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Effect of Insulin in vitro on the Isolated, Perfused Alloxan-Diabetic Rat Liver

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Summary. Withdrawal of exogenous insulin and a subsequent fast (24 h) of alloxan diabetic rats stimulated rates of gluconeogenesis, ureogenesis, ketogenesis, and amino acid release by in situ perfused livers when compared to those from normal, fasted rats. The contribution of liver glycogen to the high rates of gluconeogenesis observed with the diabetic liver could be excluded. Perfusate lactate concentrations remained constant during the period when the elevated rate of gluconeogenesis was observed with diabetic liver. Addition of insulin as a bolus (750 mU) and continuous infusion (12.5 mU/min) to the perfusion medium of diabetic livers resulted in constant perfusate levels of glucose, urea and α-amino nitrogen indicating a suppression of the catabolic processes present in the fasted, diabetic liver. The rate of ketogenesis was also slowed by insulin to about half the rate prior to addition of the hormone. These data indicate that insulin has an immediate anti-catabolic effect in the perfused, diabetic liver.

Key words: Diabetes, liver, perfusion, insulin, gluconeogenesis, ureogenesis, ketogenesis, α-amino nitrogen, triglyceride.

The demonstration of a direct effect of insulin on the isolated perfused rat liver has shown that the hormone decreases glucose [1, 2], urea [1, 3] and amino acid production [3]. Recently, Mortimore and Mondon [4] have reported that insulin had an inhibitory effect on the release of valine from protein but failed to show any influence of the hormone on its incorporation into liver protein in the isolated perfused rat liver. One might expect that the diabetic liver, with its high rates of catabolism [5, 6], might be particularly responsive to insulin addition in the isolated liver perfusion system.

Insulin treatment in vivo as short as 0.5 h before isolating the liver from diabetic rats has been shown to reverse metabolic alterations in the isolated, perfused diabetic liver [6]. Although insulin addition to the medium perfusing the alloxan diabetic liver has been shown to decrease amino acid production [7], there is no consistent view on the effect of insulin in vitro on urea and glucose production of the isolated, diabetic rat liver [7–11].

In the present study, we have investigated the addition of insulin in vitro to the perfused, fasted diabetic rat liver in order to resolve these problems.

Materials and Methods

Materials

Male albino rats of the Sprague-Dawley strain were obtained from ARS Sprague-Dawley, Madison, Wisconsin. Bovine fraction V albumin was purchased from Miles Laboratories, Inc., Elkhart, Indiana and purified as described below. Outdated, packed human erythrocytes were supplied by the Milwaukee Blood Center, Milwaukee, Wisconsin, and were used within five days of their expiration. Alloxan monohydrate was obtained from Nutritional Biochemicals, Cleveland, Ohio. Lente insulin (Eli Lilly, Indianapolis, Indiana) was utilized for maintenance of diabetic rats. Porcine insulin (0.002% glucagon) used in the perfusion studies was a gift

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from Dr. Mary A. Root, Indianapolis, Indiana (Lot No. 615-D63-10). The enzyme and co-factors necessary for the analysis of perfusate samples were obtained either from Boehringer Mannheim Corporation, New York, New York, or Sigma, St. Louis, Missouri. All other chemicals were of reagent grade.

Alloxan Diabetes

Fed, male rats, weighing approximately 170 g, were lightly anaesthetized with ether and the femoral vein was exposed. Alloxan monohydrate (60 mg/kg) in 9 g/l NaCl was injected as a bolus IV, followed by an IP injection of 5 ml of 9 g/l NaCl. Controls received only the sodium chloride in amounts similar to alloxan-treated rats. Approximately 36 h later, 6 U of Lente insulin SC were given and this continued daily. Alloxan diabetic rats were maintained on insulin replacement therapy for at least 2 weeks, at which time they reached a weight (approximately 250 g) similar to the control rats. The last insulin injection was given 48–54 h before the livers were to be perfused. Twenty-four to 30 h before the perfusion time, diabetic and normal rats were placed in metabolism cages without food. All diabetic rats exhibited polyuria, glucosuria (Diastix®, Ames Company, Elkhart, Indiana) and in most cases ketonuria (Acetest®, Ames Company, Elkhart, Indiana).

Liver Perfusion

The in situ isolated rat liver perfusion as described by Hems et al. [12] was used. The perfusion medium (100 ml) consisted of: washed, outdated human erythrocytes to give a final hemoglobin concentration [13] of approximately 4.5 g; 2 g of dialyzed, lypholized bovine serum albumin and Krebs-Henseleit [14] bicarbonate buffer-pH 7.4, previously gassed with 95% O_2: 5% CO_2. The perfusion medium was prepared without added glucose to enable the detection of small changes in perfusate glucose, since no exogenous gluconeogenesis substrate such as alanine was added.

Criteria used in determining success of perfusion were liver color, flow rates (1.2 ml/g wet wt/min with 15 to 20 cm H_2O hydrostatic pressure) and wet wt/dry wt ratio (3.5 to 3.75). The wet wt/dry wt ratio was determined by drying a weighed piece of liver at 125° C for at least 6 h and reweighing. The values obtained were in good agreement with those reported by Hems et al. [12].

Both normal and diabetic livers were perfused for a 30 min equilibration time before perfusate and/or tissue samples were obtained for analysis. Perfusate samples were then obtained from the reservoir at 5 min intervals for an additional 60 min and protein-free filtrates were prepared with perchloric acid. In some experiments a liver biopsy (~200 mg) was taken after 30 min equilibration and immediately frozen with Wollenberger clamps [15], precooled with liquid N_2. All tissue samples were powdered with a percussion mortar and stored at liquid N_2 temperature until the glycogen and triglyceride analyses were performed.

The effect of insulin on diabetic liver metabolism was studied by adding 750 mU of the hormone in 1 ml of 2 g/100 ml albumin after 30 min of the experimental period and an infusion of 12.5 mU/min for 30 min was continued until the end of the perfusion. The normal and diabetic livers without insulin received the same amounts of albumin alone.

Determination of Perfusate and Liver Constituents

Glucose [16], lactate [17], acetoacetate [18], 3-hydroxybutyrate [19], and urea [20] in protein-free filtrates of perfusion medium were determined by enzymatic methods. Perfusate α-amino N was analyzed by the ninhydrin procedure of Rosen [21]. Glycogen and triglyceride in frozen powder of liver samples were determined as described by Huijing [22] (results expressed as glycogen-glucose) and Laurell [23], respectively. All perfusate and liver constituents were expressed as μmol/g wet liver. Total ketone bodies in the perfusate are the sum of acetoacetate and 3-hydroxybutyrate. Rates of metabolite production were calculated for the 30–60 min and 60–90 min periods using linear least squares regression analysis. The correlation coefficient was always greater than 0.9 for these fits. Upon addition of insulin to the perfusate of diabetic livers, rates for gluconeogenesis and ureogenesis could not be calculated as the hormone completely suppressed these processes. Significance of differences between experimental groups of perfused livers were tested by Student's t-test.

Results

The rates of gluconeogenesis, ureogenesis and ketogenesis over two 30 min perfusion intervals of perfused livers from normal rats fasted for 24 h were determined (Table 1). The rates of ureogenesis and ketogenesis were similar over both intervals, while the production of glucose by the liver from normal rats was approximately twice as high ($P<0.02$) during the first interval (30–60 min) than the second (60–90 min). The difference in the rate of glucose production between the two perfusion in-

tervals can be accounted for by the decrease in perfusion medium lactate concentration from 1.2 to 0.6 mmol/l which occurred during the period 30 to 60 min. This rate of lactate uptake (0.25 μmol/g wet liver/min) during the period of 30 to 60 min could account for most of the difference in the rates of gluconeogenesis observed, if one assumes stoichometric conversion of lactate to glucose. Liver glycogen content were extremely low in perfused livers from normal, fasted rats after 30 min of perfusion (approximately 1 μmol glycogen-glucose/g wet liver). Thus, the possible contribution of liver glycogen to the observed rate of gluconeogenesis was negligible. During the perfusion interval 60–90 min, medium lactate content remained constant at 0.4 mmol/l with perfused livers from normal, fasted rats. The production of α-amino N (Table 2) and rates of ureogenesis (Table 1), the values of which were constant during both intervals, indicated that endogenous liver protein breakdown provided the major share of the carbon necessary for the rate of gluconeogenesis observed, especially when perfusate lactate remained constant during 60–90 min.

The goal of the present study was to investigate if the addition of insulin to the perfusion medium of perfused diabetic livers would alter the metabolic aberrations observed with such livers. Perfusion of livers from fasted, diabetic rats in the absence or presence of insulin in vitro, under conditions exactly similar to those for normal rat livers except for the added variable of insulin infusion (60–90 min), was performed. During the perfusion interval (30–60 min) before insulin addition to the medium was made, the rates of ureogenesis and ketogenesis of diabetic liver were 2.5 and 4.5 times higher ($P<0.01$) respectively than the normal liver (Table 1). The almost four fold higher rate of α-amino N production with perfused diabetic livers when compared to normal ($P<0.01$) was also indicative of high rates of liver protein catabolism (Table 2). The perfusion medium lactate had already reached a constant low value of 0.5 mmol/l with diabetic liver after 30 min of perfusion, similar to the value reached with normal livers after 60 min. Consequently, the observed rate of glucose production was due directly to endogenous liver protein catabolism. Thus, if one corrects for the probable contribution of lactate uptake in the rate of gluconeogenesis observed with the normal fasted liver, the rate found with diabetic liver is approximately 1.5 times higher (Table 1).

The rate of gluconeogenesis observed with diabetic livers over the perfusion interval 30–90 min (0.37 μmol/g/min) indicated that the increased rates indicated above for the interval 30–60 min (0.31 μmol/g/min) were maintained and linear throughout the experiment. Thus, the effect of insulin addition to the medium at 60 min of perfusion time was studied over the next 30 min. The results of insulin addition at 60 min continuous infusion over the next 30 min interval with the same group of diabetic rats and under exactly the same perfusion conditions as in the absence of insulin are summarized in Figure 1 and Table 1. There was an immediate fall in the accumulation of medium glucose and urea upon addition of insulin to the perfusion

Table 1. Rates of gluconeogenesis, ureogenesis and ketogenesis with perfused livers from normal and diabetic rats

	Insulin addition to perfusion medium*		Perfusion interval	
Group	30 to 60 min	60 to 90 min	30 to 60 min	60 to 90 min
			Gluconeogenesis (μmol/g wet liver/min)	
Normal (7)	−	−	0.33±0.06	0.14±0.02
Diabetic (4)	−	+	0.31±0.07	(See Fig. 1)
			Ureogenesis (μmol/g wet liver/min)	
Normal (7)	−	−	0.21±0.05	0.21±0.06
Diabetic (4)	−	+	0.52±0.17	(See Fig. 1)
			Ketogenesis (μmol/g wet liver/min)	
Normal (7)	−	−	0.12±0.03	0.15±0.03
Diabetic (4)	−	+	0.54±0.02	0.27±0.07

After a 30 min equilibration time, perfusate samples were obtained and concentration of metabolites measured as described in the text. Rates of gluconeogenesis, ureogenesis, and ketogenesis are given as the mean ± SEM for the number of perfusions which are listed as the figure in the parenthesis. *Addition of the 750 mU insulin was made at 60 min and an infusion of 12.5 mU/min was continued over the next 30 min with the diabetic liver

Table 2. Production of α-amino N with perfused livers from normal and diabetic rats

	Insulin addition to perfusion medium*		Release of α-amino N Perfusion interval	
Group	30 to 60 min	60 to 90 min	30 to 60 min	60 to 90 min
			μmol/g wet weight	
Normal (6)	−	−	2.1±0.9	2.5±2.1
Diabetic (4)	−	+	7.9±1.4	0.0±1.1

Perfusate α-amino N was determined as described in the Materials and Methods. Details of treatment of rats and perfusion conditions are given in the text. Values given are the mean ± SEM for the number of perfusions which is given in parenthesis. *Addition of 750 mU insulin was made at 60 min and an infusion of 12.5 mU/min was continued over the next 30 min with the diabetic liver

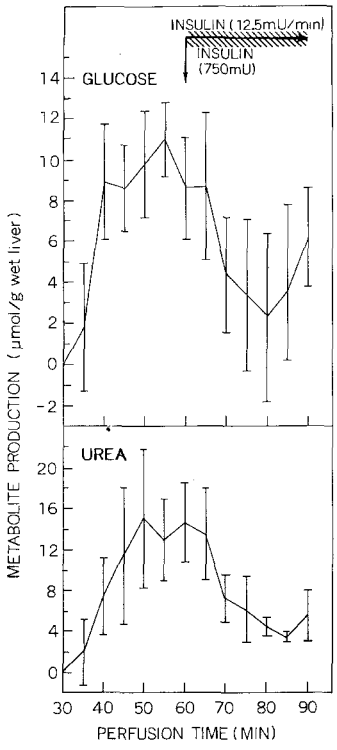

Fig. 1. Effect of insulin infusion (750 mU at 60 min + 12.5 mU/min for the period 60–90 min), on the production of glucose and urea in perfused livers from fasted, diabetic rats (24 h). Perfusate metabolites were determined as described in the Materials and Methods. Values represent mean ± SEM

medium (Fig. 1). The rate of ketogenesis with perfused diabetic liver after addition of insulin was only one-half the value of that without the hormone ($P<0.01$; Table 1). Determination of α-amino N levels in the perfusate further indicated that addition of insulin to the perfusion medium completely blocked endogenous liver protein breakdown ($P<0.01$; Table 2). The approximately 2 fold higher level of triglyceride in the diabetic liver (36.6 μmol/triglyceride-glycerol/g wet liver), when compared to the value for normal (18.7 μmol/g) at 30 min of perfusion, correlates well with the high rates of ketogenesis observed with the perfused diabetic liver (Table 1).

Discussion

In the present study, the rates of gluconeogenesis, ureogenesis and ketogenesis were elevated several-fold in isolated fasted diabetic liver over those found with the normal fasted liver. The acute diabetic state was produced by giving the last insulin injection 48–54 h before liver perfusion and superimposing a fast during the last 24 h. In most reported studies the perfusion of livers from diabetic rats in the fed state have allowed only the measurement of glucose balance, since significant shifts in liver glycogen content occur during the interval of perfusion [7, 9, 11, 24]. The use of [^{14}C]-lactate as gluconeogenic substrate in the experiments conducted by Exton et al. [6] with isolated livers from fed alloxan-diabetic rats led to the conclusion that the rate of gluconeogenesis with these livers was twice that found with livers from fed normal rats.

The relative contribution of counter-regulatory hormones such as glucagon [25, 26] or glucocorticoids [27] in the observed elevated rates of gluconeogenesis, ureogenesis, ketogenesis, and amino acid production by the isolated perfused diabetic liver was not ascertained in the present study. However, addition of insulin to perfusion medium resulted in an immediate reversal of the catabolic processes just mentioned. The cessation of amino acid release and urea production by isolated diabetic liver was observed upon insulin addition to the perfused liver from diabetic rats.

Although stimulation of protein synthesis by insulin could explain our observations, Mortimore and Mondon [4] have shown that a major role of insulin in protein turnover is in the control of proteolysis. The simultaneous decrease in the rate of gluconeogenesis and protein catabolism occurring with the isolated perfused diabetic liver in the presence of insulin supports the conclusion that liver protein breakdown during perfusion of the diabetic liver provides the carbon source for gluconeogenesis under these conditions.

In any case, addition of insulin to the perfusate reversed the increased catabolism observed with the perfused livers from fasted diabetic rats in the present study. Although insulin action on liver, especially its interaction with glucagon on the organ, was postulated to involve cyclic AMP almost ten years ago [25, 26], the exact locus of this effect remains controversial. Furthermore, the mechanism by which insulin exerts its acute anticatabolic effect remains to be elucidated.

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