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THE USE OF INSULIN IN THE PRODUCTION OF L-LEUCINE-INDUCED HYPOGLYCEMIA IN NORMAL DOGS *

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The initial observation of Cochrane, Payne, Simpkiss and Woolf, who described the hypoglycemic effect of L-leucine in certain patients with idiopathic infantile hypoglycemia (1), has been repeatedly confirmed (2–6). L-leucine has also produced a hypoglycemic effect in certain patients with functioning islet cell tumors of the pancreas (7). Not all patients with these clinical syndromes respond to leucine, and the phrase "leucine-sensitive" has been used to designate those patients in whom a hypoglycemic response to leucine can be demonstrated. Previous studies have been unable to demonstrate a lowering effect of L-leucine on blood glucose in normal subjects, and experimental studies of leucine-induced hypoglycemia have of necessity been limited to a few patients with relatively rare diseases. Since hyperinsulinism or hypoglycemia, or both, appeared to be possible necessary prerequisites for the induction of leucine hypoglycemia, an attempt was made to produce experimentally "leucine sensitivity" in normal dogs by pretreatment with insulin. The present report describes the lowering effect on blood glucose that follows L-leucine administration to normal dogs treated with insulin in a variety of ways. In addition, the effect of L-leucine on the magnitude of femoral artery-femoral vein glucose concentration differences after acute insulin-induced hypoglycemia is discussed.

METHODS

Normal mongrel dogs of both sexes were used in these experiments; all were anesthetized with pentobarbital (Nembutal). Blood glucose was determined by Nelson's modification of Somogyi's method (8). Leucine was administered as 30 mmoles of L-leucine in 200 ml distilled water. An equal volume of 0.9 per cent NaCl was used as a control solution. The protocols of the experiments performed are as follows.

A. Acute administration of crystalline zinc insulin. Glucagon-free insulin, 0.1 U per kg of body weight, was administered to dogs by rapid intravenous injection. Blood was withdrawn for glucose determination before the insulin was given and at 15-minute intervals for 2 hours. Paired experiments were done on each animal. On one day animals received insulin plus L-leucine; on another day they were given the same amount of insulin plus saline. Maximum hypoglycemia was observed 30 minutes after the administration of insulin. The degree to which L-leucine prolonged the hypoglycemic response to a standard dose of insulin was calculated by dividing the rise in glucose concentration from 30 minutes to 120 minutes by the fall in blood glucose concentration during the first 30 minutes: per cent recovery = rise in glucose concentration from 30 to 120 minutes/fall in glucose concentration from 0 to 30 minutes.

B. Acute plus sustained infusion of insulin. A total of 0.3 U of glucagon-free insulin per kg body weight was given to each experimental animal. One-tenth U per kg was given at the beginning of the experiment by rapid intravenous injection, and 0.2 U per kg was given as a sustained infusion over the next 90 minutes. The insulin used for the sustaining infusion was added to 200 ml of either L-leucine or saline in paired experiments in the same animal. Blood was withdrawn for glucose before the insulin was given and every 15 minutes thereafter. After 90 minutes the sustaining infusion of L-leucine plus insulin, or saline plus insulin was stopped, and the animal was permitted to recover from the prolonged period of hypoglycemia during a 60-minute postinfusion period. The degree to which L-leucine prolonged the return of blood glucose concentration to baseline values during this period of recovery was calculated as follows: per cent recovery = rise in glucose concentration during recovery period/fall in glucose concentration during hypoglycemic period.

C. Prior administration of ultralente insulin. Ultralente insulin (0.75 U per kg body weight) was injected subcutaneously into dogs. The animals were anesthetized 14 hours after the injection, blood was drawn for baseline glucose value and, in paired experiments, either L-leucine or saline was administered by rapid intra-

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venous infusion. Blood was then withdrawn for glucose determinations every 15 minutes for 1 hour. The per cent drop in glucose concentration was determined by dividing the value for blood glucose 1 hour after the administration of L-leucine or saline by the initial value.

D. Femoral artery-femoral vein glucose concentration differences after the acute administration of crystalline zinc insulin. The method of these experiments was identical with that described in Section A, except that blood was withdrawn simultaneously from the femoral artery and the femoral vein, rather than from the venous side alone.

RESULTS

Normal dogs, when given an intravenous injection of 0.1 U of glucagon-free insulin per kg body weight, demonstrated a uniform hypoglycemic response. Blood glucose concentration fell to its lowest points 30 minutes after the insulin was given and usually returned to baseline levels by 90 minutes. The concurrent administration of insulin plus L-leucine significantly prolonged the period of hypoglycemia. The degree of prolongation varied from almost complete hypoglycemic unresponsiveness (Figure 1) to varying degrees of delayed recovery of glucose concentration to the initial value. Results in the seven dogs studied are seen in Table I. It is apparent that L-leucine prolonged the period of hypoglycemia in every paired experiment. With a nonparametric sign test (9), the probability that the effect of L-leucine is due to chance is 0.015.

In order to demonstrate a more pronounced effect with L-leucine, a longer period of hypoglycemia was produced by a sustained infusion of insulin. The effect of L-leucine on recovery from this longer period of hypoglycemia is depicted in Table II. It is apparent that in every paired experiment L-leucine inhibited recovery from hypoglycemia during the 60-minute recovery period. If the results of this series of experiments are compared with those shown in Table I, it appears that L-leucine had a greater effect during a period of sustained hypoglycemia. The differences between L-leucine and the control solution are significant at the 0.015 level.

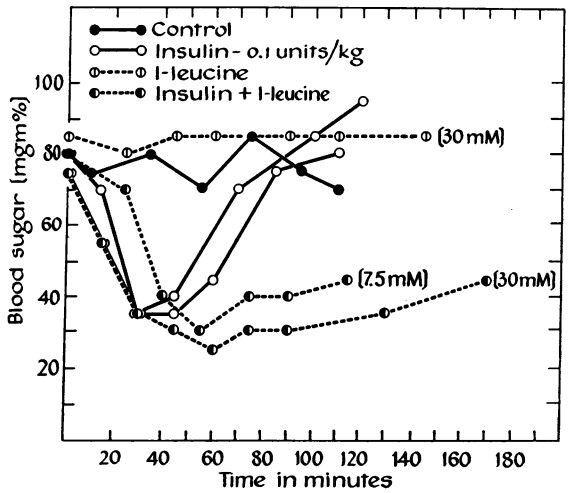

FIG. 1. EFFECT OF L-LEUCINE ON PROLONGATION OF THE PERIOD OF HYPOGLYCEMIA PRODUCED BY CRYSTALLINE ZINC INSULIN. All of the experiments were performed on the same dog.

TABLE I

Effect of L-leucine on per cent recovery from acute period of hypoglycemia

Dog	L-leucine				Saline			
	Blood glucose				Blood glucose			
	Baseline	30 min	120 min	Recovery*	Baseline	30 min	120 min	Recovery*
	mg/100 ml			%	*mg/100 ml*			%
M.A.	80	40	44	10	85	35	85	100
M.A.	75	35	35	0	75	35	80	113
M.A.	70	30	35	13	80	28	75	90
W.	95	40	65	45	90	30	80	83
Z.Z.	72	38	54	47	88	48	85	93
B.	90	33	60	47	85	33	75	81
B.O.	91	36	60	44	78	28	63	70
M.M.	75	32	49	40	80	33	74	87
G.	95	42	58	30	92	38	88	92
Average				31				90

* Per cent recovery = rise in blood glucose from 30 to 120 min/fall in blood glucose from 0 to 30 min.

TABLE II

Effect of L-*leucine on per cent recovery from prolonged period of hypoglycemia*

Dog	L-leucine				Saline			
	Blood glucose				Blood glucose			
	Baseline	End of hypoglyc. period	End of recov. period	Recovery*	Baseline	End of hypoglyc. period	End of recov. period	Recovery*
	mg/100 ml			%	*mg/100 ml*			%
70	83	37	44	15	88	31	50	33
77	82	36	36	0	73	26	52	55
91	85	30	35	9				
99					98	46	88	81
137	90	41	49	16	93	33	73	67
143	73	20	25	6	75	30	60	67
144	99	43	50	12	99	49	89	80
146	92	25	45	35	88	28	72	73
147	82	21	38	28	80	23	80	100
Average				15				70

* Per cent recovery = rise in blood glucose during recovery period/fall in blood glucose during hypoglycemic period.

In an effort to produce a drop in the blood glucose, rather than simply a delayed recovery from hypoglycemia, L-leucine was given to normal dogs 14 hours after subcutaneous injection of ultralente insulin (0.75 U per kg body weight). The experimental results are seen in Table III. It is apparent that none of the dogs was hypoglycemic before either L-leucine or saline was administered. In every paired experiment the administration of L-leucine resulted in a drop in blood glucose concentration. The same volume of the control solution never produced a fall in glucose concentration. With the same nonparametric procedure, the probability that the fall in blood glucose is due to chance rather than L-leucine is 0.0075.

Finally, in an attempt to gain some insight into the mode of action of L-leucine, femoral artery-femoral vein blood glucose differences were compared for 120 minutes after the intravenous injection of short-acting insulin. The results of four such paired experiments are seen in Table IV. Although arteriovenous glucose differences are usually assumed to reflect peripheral glucose uptake, it has recently been suggested that this is completely true only during periods of rapidly falling glucose concentration, and that during the re-

TABLE III

Effect of L-*leucine on blood glucose of dogs given ultralente insulin 14 hours prior to experiment*

Dog	L-leucine			Saline		
	Blood glucose			Blood glucose		
	Before leucine	60 min after leucine	Change	Before saline	60 min after saline	Change
	mg/100 ml		%	*mg/100 ml*		%
60	84	69	−18	80	85	+ 5
70	85	71	−16	95	95	0
77	66	51	−23	63	63	0
143	80	59	−26	75	84	+12
137	85	73	−14	83	98	+18
144	90	72	−20	84	98	+17
145	88	71	−19	72	78	+ 7
	95	71	−25	89	95	+ 7
	90	68	−24	91	99	+ 9
	100	70	−30	78	82	+ 5
282	90	70	−22	93	95	+ 2
Average			−22			+ 7

TABLE IV

Effect of L-*leucine on femoral artery(FA)-femoral vein (FV) glucose differences after administration of intravenous insulin*

		L-leucine							Saline						
Dog	Minutes:	0	15	30	45	60	90	120	0	15	30	45	60	90	120
Z.Z.															
	FA gluc., *mg* %	80	47	40	49	52	56	60	95	70	50	69	85	89	95
	FV gluc., *mg* %	72	46	38	45	50	54	54	88	64	48	58	70	79	85
	FA-FV, *mg*	8	1	2	4	2	2	6	7	6	2	11	15	10	10
B.O.															
	FA gluc., *mg* %	98	48	38	40	54	59	69	88	48	38	41	56	75	71
	FV gluc., *mg* %	91	45	36	36	50	55	60	78	46	36	33	46	65	63
	FA-FV, *mg*	7	3	2	4	4	4	9	10	2	2	8	10	10	8
MA															
	FA gluc., *mg* %	78	68	31	33	38	41	51	83	61	36	51	60	75	80
	FV gluc., *mg* %	73	64	32	35	35	38	47	77	57	34	47	52	64	72
	FA-FV, *mg*	5	4	−1	−2	3	3	4	6	4	2	4	8	11	8
M.M															
	FA gluc., *mg* %	78	54	36		43	54	59	85	48	50	54	73	88	90
	FV gluc., *mg* %	75	50	32		40	49	51	80	45	33	43	62	71	74
	FA-FV, *mg*	3	4	4		3	6	8	5	3	17	11	11	17	16
Average															
	FA gluc., *mg* %	84	54	36	41	47	52	60	88	57	44	54	68	82	84
	FV gluc., *mg* %	78	51	34	39	44	49	53	81	53	38	46	57	70	74
	FA-FV, *mg*	6	3	2	2	3	3	7	7	4	6	8	11	12	10

covery phase homeostatic mechanisms come into play, with the result that arteriovenous glucose differences become a function of both hypoglycemic factors (10). With this thought in mind, we have analyzed the results by dividing the experiment into two periods: 0 to 30 minutes, which corresponds to the period of rapidly increasing hypoglycemia: and 30 to 120 minutes, which represents the recovery period. There is no significant effect of L-leucine on either glucose concentration or on the magnitude of femoral artery-femoral vein glucose concentration differences during the first 30 minutes, and consequently no evidence that L-leucine acts to increase peripheral uptake of glucose. It is apparent, however, that during the recovery period the administration of L-leucine consistently decreased arteriovenous glucose differences. A mixed model analysis of variants technique (11) showed that the hypothesis that L-leucine diminished arteriovenous glucose differences during the recovery period was significant at the 0.025 level, and barely missed being significant at the 0.01 level. It can also be seen that L-leucine prolonged the return of glucose concentration to the baseline value. The combination of a prolonged recovery period accompanied by smaller arteriovenous glucose differences suggests that L-leucine inhibited the hyperglycemic response to hypoglycemia.

DISCUSSION

The experimental results presented demonstrate that L-leucine is capable of prolonging hypoglycemia produced by short-acting insulin, and of lowering blood glucose concentration following long-acting insulin. The production of "leucine sensitivity" in a series of normal dogs treated with insulin suggests that the hypoglycemic effect of L-leucine is not unique to any individual, but may represent a physiological effect of L-leucine in a variety of situations characterized by hypoglycemia or hyperinsulinism, or both.

The manner in which L-leucine lowers blood glucose concentration in patients with various hypoglycemic syndromes is still not clear. In this regard Yalow and Berson have demonstrated that hypoglycemia associated with L-leucine administration was accompanied by a rise in plasma insulin levels in four of six patients studied (12). Although there is no reason to assume that L-leu-

cine necessarily acts the same way in man and dog, it is difficult to explain our observations on the theory that L-leucine increases endogenous insulin secretion. Since L-leucine alone has little, if any, effect on glucose concentration of normal dogs, the amount of endogenous insulin being secreted must be quite small. However, when large amounts of exogenous insulin are given to these same dogs, the hypoglycemic effect of L-leucine is quite apparent. It seems unlikely that the concomitant administration of L-leucine plus significant amounts of exogenous insulin would result in greater stimulation of endogenous insulin than does the administration of L-leucine alone. Consequently, the possibility arises that the effect of L-leucine in normal dogs treated with insulin is due to some other mechanism.

If L-leucine does not produce its hypoglycemic effects in normal dogs by stimulating endogenous insulin secretion, it might act either by increasing peripheral uptake of glucose or by inhibiting hepatic glucose output. In these instances the exogenous insulin necessary to demonstrate the hypoglycemic effect of L-leucine in normal dogs would be acting in a permissive role. The observation that L-leucine did not increase arteriovenous glucose differences in dogs during insulin-induced hypoglycemia is evidence against the action of L-leucine to increase peripheral utilization of glucose. Furthermore, L-leucine did not significantly increase glucose uptake when incubated with rat diaphragm *in vitro* (13). Demonstration that L-leucine produces smaller arteriovenous glucose differences as it prolongs recovery from insulin-induced hypoglycemia suggests that L-leucine decreases the hyperglycemic response to hypoglycemia—specifically, hepatic glucose output. Since it has been shown that insulin can directly inhibit hepatic glucose output (14), L-leucine may be simply potentiating this action of insulin. It is also possible that L-leucine may inhibit the effects of glucagon or epinephrine, or both, on the activation of phosphorylase (15) and decrease hepatic glucose output in this manner. Finally, L-leucine may inhibit hepatic glucose output in a variety of ways, all independent of these other mechanisms; for example, by decreasing glucose-6-phosphatase activity. It is apparent that there are many possible explanations of this interesting effect of an amino acid on glucose homeostasis. It is anticipated that further investigation of the effect of L-leucine in normal dogs prepared with insulin will provide useful information in our efforts to understand this phenomenon.

SUMMARY

The administration of L-leucine to normal dogs has both prolonged a period of insulin-induced hypoglycemia and lowered blood glucose from euglycemic levels. The observation that a lowering effect of L-leucine on blood glucose could be demonstrated after the administration of large amounts of exogenous insulin makes it difficult to explain the action of L-leucine in this situation on the basis of stimulating the secretion of endogenous insulin. Measurement of femoral artery-femoral vein glucose differences during recovery from acute insulin-induced hypoglycemia has suggested that L-leucine may produce its hypoglycemic effect in normal dogs by inhibiting hepatic response to hypoglycemia. The methods described for producing L-leucine hypoglycemia in normal dogs should provide a useful tool for experimental investigation of the phenomenon of L-leucine-induced hypoglycemia.

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REFERENCES

1. Cochrane, W. A., Payne, W. W., Simpkiss, M. J., and Woolf, L. I. Familial hypoglycemia precipitated by amino acids. J. clin. Invest. 1956, **35**, 411.
2. Cochrane, W. A. Studies in the relationship of amino acids to infantile hypoglycemia. J. Dis. Child. 1960, **99**, 476.
3. Woolf, L. I. The hypoglycaemic effect of leucine and isovaleric acid. Clin. chim. Acta 1960, **5**, 327.
4. Grumbach, M. M., and Kaplan, S. L. Amino acid and alpha-keto acid-induced hyperinsulinism in the leucine-sensitive type of infantile and childhood hypoglycemia. J. Pediat. 1960, **57**, 346.
5. Mabry, C. C., DiGeorge, A. M., and Auerbach, V. H. Leucine-induced hypoglycemia. I. Clinical observations and diagnostic considerations. J. Pediat. 1960, **57**, 526.

6. Hartmann, A. F., Sr., Wohltmann, H. J., Holowach, J., and Caldwell, B. M. Studies in hypoglycemia. J. Pediat. 1960, **56**, 211.
7. Flanagan, G. C., Schwartz, T. B., and Ryan, W. G. Studies on patients with islet-cell tumor, including the phenomenon of leucine-induced accentuation of hypoglycemia. J. clin. Endocr. 1961, **21**, 401.
8. Nelson, N. A photometric adaptation of the Somoygi method for determination of glucose. J. biol. Chem. 1944, **153**, 375.
9. Dixon, W. J., and Massey, F. J., Jr. Introduction to Statistical Analysis. New York, McGraw-Hill, 1951, p. 247.
10. Frawley, T. F., Shelley, T. F., Runyan, J. W., Jr., Margulies, E. J., and Cincotti, J. J. Further studies on the significant role of the liver in sulfonylurea hypoglycemia. Ann. N. Y. Acad. Sci. 1959, **82**, 460.
11. Anderson, R. L., and Bancroft, T. A. Statistical Theory in Research. New York, McGraw-Hill, 1952.
12. Yalow, R. S., and Berson, S. A. Immunoassay of endogenous plasma insulin in man. J. clin. Invest. 1960, **39**, 1157.
13. Johnson, P., Herring, B., and Field, J. B. Effects of L-leucine and its metabolites on glucose uptake by rat diaphragm. Metabolism 1961, **10**, 415.
14. Madison, L. L., Combes, B., Adams, R., and Strickland, W. The physiological significance of the secretion of endogenous insulin into the portal circulation. III. Evidence for a direct immediate effect of insulin on the balance of glucose across the liver. J. clin. Invest. 1960, **39**, 507.
15. Sutherland, E. W., and Rall, T. W. The relation of adenosine-3′,5′-phosphate and phosphorylase to the actions of catecholamines and other hormones. Pharmacol. Rev. 1960, **12**, 265.