Journal of Clinical Investigation
Vol. 42, No. 11, 1963

EVIDENCE THAT INSULIN RELEASE IS THE MECHANISM FOR EXPERIMENTALLY INDUCED LEUCINE HYPOGLYCEMIA IN MAN *

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(Submitted for publication April 15, 1963; accepted July 18, 1963)

In a previous report (1), we have described studies dealing with the experimental induction in man of sensitivity to leucine hypoglycemia. In healthy subjects, marked sensitivity to leucine hypoglycemia was consistently induced by prior administration of sulfonylureas, compounds that are known to stimulate islet-cell activity. On the other hand, after pretreatment with Novo Ultralente insulin, administration of leucine produced decreases in blood glucose levels that, although significant, were small and inconsistent. A modest but significant hypoglycemic effect was also produced in some healthy subjects by administering leucine without prior administration of hypoglycemic agents (1, 2). The magnitude of these decreases in blood glucose levels was similar to that observed after administration of leucine to subjects pretreated with Ultralente insulin. From the results of these studies, we concluded that in man release of additional insulin is the primary mechanism by which leucine decreases the blood sugar in experimentally induced sensitivity to leucine hypoglycemia. Earlier reports had demonstrated an increase in concentration of plasma insulin during leucine-induced hypoglycemia, both in infants with idiopathic hypoglycemia (3–5) and in patients with islet-cell tumors (1, 6–8).

The present study, employing an immunoassay for insulin, was undertaken to determine the relationship between endogenous insulin and the induction of experimentally induced leucine hypoglycemia in man. The results indicate *1*) that significant and consistent increases in peripheral plasma levels of insulin precede and accompany experimentally induced leucine hypoglycemia and *2*) that release of additional insulin is the mechanism by which leucine causes hypoglycemia.

METHODS

Details of testing procedures, analytical methods, and methods of pretreatment with sulfonylurea compounds or Ultralente insulin have been described previously (1). L-Leucine [1] (0.2 g per kg body wt) was administered either orally as a suspension or intravenously as a solution. Control tests consisted of oral administration of an equal volume of tap water or iv administration of an equal volume of normal saline. Samples of peripheral venous blood were usually obtained for determination of blood glucose and plasma insulin 30 minutes before administration of the test material and at appropriate intervals for 2½ to 3½ hours. Blood samples for immunoassay of insulin, with a small amount of powdered heparin added, were kept chilled before centrifugation at 4° C. Subsequently the plasma was separated and stored frozen. Radioimmunoassay was usually performed several days to several weeks after the testing procedure. Samples for one test [J.S. (a), Table I] were assayed 10 months after the experiment. A plasma sample reassayed several times over a period of 8 months showed no detectable loss of immunological potency.

Radioimmunoassay of plasma insulin was performed by the method of Yalow and Berson (3). Radioiodination of crystalline beef insulin [2] with NaI^{131} was performed as described by McCall, Timm, Eisentraut, and Unger (9). In most instances, cellulose column purification was performed immediately after dialysis. The SA of various preparations of insulin I^{131} ranged from

* Presented in part before the 44th annual meeting of the Endocrine Society, Chicago, Ill., June 21–23, 1962. This work was supported in part by U. S. Public Health Service grants AM 02244, AM 00888, and 2A-5001, National Institute of Arthritis and Metabolic Diseases, Bethesda, Md.

[1] Some of the leucine used in these studies was kindly supplied by Dr. Domenic G. Iezzoni, Chas. Pfizer & Company, Inc., New York, N. Y.

[2] Kindly supplied by Dr. W. R. Kirtley, Eli Lilly Research Laboratories, Indianapolis, Ind.

TABLE I

Levels of plasma insulin and blood glucose after administration of leucine to chlorpropamide-pretreated healthy subjects

Test	Subject	Plasma insulin at time in minutes															Blood glucose at time in minutes												
		−30	−15	−1	0	10	15	20	30	40	50	60	75	90	120	180	−30	−15	−1	0	10	15	20	30	40	50	60	75	90	120	180
		μU/ml															*mg/100 ml*													
Leucine (oral)	W.B.	8	14	7	10		11	20	53	100	104	96	106	28	17	29	72	75	76	76		71	71	65	58	46	39	28	35	42	68
	J.W.	18	10	10	19	50		60	53			75	36	31	53		77	80	76	75	73		71	69			42	36	38	44	64
	R.H.			26			42		74	50	58	55		55	19	10		74	74			69		60	52	45	36		45	52	68
	J.S.(a)	26		0			10	16	38	43	61	78		54			64		67			77	72	70	63	56	58		39	38	64
	J.S.			18			20	19	18	38	25	50		20	31				74			74	70	71	67	65	52		62	54	73
Water (oral)	W.B.		0	0	0		4	3	3	1	1	1	3	3	0	4		82	82	80		80	77	77	76	77	76	73	71	77	93
Leucine (iv)	W.B.		8	14		29			53	182		50			11		74	77	76	79	54			48	32	26	32			60	
	W.B.*					42		61	63	120		54		23	20						63		55	48	43		37		49	60	
	W.B.	96		79	96	104		113	114	149	170	140	127	144	77	97	40	32	34	35	34		29	27	27	28	26	25	35	29	29
	J.W.	41	55	53	55	67		82	91	84	77	70	54			34	70	69	71	74	38		61	57	57	48	45	44	53	60	
	J.W.	43	24	38	41	43		79	103	90				34	19		71	73	72	72	72		62	59	50	43	28	45	49	61	
	J.W.*					49		55	78	74		38		42							58		51	51	51	47	45	46	51	58	
	B.B.	12		16	10	84		43	62	38	18	14			0		75		77	77	71		59	47	39	43	59	61	67	68	
	B.B.*					0		36	36	24	22	19		2	0						71		64	59	56	51	56		59	71	
	B.B.	18	19	21		123		111	159	86	44	16		11	10		86	92	87	88	82		71	66	53	52	49	65	80	83	87
	C.S.	3	5	1		24		7	31	40	24	15		1	0		70	73	72		71		67	56	54	46	43	51	60	63	77
	L.D.†	8	15	17		44		88	89	45	25	11		12	19		73	74	76	74	71		63	56	49	43	49	72	83	77	80
	L.D.‡	11	23	14	13	65		22		16	10	13		5	12		70	68	72	69	69		53	45	42	58	63	73	77	73	69
Saline (iv)	J.W.	6	13	14	11	9		0	5	0	0	0		0	1		76	76	76	77	75		75	77	75	73	69		73	73	
	B.B.			43		41		47	36	29	35	36		29	26				70		75		77	76	72	76	80		76	77	
	W.B.	10	12	14	14	13		11		11		19			18		78	79	80	73	80		72	75	73	76	78		75	74	

* Second infusion of leucine started at 2 hours.
† Leucine, 0.15 g/kg.
‡ Leucine, 0.075 g/kg.

80 to 300 mc per mg.[3] SA was estimated by monitoring radioactivity during the stages of the iodination process, by analyzing chromatoelectrophoretograms of the iodination reaction mixture, and by measuring the degree of binding of insulin I^{131} to antiserum of known potency. Guinea-pig antibeef insulin antiserum of a single plasma sample from one animal was used for these studies. Final antibody concentrations ranged from 1:9,000 to 1:12,000. The insulin standard was human insulin[4] lyophilized in 0.5-U amounts.

The standard insulin was dissolved in 0.1 ionic strength barbital buffer containing 0.25% human serum albumin. Samples frozen for future use demonstrated no detectable loss in immunological potency when compared up to one year later to freshly dissolved 0.5 U of the same lot. This Lilly insulin, assayed by the standard U.S.P. rabbit method with 96 rabbits, was found to contain 6.1 international U per mg (95% confidence limits of ± 0.45 U per mg) (10) and was considered to be 25.4% pure insulin by weight. Tietze insulin[5] was previously considered (3) to be 6.45% pure insulin by weight. When we compared the two preparations by weight, immunoassay showed no differences. Neither preparation was so efficacious in lowering the bound to free (B/F) ratio as crystalline beef insulin.

Most of the samples were assayed in duplicate. To ascertain the variability of results within a single assay, we performed 10 determinations on each of 2 plasma samples of differing concentrations. The means ± SE of the means were 25 ± 2 and 159 ± 5 μU per ml, respectively. Also determined was the variability of the results obtained with 2 samples of plasma assayed repetitively over a period of months. The sample of plasma above at the lower concentration was assayed 7 separate times over 4 months, and a third sample at a high concentration was assayed on 8 separate occasions over 6 months. The means ± SE of the means were 27 ± 4 and 89 ± 6 μU per ml, respectively. Each assay employed different lots of trace insulin-I^{131}. Two vials of human insulin standard from a single lot, but dissolved several months apart, were used during this period.

RESULTS

Leucine tests in healthy subjects after pretreatment with sulfonylurea compounds. After administration of chlorpropamide[6] to healthy subjects, leucine produced large decreases in blood glucose levels consistently in each of 38 experiments after its oral administration (mean of maximal decreases, 29 mg per 100 ml) and in each of 13 experiments after its iv administration (mean of maximal decreases, 38 mg per 100 ml) (1). Levels of plasma insulin were measured during 5 oral and 12 iv tests (Table I). Significant increments in plasma levels of insulin occurred in each of the 17 tests. The mean of the maximal increases in plasma insulin was 63 μU per ml for the oral tests and 87 μU per ml for the iv tests. During 3 experiments a second infusion of leucine, begun 2 hours after the start of the first infusion, again produced significant increments in plasma insulin and corresponding decreases in blood glucose levels (Table I). During the second leucine infusion, the plasma insulin increases and blood glucose decreases were of lesser magnitude than those in the first infusion. An increment in insulin concentration was evident within 10 minutes in 9 of 12 iv tests and by 20 minutes in the other 3. During control tests with oral tap water or iv saline, there were no significant increments in concentration of plasma insulin (Table I). Two of the 4 control tests were chosen for immunoassay because of minor, though exceptional, decreases in blood glucose levels.

The *in vitro* addition of leucine to the plasma of a subject pretreated with chlorpropamide did not significantly alter the concentration of insulin as determined by immunoassay. The mean value of 3 plasma samples was 58 μU per ml before as well as after addition of leucine up to a concentration of 200 μg per ml of plasma.

Leucine tests in healthy subjects after pretreatment with Ultralente insulin. After administration of Ultralente insulin to healthy subjects, oral administration of leucine produced a significant hypoglycemic effect in only 21 to 46 experiments (mean of maximal decrease in blood glucose levels, 5.6 mg per 100 ml) (1). After iv administration of leucine to six Ultralente insulin-pretreated subjects, the mean of the maximal decreases in blood glucose was 11 mg per 100 ml (1). Plasma levels of insulin were measured during 3 oral and 3 iv tests (Table II). The mean of the maximal increases in plasma insulin was 13 μU per ml for the oral tests and 16 μU per ml for the iv tests (Table II).

During the 3 oral leucine tests shown in Table

[3] Some of the insulin I^{131} used in these assays was supplied through the courtesy of Dr. Howard Glenn, Abbott Laboratories, Oak Ridge, Tenn.

[4] Supplied through the courtesy of Dr. Mary Root, Eli Lilly Research Laboratories, Indianapolis, Ind.

[5] Supplied through the courtesy of Dr. Frank Tietze, National Institutes of Health, Bethesda, Md.

[6] 1-Propyl-3-(*p*-chloro-benzenesulfonyl) urea.

TABLE II

Levels of plasma insulin and blood glucose after administration of leucine to healthy subjects a) following pretreatment with Ultralente insulin and b) without pretreatment

Test	Subject	Plasma insulin at time in minutes														Blood glucose at time in minutes													
		−30	−15	−1	0	10	15	20	30	40	50	60	75	90	105	120	150	180	−30	−15	−1	0	10	15	20	30	40	50	60	75	90	105	120	150	180
		μU/ml																	*mg/100 ml*																
		a) Ultralente pretreatment																																	
Leucine (oral)	C.S.	10	11					10	3	10	5	5	17	11		15			63	63	60	61	63		63	65		63	62	61	62		64		66
	R.D.	14	22	18	24		29	18	40	43	49	13	8	0		0			41	48	55	55		54	58	55	52	54	51	46	47		53		55
	B.B.	46	42	53	50			41	48	50	41	54	37	54		48		44	56	60	60	62			61	56	59	64	55	55	55	47		60		66
Leucine (iv)	B.B.	28		22	22			29	42	40	16	28	11	23		17		20	71	68	67	67	65		55	48	44	62	64	70	67		72		
	B.B.	36	46	43	43	43		43	52	46	50	31				25			74	70	68	66	70		63	57	56	51	54	59	65		67		
	C.S.	46	58	62	53	74		77	41	43	43	38	41	48		55			67	64	61	60	55		51	53	41	43	52	52	53		58		
Saline	B.B.		32	27	25	15		10	19	6	10	6	0	7		15			77	73	74	75	68		70	63	59	61	64	70	73		67		
		b) No pretreatment																																	
Leucine (oral)	W.B.	23	18	18	20		14		18	30		26	26	14		10		6	82	79	78	79		80	79	78	75	76	74	69	68		68		
	J.V.	14	14	14				18	12	17	18	17	16	27	20	21			84	87	87	83			89	87	82	81	81	79	78	77	74	83	
Leucine (iv)	W.B.	5	0	4	0	16		20			19	22	19	13		14		86	91	89	89	87	89		86	82	78	77	75	77	84		79		
	J.W.	30	41	38	37	50		66	66	43	34	38	14			8			83	84	85	85	83		80	75	70	69	68	65	69		70		
	R.B.		5	5	19	19		28	33	32	31	18	14	17	11	9	7	15		87	92	89	86		82	78	71	71	70	68	66	69	69	77	79
	B.B.	1	1	0	1	5		6	8	7	4	2	4	4	0	2			80	81	82	82	82		78	76	66	69	69	71	71	73	77		
Saline (iv)	W.B.	53	43	54	60	46		49	29	0	10	19	7	5		13			87	87	83	87	87		87	87	86	83	83	85	85		85		89

II, there occurred an increase in insulin and a decrease in glucose levels in one, no change in either insulin or glucose levels in another, and a decrease in blood glucose without an increase in plasma insulin in the third. Increases in plasma insulin were always accompanied by decreases in blood glucose. During the 3 iv tests, decreases in blood glucose were accompanied by small increases in plasma insulin levels. A saline control test, chosen because of the exceptional decrease in blood glucose of 15 mg per 100 ml, showed no increment of plasma insulin concentration (Table II).

Leucine tests in healthy subjects without pretreatment. Administration of leucine to subjects not pretreated with hypoglycemic agents caused a significant decrease in blood glucose during 8 of 23 oral tests and during 12 of 16 iv tests (1). Six of these leucine tests (2 oral and 4 iv tests) showing significant decreases in levels of blood glucose were selected for insulin assay. The mean of the maximal increases in plasma insulin was 12 μU per ml for the oral tests and 20 μU per ml for the iv tests. A control test with iv saline showed neither an increment of insulin levels nor a change in blood glucose concentration (Table II).

Comparison of results. Results obtained by each of the testing procedures are summarized in Table III.

TABLE III

Comparison of results

Pretreatment	Test material and route of administration	No. tests used for calculating mean of maximal increases	Plasma insulin, mean of maximal increases
			μU/ml
Chlorpropamide	Leucine (oral)	5	63
	Leucine (iv)	7*	87
Ultralente insulin	Leucine (oral)	3	13
	Leucine (iv)	3	16
None	Leucine (oral)	2	12
	Leucine (iv)	4	20
Chlorpropamide [4]† Ultralente insulin [1] None [1]	Water (oral) Saline (iv)	6	2

* Only the 7 standard leucine tests were used for calculation of mean of maximal increases. The additional 5 tests not used are identified by footnotes in Table I.

† Numbers in brackets refer to number of tests.

DISCUSSION

Our previous studies showed that sensitivity to leucine hypoglycemia could be induced consistently in healthy subjects after administration of sulfonylurea compounds (1). Our present study demonstrates that large increases in plasma levels of insulin are associated with the major decreases in blood glucose levels that follow the administration of leucine to healthy subjects pretreated with chlorpropamide. We found the mean of the maximal increases in plasma insulin to be 63 μU per ml for the oral tests and 87 μU per ml for the iv tests. In chlorpropamide-pretreated subjects, the mean of the maximal decreases in blood glucose levels was 29 mg per 100 ml for the oral tests and 38 mg per 100 ml for the iv tests (1). Leucine produced much greater increases in plasma levels of insulin and much greater decreases in blood glucose levels in subjects pretreated with chlorpropamide than in subjects without pretreatment or pretreated with Ultralente insulin. This indicates that sulfonylurea compounds, known to stimulate islet-cell activity, sensitize normal subjects to the insulin-releasing effect of leucine.

In healthy subjects, without prior administration of hypoglycemic compounds, administration of leucine produced modest and less consistent decreases in blood glucose levels (1, 2). Subjects in whom leucine did produce decreases in blood glucose (mean of maximal decrease, 10 mg per 100 ml) had significant increases in plasma insulin. The mean of the maximal increases in plasma insulin was 12 μU per ml for the oral tests and 20 μU per ml for the iv tests. This contrasts with the control tests, during which there were no significant increases in plasma insulin (mean of maximal increases, 2 μU per ml). Evidently, the absence of a decrease in blood glucose after administration of leucine to some normal subjects is due to failure of leucine to induce *1*) any insulin release or *2*) sufficient insulin release to cause reduced blood glucose.

Administration of leucine to subjects pretreated with Ultralente insulin produced decreases in blood glucose similar in frequency and magnitude to those in subjects not pretreated with hypoglycemic agents. In subjects pretreated with Ultralente insulin, the mean of the maximal increases in plasma insulin was 13 μU per ml for the oral tests and 16 μU per ml for the iv tests. Thus the increases in plasma insulin after administration of leucine to this group were comparable with those in subjects without pretreatment. This finding supports earlier studies that failed to demonstrate any potentiating activity of leucine upon the hypoglycemic action of exogenous insulin in man (1).

The results of leucine tests in the 3 experimental groups of subjects indicate that plasma insulin begins to increase before or at the onset of the fall of blood glucose. Peak insulin concentration preceded the maximal blood glucose depression by 10 to 30 minutes. The return to preinfusion levels of plasma insulin preceded that of blood glucose by about 30 minutes. The magnitude of the mean of maximal increases in plasma insulin for each of the 3 groups was commensurate with and proportional to the mean of the maximal decreases of their blood glucose values. This relationship between the magnitude of changes in plasma insulin and blood glucose as well as their temporal relationships after administration of leucine indicates that increased release of insulin is the cause of the decreases in blood glucose levels.

Occasionally we failed to detect an increase in the level of plasma insulin in peripheral venous blood after administration of leucine, even though a reduction of blood glucose occurred. This dissociation is interpreted in the following way. Up to 50% of endogenous insulin passing through the liver can be removed by this organ for over 1 hour (11), and more than 50% of insulin I^{131} rapidly injected into the portal vein is removed by the liver during one transhepatic passage (12). Thus, small amounts of endogenously released insulin may exert an hepatic effect without being detectable in peripheral blood. One major effect of insulin is the reduction of hepatic glucose output (13). We suggested previously that any additional insulin released from the pancreatic islets after administration of leucine would produce a major part of its hypoglycemic effect by decreasing hepatic glucose output (1). The demonstration by Lucas and Reaven (14) of a decrease in hepatic glucose output during and after leucine infusion to dogs pretreated with chlorpropamide confirms a hepatic role in the genesis of the ob-

served hypoglycemia. The present data, showing large increases of insulin in peripheral venous (posthepatic) blood, adequately account for this reduction in hepatic glucose output by a direct effect of endogenously released insulin on the liver. In addition, however, the increases in plasma levels of insulin in peripheral venous blood, and the decreases in plasma levels of FFA after leucine administration (1), indicate that increased peripheral carbohydrate utilization, mediated by increased release of endogenous insulin, contributes to the hypoglycemia induced by leucine.

SUMMARY

Plasma levels of insulin were measured by immunoassay before, during, and after the administration of leucine to healthy subjects *a*) after pretreatment with chlorpropamide, *b*) after pretreatment with Ultralente insulin, and *c*) without previous administration of hypoglycemic agents. Appropriate control tests were performed by administration of water or saline to similar groups of subjects.

In chlorpropamide-pretreated subjects, administration of leucine produced large and consistent increases in plasma levels of insulin and large decreases in blood levels of glucose. When leucine was administered to subjects either pretreated with Ultralente insulin or without pretreatment, plasma insulin increases were considerably smaller, and blood glucose decreased less. In the three groups of subjects, there was an excellent correlation between the magnitude and the temporal relationships of changes in plasma insulin and blood glucose.

We conclude that increased release of additional insulin is the primary mechanism by which leucine causes hypoglycemia in healthy subjects whether or not they are pretreated with sulfonylurea compounds. We suggest that endogenously released insulin exerts its hypoglycemic action by decreasing hepatic glucose output as well as by increasing peripheral utilization of carbohydrate.

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