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Acidosis rather than Ketosis Reduces Insulin Action during Metabolic Ketoacidosis: An Investigation by Glucose Clamp Study in the Rat

Mitsuo MATSUMOTO, Masahiko IGARASHI, Makoto TOMINAGA, Makoto DAIMON, Norio FUKASE, Kazuhiko SUGIYAMA, Atsushi OGAWA, Keiichi YAMATANI and Hideo SASAKI

Department of Internal Medicine III, Yamagata University School of Medicine, 2-2-2 Iida-nishi, Yamagata 990-23, Japan

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Summary. **To elucidate the occurrence of insulin resistance in metabolic ketoacidosis, we examined the hepatic glucose output (HGO) and glucose disappearance rate (Gd) by a euglycemic insulin clamp study on male Wistar rats under experimental metabolic ketosis and acidosis. Metabolic ketosis and acidosis were induced by the continuous intravenous infusion of either 3-hydroxybutyrate or ammonium chloride, respectively. Arterial blood gas analysis was performed by use of a blood gas analyzer. Insulin was infused at a rate of 3 mU/kg/min (low dose) or 20 mU/kg/min (high dose) during the glucose clamp course. Insulin sensitivity was evaluated by the mean of steady state glucose infusion rate (GIR) during the last 20 min. HGO and Gd were measured using 3-[^{3}H]-glucose. Steady state GIR of acidotic rats was 2.65 ± 0.25 mg/kg/min with low dose insulin and 7.22 ± 0.42 mg/kg/min with high dose insulin. Thus, steady state GIR in both condition was significantly lower than those of the control rats. Steady state HGO of acidotic rats was not suppressed under low dose insulin infusion, while HGO of the normal control rats was completely suppressed. On the other hand, neither the GIR and HGO of the ketotic rats differed from those of the control. Our results suggest that metabolic acidosis rather than ketosis is responsible for insulin resistance in metabolic ketoacidosis.**

INTRODUCTION

Marked insulin resistance is usually found in diabetes mellitus during ketoacidosis.[1-7] Several investigators have reported that insulin resistance in metabolic ketoacidosis may be caused by acidosis,[1,4] and counterregulatory factors such as growth hormone[2] and free fatty acid.[3] Whittaker et al. speculated that the pH dependance of the insulin binding to adipocytes from ketoacidotic streptozocin diabetic rats contributed to insulin resistance.[5] *In vivo*, Barrett et al. reported that impaired glucose disposal by peripheral tissues was one important site of insulin resistance in diabetic ketoacidosis patients.[6] On the other hand, the euglycemic glucose clamp technique originally described by Andres et al.[8] has provided us the most precise *in vivo* method for evaluating insulin action in this milieu. Yki-Järvinen et al. used the euglycemic clamp technique to study the insulin sensitivity of newly diagnosed post-ketoacidotic diabetic patients and speculated that intracellular defects in peripheral tissues results from insulin resistance.[7] However, their experiment was performed after the disappearance of ketosis and after 3 months of insulin therapy. The main cause of the insulin resistance remains poorly understood.

The lack of knowledge about the occurrence of insulin resistance during ketoacidosis prompted us to systematically study the acute effects on glucose handling *in vivo*. The aim of this study was to determine by the glucose clamp technique in rats *in vivo* whether insulin resistance in metabolic ketoacidosis results from ketosis or acidosis.

METERIAL AND METHODS

Animals and treatment

Male Wistar rats (Funabashi Farm, Chiba) weighing 230-250 g were initially divided into three respecture groups of control, acidosis and ketosis. After fasting the animals for 20 h, the following treatments were performed. In the acidotic group, after 0.35 g/kg ip

chloral hydrate (Nakarai Chemical, Kyoto) anesthesia, a Silastic catheter (Dow-Corning, Midland, MI, USA) was cannulated into the right jugular vein. According to the method by Mackler et al.[9] ammonium chloride (Wako Pure Chemical Industries, Osaka) diluted with 0.9% saline was infused in 0.3 ml/h with a syringe pump (Terufusion, Terumo, Tokyo) for 12 h to produce metabolic acidosis. Ketosis was induced by the method by Balasse et al.[10] After the same anesthesia, a Silastic catheter was cannulated into the left femoral vein of the rat, and 3-hydroxybutrate (Wako Pure Chemical Industries, Osaka), which is an intermediate metabolite of ketone bodies, was infuse at a rate of 0.5 ml/hr with syringe pump for 2 h. In both groups, each infusion was continued until the end of the glucose clamp experiment.

Euglycemic glucose clamp study and calculation

After these treatments, the rats were subjected to the euglycemic glucose clamp according to the modified method[11,12] by Andres et al.[8] and DeFronzo et al.[13] Insulin infusion rates (IIR) of the normal control rats were 3 and 20 mU/kg/min. Insulin was infused into the acidotic rats at the same rate as the control (3 and 20 mU/kg/min). However, in the ketotic rats, only 3 mU/kg/min insulin was infused. Under 0.35 g/kg ip chloral hydrate anesthesia, two Silastic catheters were cannulated into the left femoral vein for 3-[^{3}H]-glucose (New England Nuclear, Boston, MA, U. S. A.) and 20% glucose infusions, and the other catheter into the right femoral vein for the diluted insulin infusion. One more Silastic catheter was inserted into the left jugular vein for sampling heparinized blood to measure glucose by a Glucose Monitor (Kyoto Daiichi Kagaku, Kyoto). Another Silastic catheter was cannulated into the left carotid artery for blood sampling to determine the specific activity of 3-[^{3}H]-glucose as well as the insulin level and for blood gas analysis.

After the surgical procedure and while the rats were still under the same anesthesia, 5 μCi 3-[^{3}H]-glucose was infused as a bolus, followed by continuous infusion at a rate of 0.05 μCi /min using a peristaltic roller pump (Miniplus 2, Gilson, France) until the end of the experiment. Porcine insulin (Actrapid MC, Novo Research Institute, Bagsvaerd, Denmark) diluted with saline containing 0.25% bovine serum albumin (BSA, Fraction-V, Sigma Chemical, St. Louis, MO, U. S. A.) was continuously infused using a syringe pump after a 10-min priming infusion until the end of the following experiment. Blood glucose levels were maintained within 10% variation of the basal levels for 60 min by variable infusion rates of 20% glucose which were calculated from the levels of, and changes in, glucose concentrations as measured by the Glucose Monitor every 2 min. A modified algorithm to determine the glucose infusion rate (GIR), originally described by DeFronzo et al.,[13] was employed as previously described.[11] Briefly, the calculation using programmable calculator was as follows:

$$GIR = 0.2 \times (GD - BG) + GC$$

$$GC = GC_{-1} - [BG - GD \times 4(BG - BG_{-1})]/64$$

where GD is the target blood glucose level, BG the blood glucose level, and GC a constant determined during the clamp.

Blood samples were taken at 55 and 60 min of the glucose clamp and were immediately mixed with $ZnSO_4$ and $Ba(OH)_2$ and centrifuged to measure insulin levels and the specific activity of 3-[^{3}H]-glucose. The specific activity of 3-[^{3}H]-glucose in the deproteinated supernatant was measured with a liquid-scintillation counter. Glucose disappearance rate (Gd) and hepatic glucose output (HGO) were determined by Steele's equation.[14] These are the parameters for insulin action in addition to the mean glucose infusion rate (GIR) during the last 20 min clamp. In the basal state before the porcine insulin infusion, basal HGO was estimated to be the same as the basal Gd. HGO during the clamp was determined by subtracting GIR from the clamp total Gd. if HGO was a minus datum, the HGO was regarded as zero.

Other measurements

Blood glucose and insulin levels in the blood sampled at the end of the clamp were measured by the glucose oxidase method with the Glucose C test kit (Wako Pure Chemical Industries, Osaka) and radioimmunoassay[15] using porcine insulin as a standard, respectively. Blood gas analysis was immediately performed with an automatic blood gas analyzer. 3-hydroxybutyrate were measured with Ketone test B (Sanwa Chemicals, Nagoya).

Statistical analysis

Results were expressed as the means $\pm$ SEM, and statistical significance was assessed by non-paired Student's t-test.

RESULTS

Blood glucose and insulin levels

As shown in Table 1, there was no significant difference in the mean blood glucose levels between the basal and steady states. Thus, euglycemic states were confirmed in all cases. Insulin levels among the three groups' infused insulin at 3 mU/kg/min were not different. However, the insulin level in acidotic group infused insulin at 20 mU/kg/min was significantly higher than that of the corresponding control ($p<0.005$).

Concentration of 3-hydroxybutyrate

The concentration of 3-hydroxybutyrate in the ketosis group was significantly higher than that in the control ($p<0.005$) (Table 1).

Blood gas analysis

PHs of the acidotic groups (Acidosis; 6.858 ± 0.065 at 3 mU/kg/min of IIR and 6.800 ± 0.026 at 20 mU/kg/min of IIR) were significantly lower than those of the controls (Control; 7.310 ± 0.029 at 3 mU/kg/min of IIR and 7.327 ± 0.027 at 20 mU/kg/min of IIR) ($p<0.001$). The values of base excess and HCO_3^- in the acidotic groups were remarkably lower than those in the controls, confirming metabolic acidosis. Here, pH in the ketotic group was more alkalotic than that in the control group presumably due to a buffering system ($p<0.001$) (Table 2).

GIR

As shown in Fig. 1, the mean values of steady state GIR during the last 20 min of glucose clamp study, a parameter of insulin sensitivity, were significantly lower in the acidotic group than in the control (Control; 8.29 ± 0.69 mg/kg/min vs. Acidosis; 2.65 ± 0.25 mg/kg/min at 3 mU/kg/min of IIR, and Control; 20.61 ± 0.97 mg/kg/min vs. Acidosis; 7.22 ± 0.42 mg/kg/min at 20 mU/kg/min of IIR, respectively) ($p<0.001$). That of the ketotic group was not significantly different from the control. In addition, a significant

Table 1. Basal and steady state blood glucose, steady state serum 3-hydroxybutyrate and steady state insulin concentrations

Group	Insulin infusion rate (mU / kg / min)	Blood glucose (mg/dl) Basal	Blood glucose (mg/dl) Steady state	Steady state serum 3-hydroxybutyrate (μmol/l)	Steady state serum insulin (μU/ml)
Control	3(n=5)	118.8±3.4	108.7±3.6	276.6±15.4	234.3±25.5
	20(n=5)	122.2±5.2	106.2±6.7		930.6±229.7
Acidosis	3(n=5)	116.6±8.3	115.5±8.3		290.5±63.9
	20(n=5)	115.4±11.2	108.5±12.2		3990.5±750.4**
Ketosis	3(n=5)	115.3±6.4	104.1±6.8	4939.3±1193.3*	176.3±50.8

*$p<0.005$ compared with control (IIR 3 mU/kg/min), **$p<0.005$ compared with control (IIR 20 mU/kg/min)
mean ± SEM

Table 2. Steady state arterial blood gas analysis

Group	Insulin infusion rate (mU/kg/min)	pH	Base excess (mEq/l)	HCO_3^- (mEq/l)
Control	3(n=5)	7.310±0.029	−9.9±1.1	15.8±0.9
	20(n=5)	7.327±0.027	−10.4±0.6	14.4±0.8
Acidosis	3(n=5)	6.858±0.065*	<−30*	2.2±0.5*
	20(n=5)	6.800±0.026**	<−30**	2.2±0.4**
Ketosis	3(n=5)	7.546±0.036*	12.3±2.4*	37.2±2.3*

*$p<0.001$ compared with control (IIR 3 mU/kg/min),
**$p<0.001$ compared with control (IIR 20 mU/kg/min)
mean ± SEM

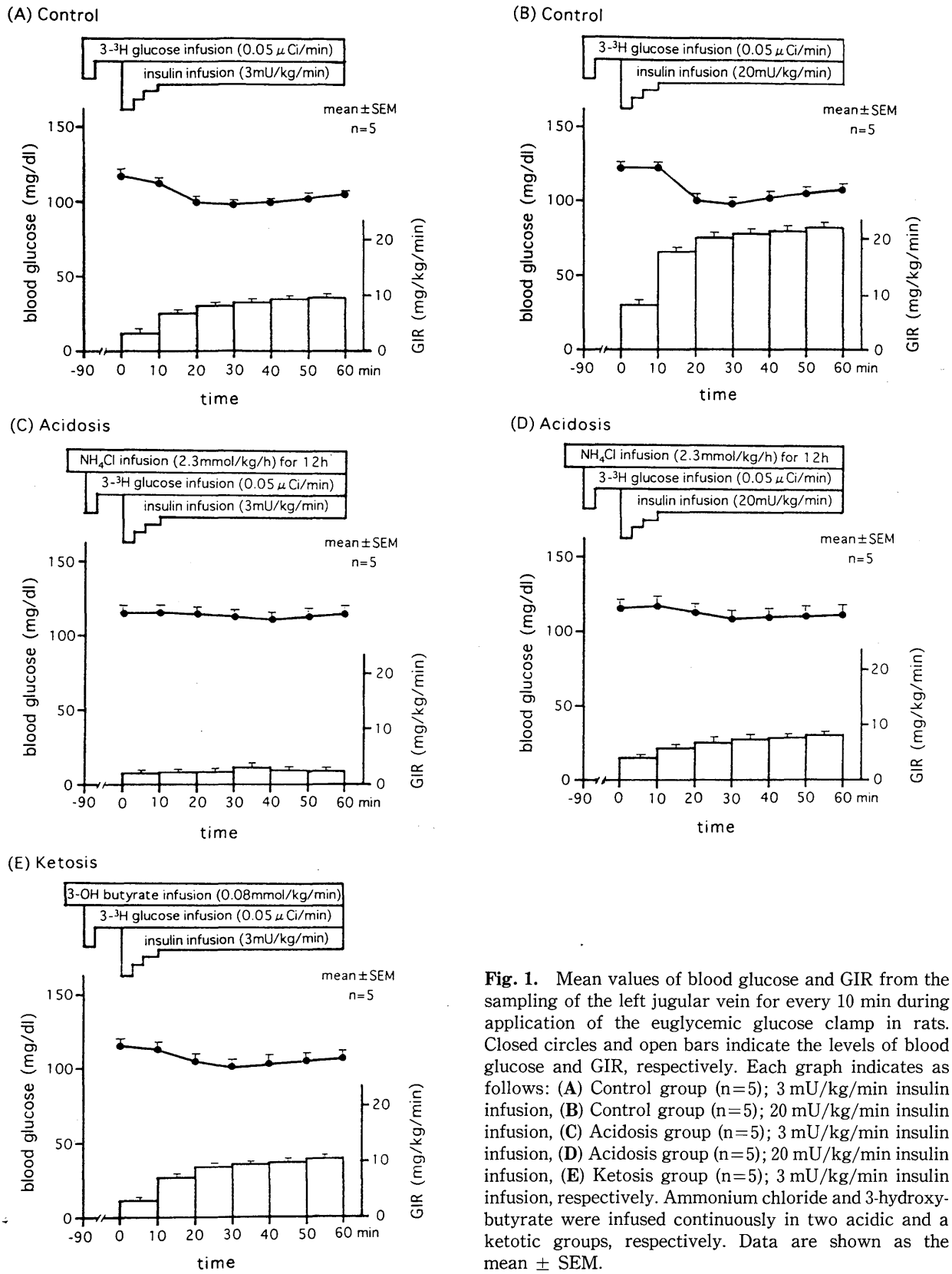

Fig. 1. Mean values of blood glucose and GIR from the sampling of the left jugular vein for every 10 min during application of the euglycemic glucose clamp in rats. Closed circles and open bars indicate the levels of blood glucose and GIR, respectively. Each graph indicates as follows: **(A)** Control group (n=5); 3 mU/kg/min insulin infusion, **(B)** Control group (n=5); 20 mU/kg/min insulin infusion, **(C)** Acidosis group (n=5); 3 mU/kg/min insulin infusion, **(D)** Acidosis group (n=5); 20 mU/kg/min insulin infusion, **(E)** Ketosis group (n=5); 3 mU/kg/min insulin infusion, respectively. Ammonium chloride and 3-hydroxybutyrate were infused continuously in two acidic and a ketotic groups, respectively. Data are shown as the mean ± SEM.

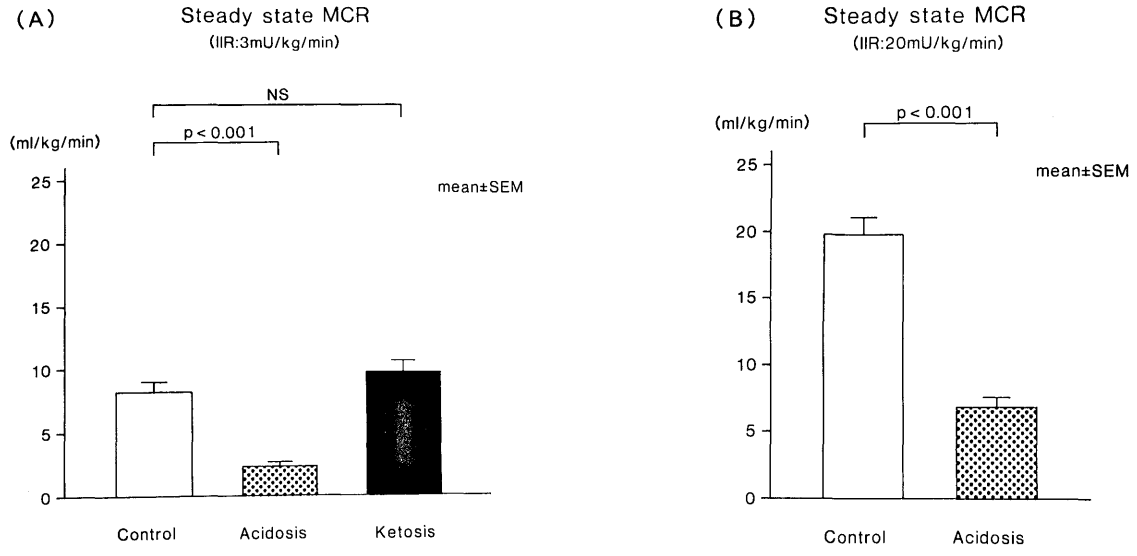

Fig. 2. MCR in the steady state, GIR being divided by each blood glucose level during the last 20 min of the euglycemic glucose clamp study. (**A**) (left) and (**B**) (right) indicate the values in the 3 mU/kg/min and 20 mU/kg/min of insulin infusion rates, respectively. Open bars show the control group, shaded bars the acidotic, and closed bars the ketotic groups. All groups consisted of five subjects and data are shown as the mean ± SEM.

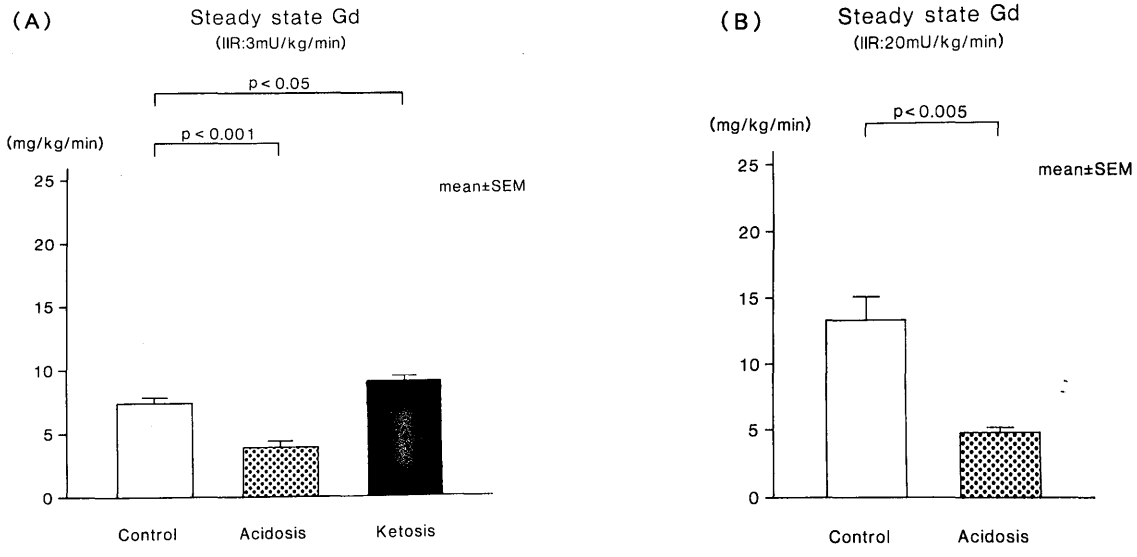

Fig. 3. Gd in the steady state, which were the mean of the values at 55 and 60 min in the euglycemic glucose clamp study. (**A**) (left) and (**B**) (right) indicate the values in the 3 mU/kg/min and 20 mU/kg/min of insulin infusion rates, respectively. Open bars show the control group, shaded, the acidotic, and closed, the ketotic groups. All groups five subjects and data are shown as the mean ± SEM.

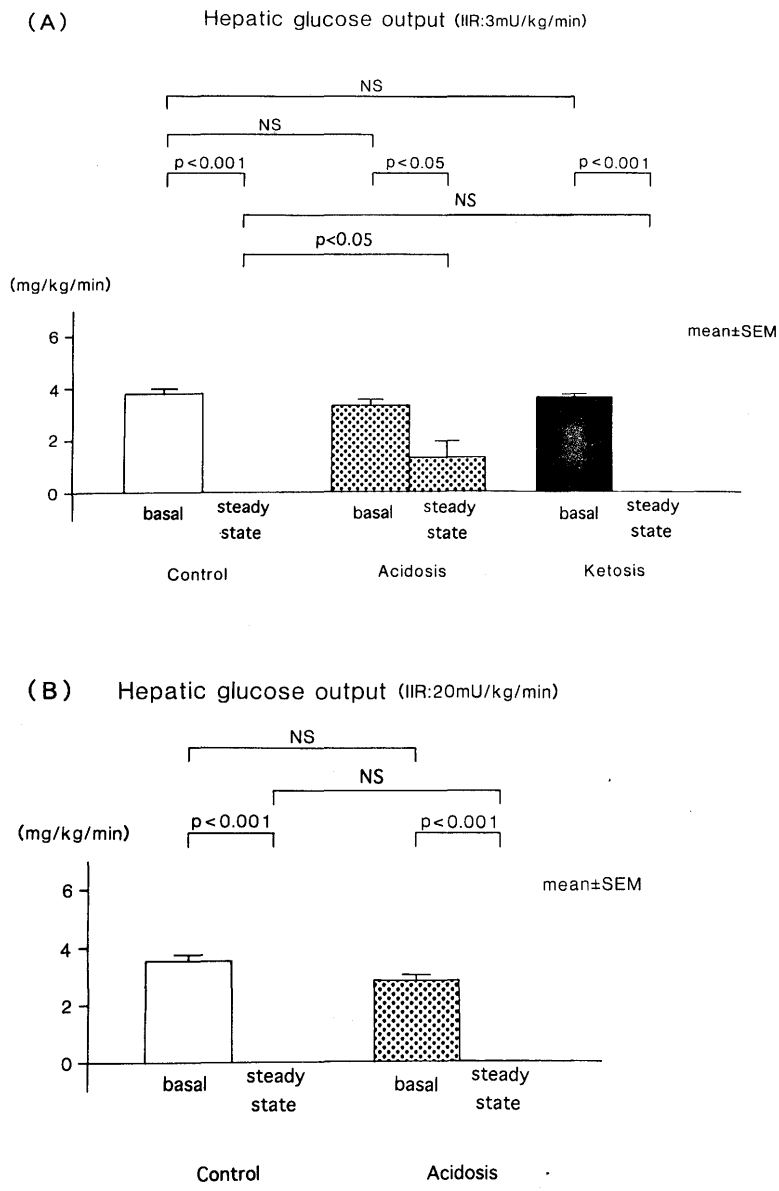

Fig. 4. Values of HGO in the basal and steady state of the euglycemic glucose clamp study. (**A**) (upper) and (**B**) (lower) indicate the values in the 3 mU/kg/min and 20 mU/kg/min of insulin infusion rates, respectively. Open bars show the control, shaded, the acidotic, and closed the ketotic groups. The values of basal HGO (each group's left) are indicated the mean of the two points before starting, and the values of steady state (each group's right), the mean of the values at 55 and 60 min in this experiments. HGO during the clamp was determined by subtracting GIR from the clamp total Gd. if HGO was found to be a minus datum, the HGO was estimated as zero. All groups had five subjects and data are shown as the mean ± SEM.

difference was observed in the steady state metabolic clearance rates (MCR), in which the influence of glucose clamp levels were excluded (Fig. 2).

Gd and HGO

The values of steady state Gd in the controls were significantly higher than those in the acidic groups at the same insulin infusion rates (Control; 7.42 ± 0.45 mg/kg/min vs. Acidosis; 3.91 ± 0.46 mg/kg/min at 3 min/kg/min of IIR ($p < 0.001$), and Control; 13.25 ± 1.76 mg/kg/min vs. Acidosis; 4.75 ± 0.29 mg/kg/min at 20 min/kg/min of IIR ($p < 0.005$), respectively). However, that of the ketotic group was higher than that of the control ($p < 0.05$) (Fig. 3).

As shown in Fig. 4, there were no significant differences in the values of basal HGO before the glucose clamp study among the control, acidotic and ketotic groups. Steady state HGO in the control and the ketotic groups was completely suppressed at 3 mU/kg/min of IIR, while HGO in the acidotic group was not suppressed completely. At 20 mU/kg/min of IIR, steady state HGO in the control and the acidotic group was completely suppressed, showing that the HGO in the acidotic group was suppressed by only high dose insulin.

DISCUSSION

With respect to insulin resistance in metabolic ketoacidosis, several studies have been performed in whole bodies[6,7] or target cells.[4,5,16,17] According to their results, the mechanism of insulin resistance in metabolic ketoacidosis may be due to intracellular defects in glucose metabolism. Andres et al.[8] and DeFronzo et al.[13] developed the euglycemic glucose clamp technique, which is capable of evaluating insulin action precisely *in vivo*. This technique has several advantages for assessing glucose metabolism: 1) because of constant plasma glucose concentration, the amount of glucose infused must be equal to the amount of glucose taken up by the whole body (unless no urinary glucose was lost); and 2) this technique has major advantages in assessing insulin sensitivity as it maintains the basal glucose concentration and avoids the influence of endogenous insulin and counter-regulatory hormones. The evaluation of insulin sensitivity by this technique in severe metabolic ketosis and acidosis in small experimental animals, such as the rat, has not been reported.

In this study, we employed euglycemic glucose clamp study to examine whether insulin resistance in metabolic ketoacidosis results from ketosis or acidosis. We produced extreme conditions of metabolic acidosis and ketosis by loading high concentrations of ammonium chloride and 3-hydroxybutyrate, respectively (Table 2). As a result, the steady state GIR and Gd, the parameters of insulin sensitivity of acidic rats, were lower than those of control and ketotic rats either with low dose insulin or high dose insulin (Fig. 3). These results indicated the presence of insulin resistance in peripheral tissues. Steady state HGO of acidotic rats was not suppressed under low dose insulin infusion, suggesting that glucose production was insensitive to insulin, or, in other words, hepatic insulin resistance (Fig. 4). Therefore, metabolic acidosis induces a reduction in insulin sensitivity in both peripheral tissues and liver. It indicates that metabolic acidosis rather than ketosis is responsible for insulin resistance in metabolic ketoacidosis.

Metabolic ketoacidosis, *in vivo*, is accompanied by increased concentrations of ketone bodies in the blood flow, which may contribute to insulin resistance. However, in our results, the value of steady state Gd in the ketotic group was higher than that in the control group (Fig. 3), despite the absence of any difference in HGO values both in the basal and steady state between the ketosis and control groups (Fig. 4). This shows that insulin sensitivity in the liver and peripheral tissues under ketosis slightly increases compared with the normal state. Other investigators have shown that ketone bodies alone were not related to insulin resistance in metabolic ketoacidosis,[16,17] and that the plasma glucose level decreased under experimental ketosis because of stimulated insulin secretion,[18] suppressed hepatic glucose production and enhanced peripheral glucose utilization.[19,20] These reports did not contradict our study. Recently, we have reported that insulin resistance in metabolic acidosis resulted from combined defects in binding affinity and the post-binding process in the insulin receptor on rat epididymal adipocytes.[21] In an acidic state (pH 6.8), the uptake of 2-deoxy-D-glucose by adipocytes was lower than those in alkaline (pH 8.0) and neutral (pH 7.4) states at the various bound insulin concentrations. On the other hand, in ketosis, the insulin receptor binding rates to adipocytes[16] and fibroblasts[22] were found to be unchanged or slightly increased compared with the neutral state. In the present study, we confirmed insulin resistance in experimental metabolic acidosis *in vivo*, using the euglycemic glucose clamp method.

In conclusion, our results suggest that metabolic acidosis *in vivo* contributes to produce insulin resis-

tance in the liver and peripheral tissues, indicating that metabolic ketoacidosis may cause the reduction in insulin sensitivity by acidosis but not by ketosis.

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Correspondence: Masahiko Igarashi, MD, Dept. of Internal Medicine III, Yamagata University School of Medicine, 2-2-2 Iida-nishi, Yamagata 990-23, Japan.