginning of intravenous insulin administration in the morning (data not shown). There was essentially no difference between the two groups during the steady state before initiation of glucose infusion in glycaemia (diabetic dogs 5.22 ± 0.18, controls 5.67 ± 0.21 mmol/l) or in glucose flux rates

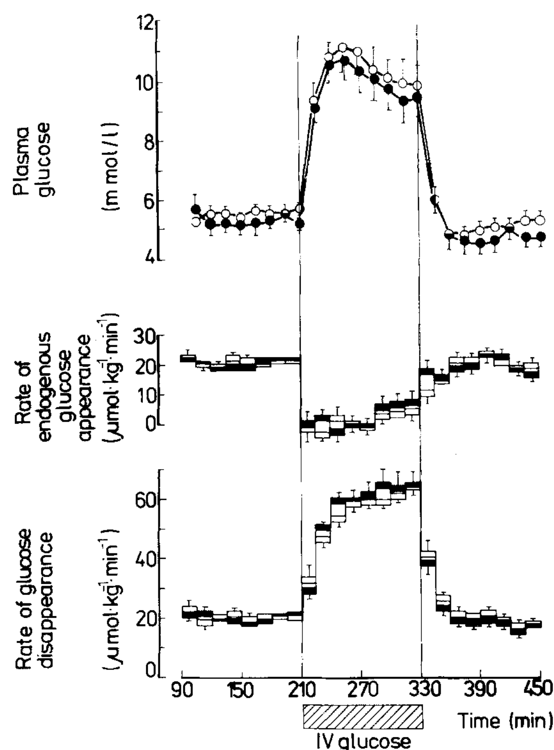

Fig. 1. Concentrations and flux rates of plasma glucose as estimated from 6-^{3}H-glucose specific activities in normal (n = 6, ○—○, □—□) and diabetic dogs on the artificial B cell (n = 5, ●—●, ■—■) before, during and after an intravenous glucose infusion test (56.67 μmol · kg^{-1} · min^{-1}, ▨). To show the endogenous appearance rate during the infusion of unlabelled glucose, the dose infused was subtracted from the estimated total glucose appearance rate. "0 min" would denote the beginning of the primed isotopic tracer infusion. In the diabetic dogs, controlled intravenous insulin supply was initiated 2 hours before the tracer infusion

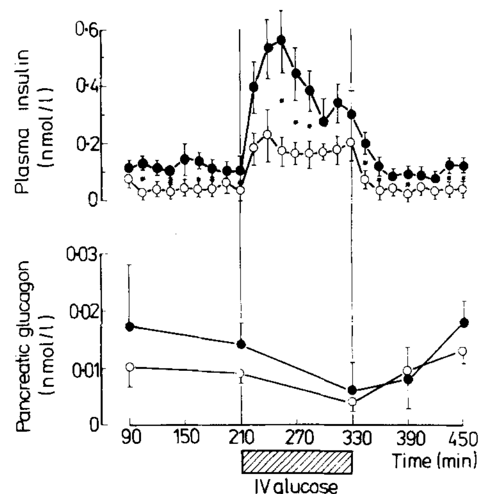

Fig. 2. Mixed venous plasma insulin and pancreatic glucagon concentrations in normal (n = 6, ○—○) and diabetic dogs on the artificial B cell (n = 5, ●—●) in the same tests as given in Figure 1. Significant differences between the groups

(diabetic dogs 20.59 ± 2.40, controls 20.44 ± 1.16 μmol · kg^{-1} · min^{-1}). This held true during the entire period when alanine metabolism was studied (Fig. 1). Furthermore, in the diabetic animals the endogenous glucose production was suppressed to a normal extent when the intravenous load of exogenous glucose was applied (Fig. 1). Moreover, the peripheral β-hydroxybutyrate levels were near-normal throughout the experiment (diabetic dogs before the glucose infusion test: 0.060 ± 0.022 mmol/l, normal dogs: 0.020 ± 0.003 mmol/l, difference not significant). However, the circulating lactate concentrations remained at a higher level in the diabetic dogs: before glucose infusion it was 0.42 ± 0.07 mmol/l in relation to 0.23 ± 0.06 mmol/l in the controls (p < 0.05).

The peripheral plasma pancreatic glucagon levels and their responses to glucose administration were also near-normal throughout the experiment (Fig. 2). There was, however, a distinct hyperinsulinaemia in the mixed venous blood during the entire period in the diabetic animals (Fig. 2). The basal levels were 3 times higher (diabetic dogs: 0.10 ± 0.03 nmol/l, controls 0.03 ± 0.01 nmol/l, p < 0.05). There was roughly the same relation during the glucose infusion test and the interval thereafter.

Basal alanine metabolism

The plasma alanine concentrations in mixed venous blood was significantly elevated in the diabetic dogs: e.g. immediately before the glucose test 0.32 ± 0.05 in relation to 0.18 ± 0.02 mmol/l in the controls. Accordingly, these animals exhibited higher rates of alanine appearance in the circulation: 8.52 ± 0.89 and 5.11 ± 0.63 μmol · kg^{-1} · min^{-1} respectively at the same time during the experiment (p < 0.05, Fig. 3). Since the plasma concentration did not change in both groups, these data also represent the alanine disappearance (i.e. hepatic utilization) and therefore show the total alanine turnover. No difference was evident in the apparent metabolic clearance rates (Fig. 3). However, in the diabetic animals under these conditions a significantly elevated amount of glucose carbon atoms originated from alanine: 15.6 ± 3.0 vs. 6.8 ± 0.8% of the rate of appearance of glucose. (Fig. 3).

The ^{14}C-activities in the lactate-containing plasma fractions were usually smaller than 30 DPM in a given sample (see Methods), i.e. the specific activities could not be determined with sufficient accuracy because they could not be differentiated from background-^{14}C-activity.

Alanine metabolism during the intravenous glucose load

There was no significant change in the plasma alanine concentrations (Fig. 3) which continued to show higher levels in the diabetic dogs. The flux rates, however, responded to hyperglycaemia and hyperinsulinaemia with a reversible increase. In the diabetics, the maximal observed alanine turnover amounted to 13.14 ± 3.06 μmol · kg^{-1} · min^{-1}, i.e. an average increase of 70% at the end of the glucose load. In the controls the related

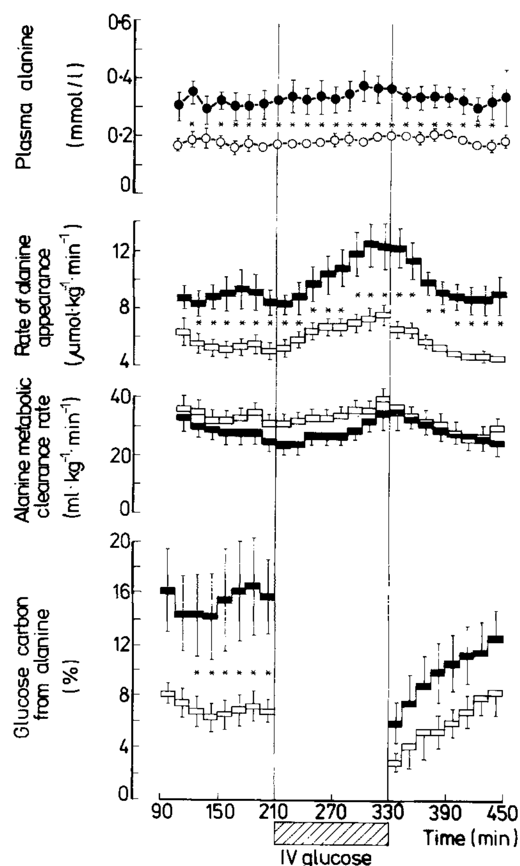

Fig. 3. Alanine metabolic patterns in normal ($n = 6$, ○—○, □—□) and diabetic dogs on the artificial B cell ($n = 5$, ●—●, ■—■) in the same tests as given in Fig. 1. Panels from top: - plasma alanine concentration; - alanine flux rates as estimated from the specific activities of plasma-U-^{14}C-alanine (since the plasma concentration was in steady state, the rates of appearance and of disappearance are virtually identical); - metabolic clearance rates of alanine from mixed venous plasma; - relative share of alanine flux rate in glucose appearance, i.e. apparently in hepatic gluconeogenesis. This estimate was not obtained for the interval when non-labelled glucose was infused, because the specific ^{14}C-activity to circulating glucose did not exclusively represent endogenous dilution of the label. Significant differences between the groups

numbers were $7.69 \pm 0.74\,\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ and 50% respectively. The relative increase in alanine flux was not significantly different between the two groups.

Since this increase in alanine turnover was not paralleled by a change in plasma alanine concentration, the apparent metabolic clearance rates of plasma alanine were also found to rise (Fig. 3). This was significant in the control animals: 31.3 ± 2.9 vs. $40.1 \pm 3.0\,\text{ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ at the end. It should, however, be noted that, in general, there was no difference in the metabolic clearance rates of alanine between the two groups of animals.

Although the exogenous glucose load did suppress the endogenous glucose appearance to negligible rates (Fig. 1) and no rates of glucose formation from alanine carbon could be calculated, there was still a significant shift of the carbon label from the alanine pool to circulating glucose. This could be reproduced in all animals of both groups during the entire period of external glucose supply. Thus, precise rates of alanine conversion to glucose were again observed from the beginning of the post-glucose infusion period (Fig. 3).

Discussion

On the basis of a total lack of endogenous β-cell function, the animals investigated in this study were chronically diabetic. Their long-term treatment, employing three subcutaneous injections of short-acting insulin, was generally insufficient. This is particularly obvious when the second half of the night is considered: glycaemia was always > 20 mmol/l but peripheral insulinaemia was not measurably low. In contrast to some other reports [11, 23], the plasma alanine levels were also elevated in this situation.

In the diabetic animals the feedback-controlled intravenous insulin administration did restore to normal both glycaemia and endogenous glucose flux rates for a relatively short interval under basal conditions and during parenteral glucose administration. However, to reach these perfect "blood glucose cosmetics", hyperinsulinaemia was induced in the posthepatic circulation as described before [2, 31]. Since this peripheral plasma insulin elevation was about threefold in relation to the control animals, it may be speculated that normal amounts of insulin were available in the liver (hepatic extraction approximately 50%) (32, 33) but without restoration of the normal portal-peripheral gradient of insulin concentration. Also, the circulating glucagon concentrations were in the near-normal range during the tests. Thus the insulin: glucagon ratio in the liver, which appears most important in control of hepatic glucose production [34], is assumed to be normal.

The data reported here on alanine metabolism correspond closely to those obtained by other authors who found plasma concentrations around 0.2 mmol/l [35-37], production rates between 5 and $7\,\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ [8, 22, 35, 38] and a relative share of glucose carbon from alanine metabolism around 6% [26, 38]. The increase in alanine flux during comparable glucose loads was about 50% during comparable glucose loads in healthy men [35].

In the experimental animals of this study, the alanine-glucose cycle was working under the condition of a chronic diabetes in normoglycaemic, resting, short-term fasting subjects that were well nourished, especially without any protein-malnutrition that could have influenced the balance of proteins and amino acids in muscle.

Both under basal conditions and during parenteral glucose administration in these animals, there were elevated circulating concentrations and flux rates of alanine with a normal response to the exogenous glucose load. Since similar increases in alanine concentration have been shown in diabetic dogs during long-term in-

sulin infusion [3], it appears improbable that these alterations are simply due to an only short-term optimal glycaemic control.

Despite the reported amount of up to about 20% of alanine released from skeletal muscle which have received their carbon moieties from products of glycolysis [7], the fluxes of alanine and of glucose in muscle appear as independent processes in vitro [39, 40] and - according to findings in literature - the production of alanine in man is only negligibly influenced by variations in the metabolic rate of glucose [35, 37, 41]. In our preliminary study (see above), an insulin-induced blood glucose decrease in normal and in diabetic dogs with identical plasma insulin patterns did not influence the circulating plasma alanine concentration. Since in this investigation there were also identical metabolic patterns of glucose in both groups, it is concluded that the peculiarities in alanine flux are not caused by simple quantitative differences in glucose metabolism. Employing mathematical modelling of metabolic processes, it has been shown by Albisser et al. [42] that, in contrast to the physiological action of insulin, the hormone is mainly acting in the extrahepatic tissues when applied intravenously. In the light of these findings, the peripheral hyperinsulinaemia observed here in the diabetic animals might have caused higher amounts of amino acids [7], especially of branched-chain species, to provide amino groups and carbon moieties for alanine synthesis in muscle [39, 40, 43].

In the range observed, there is obviously no limitation of hepatic alanine uptake [7, 35]. This is also confirmed by the increase in metabolic clearance rates of alanine as shown in this study. Therefore it is concluded that the differences observed in circulating alanine concentrations between the diabetic and the control animals are not based on an insufficient utilization of alanine in gluconeogenesis. Despite a clear-cut suppression in hepatic glucose output during the glucose load, there is still an appearance of alanine-derived carbon atoms in the circulating glucose pool which could unfortunately not be quantified in this study [44].

In addition it might be speculated that the flux of alanine carbon is directed into other metabolic compartments, e.g. into glycogen [35, 45]. This might also explain the reduced rate of carbon recirculation (Cori cycle) which was found under these conditions in diabetic patients [6] and dogs [1] despite high circulating levels of alanine and lactate.

Possible consequences of these phenomena on protein and amino-acid metabolism during artificial B cell treatment remain to be investigated. Also whether perfect blood glucose control for a longer interval or sufficient hepatic and peripheral insulin supply could restore complete physiological metabolic patterns need to be studied.

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