# STUDIES ON GLUCONEOGENESIS, PROTEIN SYNTHESIS AND CYCLIC AMP LEVELS IN ISOLATED PARENCHYMAL CELLS FOLLOWING INSULIN WITHDRAWAL FROM ALLOXAN DIABETIC RATS

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SUMMARY

Alloxan diabetic rats maintained on protamine zinc insulin for two weeks were used for these studies. Isolated liver cells were prepared from these rats at various time intervals after the withdrawal of insulin (0, 48, 72, and 96 hr). A maximal increase in the rate of gluconeogenesis in the presence of 10mM alanine (14-34 fold), lactate (4-7 fold), pyruvate (4-6 fold), or fructose (2-3 fold) is observed in isolated diabetic liver cells 72 hr after the withdrawal of insulin from the donor rat. Protein synthesis in isolated diabetic liver cells as measured by the incorporation of radioactive isoleucine, valine and phenylalanine into protein decreases (5-6 fold) with time after insulin withdrawal. Glucagon ($10^{-6}$M) alone increases cyclic AMP levels five-fold, while glucagon with theophylline (1mM) increases cyclic AMP levels ten-fold in liver cells isolated from rats maintained on insulin (0 hr) or from rats withdrawn from insulin for 48 hr. The ability of glucagon to elevate cyclic AMP levels in isolated diabetic liver cells decreases at 72 hr following insulin withdrawal.

Rat liver parenchymal cells prepared by perfusion techniques have been shown by recent studies to be a good tool for studying hepatic metabolism (1-9). Isolated liver cells are useful in studying protein synthesis, gluconeogenesis (1-2) and fatty acid metabolism (3-5). Furthermore, glucagon has been shown to stimulate adenylate cyclase (6), to increase cyclic AMP levels (7) and to stimulate glycogenolysis and gluconeogenesis (7-9) in isolated liver cells.

Previous studies have shown increases in rates of gluconeogenesis (10) and in cyclic AMP levels (11) in liver slices and in isolated perfused livers

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from acute alloxan diabetic rats. In contrast to these increases protein synthesis has been shown to decrease markedly in muscle (12,10) and to decrease to a lesser degree in liver (13,10). In the present studies we report sequential changes in gluconeogenesis, protein synthesis and cyclic AMP levels in liver cells isolated from alloxan diabetic rats withdrawn from insulin.

## MATERIALS AND METHODS

Male, fed, Cox rats were used in the experiments reported here. All buffers except for the Krebs Ringer Phosphate buffer used in the cyclic AMP determinations were equilibrated with 95% $O_2$ and 5% $CO_2$. All reagents were purchased from the Sigma Chemical Co. except where indicated. Glucagon was a gift of the Eli Lilly Co. Radioactive amino acids were purchased from INC (Irvine, Calif.).

Diabetic rats were produced by the intravenous injection of alloxan (40mg/kg, Eastman Organic Chemicals, Rochester, N.Y.), after a 48 hr fast as reported previously (14). Normal rats were starved for 48 hr but were otherwise untreated. Rats exhibiting blood glucose levels greater than 300mg/100 ml three days following alloxan administration were started on insulin and maintained on 4 units of protamine zinc insulin (Lilly) for 2 weeks prior to use. These diabetic rats were used at various time intervals after the withdrawal of protamine zinc insulin (0, 48, 72, and 96 hr). Both the diabetic and normal rats weighed between 270 and 350 g when used.

Rat liver parenchymal cells were isolated using the method of Ingebretsen and Wagle (1) with 20mg/100 ml of collagenase (125 units/mg) in the perfusion media. The isolated cells were suspended in 40 ml of a 25 mM $NaHCO_3$ Umbreit Ringer buffer and were used immediately for studies on gluconeogenesis and protein synthesis. Cells used for cyclic AMP determinations were suspended in a Krebs Ringer Phosphate buffer and were used immediately.

Glucose production and the incorporation of $C^{14}$-amino acids into protein were determined as previously described (1). Cyclic AMP levels were determined

by the competitive protein kinase binding assay of Gilman (15) after a 2 min. incubation in a Krebs Ringer Phosphate buffer at 37°C (pH 7.4). Cell counts were determined using a hemocytometer. All experiments were conducted in duplicate and all values reported are the mean ± the standard error of the mean.

## RESULTS AND DISCUSSION

Data in Table 1 demonstrate the effects of insulin withdrawal on gluconeo-

TABLE 1

Net Glucose Production by Isolated Liver Parenchymal Cells from Normal and Diabetic Rats*

(umoles glucose/g wet wt cells/hr)

| | | Diabetic Rats Hours after Insulin Withdrawal | | | |
|---|---|---|---|---|---|
| 10mM Substrate | Normal Fed Rats | 0 hr | 48 hr | 72 hr | 96 hr |
| Alanine | 2.3±0.2 (4) | 1.7±0.9 (9) | 10.8±3.2 (4) | 53.3±9.0 (6) | 34.4±6.4 (5) |
| Lactate | 9.8±2.9 (4) | 21.2±1.8 (9) | 37.8±3.9 (5) | 91.3±14 (6) | 48.0±7.5 (5) |
| Pyruvate | 4.3±1.8 (4) | 11.2±1.4 (8) | 16.9±2.7 (4) | 61.0±7.8 (6) | 41.6±7.7 (5) |
| Fructose | 54.6±5.3 (4) | 57.3±3.8 (9) | 105 ± 12 (4) | 162 ± 22 (6) | 143 ± 32 (5) |

*A 1 ml aliquot of the Umbreit Ringer cell suspension was incubated in 2 ml of Umbreit Ringer buffer with various substrates. Values reported have been corrected for glycogenolysis by subtracting the glucose production in the absence of substrate from those values in the presence of substrate.

genesis in isolated liver cells. Isolated liver parenchymal cells from diabetic rats withdrawn from insulin for 48, 72, or 96 hr exhibit higher rates of gluconeogenesis than do liver cells isolated from rats maintained on insulin (0 hr) or

from normal fed rats. There is a peak rate of gluconeogenesis at 72 hr after insulin withdrawal in diabetic liver cells with all the substrates used. Tarrant and Ashmore (14), reported a peak in glycosuria at 72 hr in similarly treated diabetic rats. Spiro et al. (16), found a peak in gluconeogenesis in liver slices from diabetic rats at 20 hr after the withdrawal of a shorter acting insulin preparation. Liver cells isolated from alloxan diabetic rats withdrawn from insulin for 48 hr and from 24 hr starved rats (1) show similar rates of gluconeogenesis. The increase in rate of gluconeogenesis observed at 72 hr in the presence of alanine (14-34 fold) is far greater than the observed rates with the other substrates used (lactate, 4-7 fold; pyruvate, 4-6 fold and fructose, 2-3 fold). This supports the role of alanine in gluconeogenesis as suggested by Cahill (17).

The supression of protein synthesis in diabetes is well established.(12,13). The ability of isolated liver cells to incorporate radioactive amino acids into protein decreases with time after the withdrawal of insulin from alloxan diabetic rats (Table 2). Rates of incorporation of $C^{14}$-isoleucine, valine and phenylalanine in cells isolated from diabetic rats withdrawn from insulin for 96 hr demonstrate a 5-6 fold decrease from control values. In contrast to the effects of a lack of insulin, isolated liver cells from diabetic rats maintained on exogenously administered insulin (0 hr) exhibit higher rates of amino acid incorporation into protein than do isolated liver cells from normal rats. This observed decrease in amino acid incorporation into protein by isolated liver cells is far greater than has been previously reported using other *in vitro* liver preparation from alloxan diabetic rats.

The effects of insulin withdrawal on cyclic AMP levels are presented in Table 3. Theophylline by itself does not increase cyclic AMP levels. Glucagon ($10^{-6}$M) alone or in the presence of theophylline increases cyclic AMP levels (5 and 10 fold, respectively) in liver cells isolated from rats maintained on insulin (0 hr) and from rats withdrawn from insulin for 48 hr. This ability of glucagon or glucagon with theophylline to elevate cyclic AMP levels decreases

TABLE 2

Incorporation of $C^{14}$-Isoluecine, Valine and Phenylalanine into Protein by Isolated Liver Parenchymal Cells from Normal and Diabetic Rats*

(cpm/mg protein)

| 10mM Substrate | Normal Fed Rats | Diabetic Rats Hours after Insulin Withdrawal 0 hr | 48 hr | 72 hr | 96 hr |
|---|---|---|---|---|---|
| Isoluecine | 869 ± 58 (5) | 1477±124 (5) | 650 ± 86 (6) | 463 ± 27 (3) | 286 ± 57 (5) |
| Valine | 1009± 85 (5) | 1621±126 (5) | 1176±118 (6) | 528 ± 43 (3) | 322 ± 50 (4) |
| Phenylalanine | 764 ± 65 (5) | 1206±119 (5) | 1047±113 (6) | 435 ± 85 (3) | 200 ± 20 (4) |

*A 1 ml aliquot of the Umbreit Ringer cell suspension was incubated for 1 hr in 2 ml of Umbreit Ringer buffer containing 10mM cold amino acid, 0.5 uCi of UL $C^{14}$-amino acid and 100 mg/100 ml glucose.

in diabetic rats deprived of insulin for 72 or 96 hr. Control cyclic AMP levels show a maximal level at 72 and 96 hr after the withdrawal of insulin. Similar increases in cyclic AMP levels in livers from acute alloxan diabetic rats have been reported by Jefferson et al. (11). They also found that livers from untreated alloxan diabetic rats have higher cyclic AMP levels than do livers from treated rats.

The decrease in the ability of glucagon to increase cyclic AMP levels or the decrease in $C^{14}$-amino acid incorporation into protein in isolated diabetic liver cells after the withdrawal of insulin is not a function of cell deterioration or changes in cell counts. While the cyclic AMP response to glucagon and rates of protein synthesis deminish, rates of gluconeogenesis are elevated. Gluconeo-

TABLE 3

Cyclic AMP Levels in Liver Parenchymal Cells
Isolated from Diabetic Rats*

(Picomoles cyclic AMP/mg wet wt cells)

| | Hours after Insulin Withdrawal | | | |
|---|---|---|---|---|
| | 0 hr | 48 hr | 72 hr | 96 hr |
| Control | 0.72±0.15 (4) | 1.06±0.22 (3) | 1.77±0.59 (4) | 1.75±0.30 (4) |
| Theophylline (1 mM) | 0.70±0.08 (4) | 1.15±0.09 (3) | 2.24±0.84 (4) | 1.96±0.10 (4) |
| Glucagon ($10^{-6}$M) | 3.46±0.83 (4) | 5.83±1.59 (3) | 3.87±0.62 (4) | 2.60±0.46 (4) |
| Glu. + Theo. | 7.90±1.19 (4) | 10.20±0.64 (3) | 6.40±1.00 (4) | 4.34±0.62 (4) |

* 1 ml of the original Krebs Ringer Phosphate buffer cell suspension was incubated in 1 ml of Krebs Ringer Phosphate buffer for 2 min at 37°C. The reaction was stopped by the addition of 10% Trichloroacetic acid. The cells were then homogenized for 15 sec with a Polytron homogenizer at full speed. The preparation was extracted 3 times with diethyl ether and lyophylized. The dry tissue was redissolved in 100 mM Na-Acetate (pH 4.0) in a constant weight per volume proportion (mg/ml). Cyclic AMP was determined in 20 ul aliquot of the preparation.

genesis is a stringent test of metabolic integrity of isolated liver cells (1,3,7). Thus liver cells isolated from diabetic rats deprived of insulin show a high degree of metabolic integrity by these standards. Cell counts on preparations from rats withdrawn from insulin for 48 and 96 hr do not differ from those obtained from normal fed rats (144,000 ± 10,000, 144,000 ± 14,000 and 139,000 ± 20,000 cells/mg wet weight, respectively). However, there is a 25% increase in the cell count at 72 hr (162,000 ± 17,000) over that observed at 0 hr (126,000 ± 10,000). These changes in cell counts do not account for the metabolic alterations observed.

These studies show that isolated liver cells are more sensitive to metabolic changes than other liver preparations. Isolated liver cells should prove to be a useful tool in studying other endocrine disfunctions or disease states known to effect the liver.

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