ON THE ROLE OF INSULIN IN THE CONTROL OF GLUCONEOGENESIS

M. L'age*, H.V. Henning*, B. Ohly and W. Seubert

Institut für vegetative Physiologie, Chemisch-physiologisches Institut der Universität, Frankfurt/Main, Germany

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The diabetic state of the rat is characterized by an increased rate of gluconeogenesis from various substrates (ASHMORE et al. 1964), and by elevated levels of the enzymes pyruvate carboxylase (WAGLE 1964; PRINZ and SEUBERT 1964; FREEDMAN and KOHN 1964), phosphoenolpyruvate carboxykinase (SHRAGO et al. 1964; WAGLE and ASHMORE 1963), fructose-1,6-diphosphatase (WEBER and SINGHAL 1964), and glucose-6-phosphatase (WEBER and SINGHAL 1964). Insulin treatment of the diabetic animal brings the metabolic and enzymatic activities back to normal. On the basis of these results, WEBER et al. (1965) suggested that insulin acts as a suppressor of the biosynthesis of glucogenic enzymes. Experimental support for this attractive concept was presented by WEBER and his collaborators (1965) by showing that the action of cortisol as an inducer of glucogenic enzymes is counteracted by insulin. The distinctive actions of cortisol and insulin on gluconeogenesis would thus be the result of a positive and negative control respectively of the transscription of structural genes into the key enzymes of glucose synthesis.

If insulin acts as a suppressor on the functional genetic unit responsible for the biosynthesis of glucogenic enzymes, any situation characterized by a lack of this suppressor should result in an increase in the level of glucogenic enzymes, and in an acceleration of glucose synthesis as observed in the diabetic state. This situation is, however, not verified in the diabetic

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* Postdoctoral fellows, Deutsche Forschungsgemeinschaft

adrenalectomized rat (SHRAGO et al. 1963, WAGLE and ASHMORE 1963). From the reduced hyperglycemic response to anti-insulin serum observed in adrenalectomized rats (as compared with the effect of this serum in normal rats), STERN et al. (1963) concluded that endocrine factors other than insulin might be involved in the observed changes. LARDY et al. (1966) also deduced, from a study of phosphoenolpyruvate carboxykinase in various metabolic states, an indirect action of insulin in controlling biosynthesis of glucogenic enzymes. In order to contribute to a clarification of these apparent discrepancies, the effect of insulin on the acceleration of gluconeogenesis and the rise of pyruvate carboxylase induced by glucocorticoids in tissue slices was studied. In this system, insulin does not accelerate inactivation of glucocorticoids by reduction of the double bond in the 4,5-position (GARREN and CAHILL, Jr. 1963), which in vivo could mimic a suppressor action of insulin by lowering intracellular levels of glucocorticoids.

MATERIAL AND METHODS

Male adrenalectomized Sprague-Dawley rats, weighing 220-300 g, were kept at a constant temperature of 25° C, and fed ad libitum with rat pellets (Altromin GmbH, Lage/Lippe) and 0.9 % sodium chloride. Animals were used 3-7 days after surgery; food was removed 12 hours before death. Rats were killed under light ether anaesthesia and bled, and the kidneys were immediately removed. Kidney cortex slices were cut with a Stadie-Riggs microtome. Incubations were carried out in Warburg vessels at 38° C at a shaking rate of 140/min.

To study pyruvate carboxylase activities under the influence of the various agents, 400 mg slices were incubated in 8 ml KREBS-HENSELEIT (1933) medium containing 0.6 mg/ml peptone (Merck AG, Darmstadt) and 5 mM pyruvate. Gas phase: 95 % O_2 and 5 % CO_2. The slices were removed after incubation for 2 hours by centrifugation at 17 000 x g at 0° C for 10 min. The "soluble" portion of pyruvate carboxylase was extracted according to HENNING et al. (1966) and assayed as described by HENNING and SEUBERT (1964).

Effect in vitro of cortisol, triamcinolone, dexamethasone, insulin, and puromycin on pyruvate carboxylase and $^{14}CO_2$-fixation in tissue slices from rat

K i d n e y C o r t e x

Pyruvate carboxylase			
Experimental group		μmoles/min/g wet wt.	p
Control	(26)*	2.98 ± 0.09*	
Cortisol $2x10^{-5}M$	(19)	3.49 ± 0.15	< 0.01
Cortisol + puromycin 30 μg/ml	(8)	2.92 ± 0.18	> 0.1
Cortisol + insulin 250 mU/ml	(7)	3.68 ± 0.08	< 0.001
Fixation of $^{14}CO_2$			
Experimental group		Counts/min/g wet wt.	p
Control	(3)	4540	-
Insulin 250 mU/ml	(7)	4600 ± 115	-
Insulin + Cortisol $2x10^{-5}M$	(7)	5090 ± 75	< 0.01
Insulin + Dexamethasone $2x10^{-5}M$	(6)	5980 ± 200	< 0.001
Insulin + Dexamethasone + Puromycin 30 μg/ml	(7)	4970 ± 165	> 0.05

L i v e r

Fixation of $^{14}CO_2$			
Experimental group		Counts/min/g wet wt.	p
Insulin 250 mU/ml	(17)	496 ± 27	-
Insulin + Triamcinolone $5x10^{-5}M$	(12)	645 ± 38	< 0.005
Insulin + Triamcinolone + Puromycin 47 μg/ml	(7)	405 ± 40	> 0.05

* For experimental details see "Material and Methods".
Number of experiments is given in parentheses.
Values are expressed as means ± S.E.M.

$^{14}CO_2$-fixation by kidney cortex slices was studied as followed: 200 mg slices were incubated in 8 ml of medium

(KREBS and DE GASQUET, 1964), containing 0.6 mg/ml peptone; gas phase: O_2. After 2 hours' incubation, pyruvate, glucose and radioactive bicarbonate (0.1 µc/µmole) were injected into the incubation vessel after a second period of O_2 gassing, to give an end concentration of 20 mM, 5 mM, and 10 mM respectively.

After an additional 2 Hours' incubation the reaction was terminated by addition of 1 ml 6 % $HClO_4$. The tissue and denaturated protein were removed by centrifugation; the clear supernatant was gassed for 15 min. with CO_2 and for 10 min. with N_2. The supernatant was adjusted to pH 9 with KOH at 0° C, and a 1 ml aliquot was plated on aluminium planchettes (30 mm ø), dried and counted in a gas flow counter.

$^{14}CO_2$-fixation by liver slices was studied according to UETE and ASHMORE (1963): 200 mg of liver slices from adrenalectomized rats were incubated in 8 ml of RINGER-bicarbonate buffer with 5.5 mM glucose and 40 mM sodium pyruvate. 8 µC $Na_2{}^{14}CO_3$ (spec. act. 45 uC/umole) were placed in a side arm of a 50 ml vessel and added after equilibration with 95 % O_2 + 5 % CO_2 for 10 minutes. After 4 hours the incubation was stopped by addition of 1 ml 6 % $HClO_4$. $^{14}CO_2$ incorporated was determined as described above.

Insulin was determined by the immuno assay of C. N. HABS and P. J. RANDLE (1963).

RESULTS AND DISCUSSION

In the table the effects of cortisol, puromycin, and insulin *in vitro* on the activities of pyruvate carboxylase are summarized. Incubation of the slices under the experimental conditions described results in the presence of cortisol, in a significant increase ($p < 0.01$) of the "soluble" pyruvate carboxylase. Suppression of the cortisol effect by puromycin favours the concept already deduced from studies *in vivo* that glucocorticoids control *de novo* synthesis of enzymes. In contrast to puromycin, insulin did not suppress the cortisol-induced rise of the enzyme activity. This finding makes a negative control of the biosynthesis of glucogenic enzymes by insulin unlikely.

The above interpretation was supported by a study of CO_2-

fixation by kidney cortex and liver slices in the presence of an excess of pyruvate (20 mM and 40 mM respectively). In both systems, CO_2-fixation was significantly increased in the presence of the glucocorticoids ($p < 0.001$, $P < 0.005$). Puromycin completely abolished the effect of the hormone. Again insulin could not substitute for puromycin. Complete inactivation of insulin during the incubation period could be excluded. Immuno-assays of insulin after incubation with kidney cortex slices revealed activities far above physiological levels still to be present.

Acceleration of CO_2-fixation into glucose by triamcinolone in vitro has been reported by UETE and ASHMORE (1963) and SONGSAWADE and ASHMORE (1965). The hormonal stimulation of CO_2-fixation observed by these authors was far greater (180 % of the control) than ours (130 %) and could only partially be suppressed by puromycin or actinomycin (SONGSAWADE and ASHMORE 1965). This finding suggests several effects of the hormone to be responsible for the increased CO_2-fixation, one of which is related to de novo synthesis of an enzyme involved in gluconeogenesis. Mobilization of amino acids from endogenous sources as the cause of the additional effect of glucocorticoids on CO_2-fixation observed by the above authors is supported by the finding in this paper, that excess of substrate (40 mM pyruvate) greatly reduces the stimulatory effect of triamcinolone in liver (130 % of control). Complete suppression of the hormone effect by puromycin also indicates that under these conditions only an enzyme induction is involved in accelerated CO_2-fixation. SONGSAWADE and ASHMORE (1965) claimed on the basis of unchanged steady-state levels of amino acids, that mobilization of endogenous amino acids causing increased CO_2-fixation is not provoked by triamcinolone. However, assays of steady state levels may not be a sufficiently sensitive criterion.

The present communication negates a suppressor role of insulin. The effects of insulin on glucogenic enzymes observed in vivo may be due to secondary effects of this hormone.

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