Effect of Glucagon on Net Splanchnic Cyclic AMP Production in Normal and Diabetic Men

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ABSTRACT Glucagon activates hepatic adenylate cyclase, thereby increasing acutely the liver content of cyclic AMP (cAMP) as well as the release of cAMP into the hepatic vein. Insulin, on the other hand, antagonizes this glucagon-mediated cAMP production, thus providing a hypothetical mechanism through which insulin might correct some of the metabolic abnormalities of diabetes.

To study this hormonal interaction in man, net splanchnic cAMP production (NScAMPP) was investigated in normal and insulin-dependent diabetic men under basal conditions and in response to intravenous glucagon, 50 ng/kg/min for 2 h.

In normals ($n = 19$), basal hepatic vein cAMP concentration was 23.6±1.1 nM and NScAMPP was 1.7±0.6 nmol/min. Glucagon stimulated NScAMPP in four normal subjects to a peak of 99.6±43 nmol/min at 25 min with a subsequent fall to 12.4±5.1 nmol/min by 90 min despite continuing glucagon infusion. Endogenous insulin secretion was stimulated as indicated by rising levels of immunoreactive insulin and C-peptide (connecting peptide) immunoreactivity, raising the possibility that endogenous insulin might be responsible for the fall in NScAMPP that followed the initial spike.

In the diabetics ($n = 8$), basal hepatic vein cAMP concentration was 24.7±1.2 nM and NScAMPP was undetectable. Glucagon stimulated NScAMPP in five diabetics to a peak of 169.9±42.6 with a subsequent fall to 17.4±3.9 nmol/min by 90 min even though endogenous insulin secretion was not stimulated (no rise in C-peptide immunoreactivity). Although the mean increase in NScAMPP was greater in the diabetics, the two groups did not differ significantly.

Conclusions. In normal resting man the liver is a significant source of circulating cAMP. Diabetics do not release abnormally large amounts of hepatic cAMP under basal conditions. Glucagon markedly enhances hepatic cAMP release with a spike-decline pattern in both normal and diabetic men. The decline in hepatic cAMP release despite continuing glucagon stimulation is due to factors other than a stimulation of insulin secretion.

INTRODUCTION

Hepatocellular cyclic AMP (cAMP) [1] has been shown to play an important role in regulating the rate of hepatic glucose release (1, 2). Hepatocellular cAMP is finely controlled by several factors including glucagon and insulin which, respectively, raise and lower its concentration (2–5). The secretion of these two hormones in turn is regulated by a variety of substrates (6–8), other hormones (9), the autonomic nervous system (10, 11), and by each other (12). Thus, there exists a complex, finely regulated control of hepatocellular cAMP and hepatic glucose release. These concepts have been developed mainly in animal experiments, especially in the isolated perfused rat liver.

In intact man, however, very little is known about hepatocellular cAMP and its regulation by glucagon and

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[1] *Abbreviations used in this paper:* cAMP, cyclic AMP; C-peptide, connecting peptide; IRI, immunoreactive insulin; NSGP, net splanchnic glucose production; NScAMPP, net splanchnic cAMP production.

insulin. This is largely due to the impracticality of obtaining and studying human liver tissue. Recently, however, perfused rat liver studies demonstrated that glucagon administration resulted in the release of large amounts of cAMP into the hepatic vein (4), and that this release of cAMP was proportional to the increase of cAMP within the hepatocyte (13). These observations suggested a way whereby meaningful studies in man concerning hepatocellular cAMP and its regulation by glucagon and insulin could be performed, namely, by measuring changes in the amount of cAMP released by the liver into the hepatic vein.

Broadus et al. administered glucagon to normal men and noted a rise in circulating plasma cAMP (13). The source of this cAMP, although thought to be the liver, was not established. Rats made diabetic by alloxan and by anti-insulin serum have been shown to have elevated levels of hepatocellular cAMP. This has been attributed to the effect of glucagon acting on the liver unopposed by insulin (5). The purpose of the present study was to try to extend these observations to normal and diabetic man. Specifically, the following questions were to be answered: (*a*) Is the liver the source of the glucagon-mediated rise in circulating cAMP in man? (*b*) If so, what is the pattern and time course of this glucagon-mediated release of cAMP? and (*c*) Do insulin-dependent diabetic men differ from normal men in their responses to glucagon? To answer these questions, hepatic vein and brachial artery catheterizations were performed in normal and diabetic men and intravenous glucagon administered.

METHODS

Materials. Glucagon (Eli Lilly and Co., Indianapolis, Ind.), indocyanine green (Hynson, Westcott & Dunning, Inc., Baltimore, Md.), and Trasylol (FBA Pharmaceuticals, Inc., New York) were used in this study. Adenosine [8-^{3}H]-3′,5′-cyclic phosphate (20.8 Ci/mmol, lot XR-2010) was purchased from Schwarz/Mann Div. (Orangeburg, N. J.). Dowex 50 resin (AG 50W-X-8, 100–200 mesh hydrogen form) was obtained from Bio-Rad Laboratories (Richmond, Calif.). Phadebas Insulin Radioimmunoassay Kit was purchased from Pharmacia Fine Chemicals, Inc. (Piscataway, N. J.).

Preparation of materials. Glucagon was mixed with saline in a sterile 100 ml volumetric flask that contained 2–3 ml of the subject's blood which was added to eliminate binding of the glucagon to the glassware.

10 ml of the subject's blood was centrifuged under sterile conditions. 3 ml of the subject's plasma was then added to freshly prepared indocyanine green in order to increase its stability (14).

Protein kinase from bovine skeletal muscle was prepared according to the method of Gilman (15).

Subjects. Five normal and six insulin-dependent diabetic men participated in this study. Also included are the basal data of an additional 14 normal and 2 diabetic men who were subjects in other studies. All subjects were screened before study and were accepted for study only when found to fulfill the following criteria. None of the normal subject group had any personal history of diabetes mellitus and no member of either group had a history of other endocrine or other major diseases. Each normal subject had a normal standard 3 h oral glucose tolerance test (40 g of glucose/m^2 of body surface area) (16). Diabetics chosen for the study were insulin-dependent, ketosis-prone, juvenile-onset patients. All subjects had normal hepatic function as assessed by measurement of bromsulphthalein extraction, serum glutamic oxalacetic transaminase, serum bilirubin, and alkaline phosphatase. Each subject had a normal serum urea nitrogen and urinalysis, and cardiovascular function was normal by history, physical examination, chest X ray, and electrocardiogram. Complete blood counts were also normal in all subjects in each group.

Normal and diabetic men were matched for age, height, and weight (Table I). All normal subjects were on 300-g carbohydrate diets for 3 days before study. The diabetics adhered to their maintenance caloric intake which averaged 250 g of carbohydrate/day.

The diabetics received their last doses of insulin at least 12 h before study. All but one of the diabetics were receiving one daily morning injection of an intermediate-acting insulin preparation (Neutral protamine Hagedorn insulin [NPH] or Lente) which was given 24 h before study. One diabetic's regimen included a 6:00 p.m. dose of NPH insulin. Two diabetics received 5–10 U of regular insulin before 9:00 p.m. the evening before study to achieve comparable degrees of glycemic control. All subjects had fasted 12–14 h at the time the study began. Five of the six diabetics were hyperglycemic at the time of study (Table I).

The nature, purpose, and possible risks of the catheterization procedure were fully explained to the subjects before obtaining their voluntary consent.

Procedure. In the Vanderbilt University Hospital Cardiac Catheterization Laboratory, a Teflon catheter was inserted percutaneously in the left brachial artery. A 1 cm cutdown was then performed over a small tributary vein distal to the right antecubital fossa. A no. 8F Cournand catheter was advanced through this vein to a right-side hepatic vein and positioned 3–4 cm from the wedge position. A continuous saline infusion maintained the patency of the hepatic vein catheter without added anticoagulant. Splanchnic blood flow was measured by continuously infusing indocyanine dye (0.3 mg/m^2 of surface area/min after a loading pulse injection of 10 mg) (17). After a 30–45 min basal period, a 2 h intravenous glucagon infusion (50 ng/kg of body wt/min) was begun. Simultaneous blood samples were obtained from the brachial artery and hepatic vein throughout the study. 500 ml of blood was withdrawn over a 3 h period, the volume of which was replaced twofold with saline infused throughout the study at the rate of about 7 ml/min.

Analytical methods. All blood samples were processed immediately after being obtained. Blood glucose was measured within 4 min using the Technicon AutoAnalyzer (Technicon Instrument Corp., Tarrytown, N. Y.) employing the Hoffman ferricyanide reaction. After immediate centrifugation, plasma was chilled in ice for later determination of connecting peptide (C-peptide) immunoreactivity, (assayed by Doctors Marshall Block and Arthur Rubenstein, Chicago, Ill.) (18), immunoreactive glucagon, (assayed by Dr. Roger Unger, Dallas, Tex.) (19), and IRI. Plasma for cAMP determination was anticoagulated with heparin and EDTA and immediately precipitated with 0.3 M perchloric acid. The nucleotide was purified on Dowex 50 resin using the chromatographic technique of Butcher, Ho,

Meng, and Sutherland (20). Tritiated cAMP was employed to determine recovery. cAMP was then assayed by the protein-binding assay of Gilman (15). Plasma concentration of indocyanine green were determined in a Beckman DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) at 815 nm (17). Student's t test was used to determine statistical significance (21).

RESULTS AND COMMENTS

The first objective of our study was to determine whether the liver was the source of the glucagon-mediated rise in circulating plasma cAMP as reported by Broadus et al. (13). Therefore, four normal men underwent hepatic vein and brachial artery catheterization and received glucagon intravenously at the rate of 50 ng/kg/min, the lowest dose known to consistently raise plasma cAMP levels (13). The hepatic venous cAMP concentration rose within 2.5 min after beginning the glucagon infusion (Fig. 1). It reached a peak at 15 min, plateaued briefly, and then declined rapidly toward basal levels despite continuing glucagon administration. The brachial artery cAMP concentrations were lower throughout the glucagon infusion but followed a similar spike-decline pattern. The widening of the hepatic vein-brachial artery difference indicated an increased rate of cAMP release by the splanchnic bed. The amount of cAMP released by the splanchnic bed per minute was calculated by multiplying the hepatic vein-brachial artery cAMP difference times the splanchnic plasma flow. This net splanchnic cAMP production (NScAMPP) also rose and declined rapidly returning nearly to basal levels by 60 min (Fig. 2). These data establish the splanchnic bed and, therefore, most likely the liver as the source of the glucagon-mediated rise in circulating cAMP. The rapidity of the increase in NScAMPP is consistent with studies in perfused rat liver in which cAMP could be detected in the effluent 30 s after the addition of glucagon to the perfusate (22). In both the rat (22) and in man, augmented hepatic glucose release begins nearly simultaneously. In the present study, hepatic vein glucose concentration had risen significantly by 5 min into the glucagon infusion and insulin secretion had been stimulated as evidenced by a progressive rise in circulating immunoreactive insulin (IRI) (Fig. 2). As plasma IRI rose to its maximum there was a concomitant decline in NScAMPP in these normal subjects. Inasmuch as insulin is known to decrease hepatocellular levels of cAMP (5, 22), we wondered if the decline in NScAMPP, despite continuing glucagon infusion, could be attributed to the secondary rise in plasma IRI. To answer this question, we needed subjects who could not respond to glucagon stimulation with increased insulin secretion. Five juvenile-onset, ketosis prone, insulin-dependent diabetic men were, therefore, studied in an identical manner to the normal subjects. In these subjects, endo-

TABLE I
Clinical Data on Subjects

Subject	Sex	Age	Height	Weight	BSA*	EHPF‡	FBS§
			cm	*kg*	m^2	*ml/min/m^2*	*mg/100 ml*
Normals							
P. F.	M	25	182.5	79.3	2.00	576	85
W. E.	M	31	176	75.6	1.91	614	96
G. H.	M	40	184	75.5	1.98	519	90
D. R.	M	47	172.5	70.7	1.83	498	81
B. H.	M	25	179.7	72.8	1.90	561	84
Mean	—	33.6	178.9	74.8	1.92	554	87.2
SE	—	4.3	2.1	1.4	0.03	21	2.6
Diabetics							
D. M.	M	24	177	90.7	2.10	406	180
K. C.	M	33	161	56 2	1.59	421	265
D. W.	M	26	178.5	77.0	1.95	449	325
C. A.	M	46	178.5	81.4	2.00	426	143
R. S.	M	20	181.5	79.9	2.00	478	95
M. P.	M	18	166	57.4	1.63	371	142
Mean	—	27.8	173.8	73.8	1.88	425	191.7
SE	—	4.2	3.4	5.7	0.08	15	35.3

* Body surface area.
‡ Mean estimated hepatic plasma flow (EHPF) as milliters per minute per square meter of surface area.
§ Fasting blood glucose concentration at onset of glucagon infusion.

genous anti-insulin antibodies interfered with the measurement of insulin by radioimmunoassay. To be certain that these diabetics secreted no insulin in response to the glucagon infusion, C-peptide immunoreactivity was measured in arterial plasma (Table II). In the four normal subjects, C-peptide immunoreactivity rose progressively in response to glucagon but did not rise in the diabetics. As C-peptide is released by the beta cell in equimolar amounts to insulin, the lack of rise of C-peptide immunoreactivity in the diabetics indicated no increased insulin secretion in response to glucagon (23).

NScAMPP in these diabetic subjects, however, rose and declined in response to glucagon in a pattern similar to that of the normals. Fig. 3 compares the NScAMPP responses of the two groups. Whereas mean NScAMPP was greater in the diabetics than in the normals, the two groups did not differ significantly.

The cause of the secondary decline in NScAMPP which occurred despite continuing glucagon stimulation remains unknown. That this decline also occurs in non-insulin-secreting diabetic men indicates that the decline is not due to a concomitant increase in insulin secretion. A similar spike-decline pattern of cAMP synthesis has been induced in rat liver by epinephrine (2). It has been induced in isolated rat adipocytes by glucagon, ACTH, and epinephrine (24) and in human adipocytes by epinephrine (25).

One may only speculate as to the cause of the secondary decline in cAMP release. Perhaps a certain portion of the intracellular cAMP could exist preformed within the hepatocyte, ready for immediate activation or release upon proper stimulation. 60% of the cAMP in a rat liver homogenate has been found to be present in the particulate fractions obtained by centrifugation (22). Alternatively, an inhibitor could be formed during the initial period of stimulation, which blocks cAMP formation. Such an inhibitor has been reported (24) and is currently under further investigation.

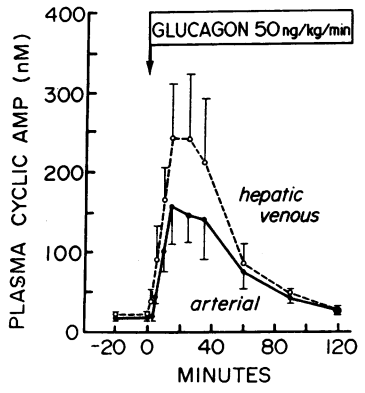

FIGURE 1 Effect of glucagon infusion on brachial artery and hepatic vein cAMP concentration in four normal men. Mean values ± SE are shown.

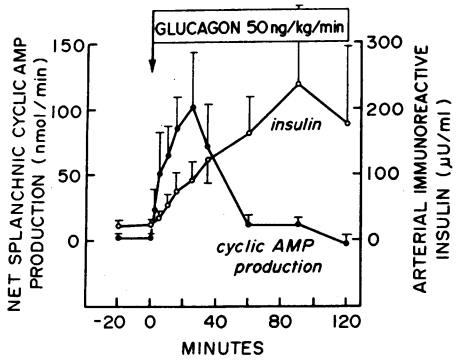

FIGURE 2 Effect of glucagon infusion on NScAMPP and arterial plasma IRI in four normal men. Mean values ± SE are shown.

Basal NScAMPP. Inasmuch as hepatocellular levels of cAMP are elevated in diabetic rats (5), diabetic men might be similarly affected. We wondered, therefore, if diabetic men might show evidence of increased hepatocellular cAMP levels by releasing increased amounts of cAMP from the liver during the basal state. We, therefore, compared the basal results of a large number of normal and diabetic men (Table III). In 19 normal subjects, there was a small but significant basal NScAMPP. In eight diabetics, however, there was no detectable basal NScAMPP. This was due to the fact that in the

TABLE II

Effect of Glucagon and Saline on Arterial C-Peptide Immunoreactivity in Normal and Diabetic Men

	Arterial C-peptide immunoreactivity						
Time, *min*....	−20	0*	5	15	35	60	120
				ng/ml			
Normals receiving glucagon							
P. F.	ND‡	ND	ND	1.5	4.7	3.3	2.0
W. E.	ND	ND	ND	2.0	3.3	6.0	10.0
G. H.	ND	ND	ND	ND	1.3	3.2	2.2
D. R.	ND	ND	ND	1.3	2.8	5.0	3.5
Normal receiving saline							
B. H.	ND	ND	ND	ND	ND	ND	ND
Diabetics receiving glucagon							
D. M.	ND	ND	ND	ND	ND	ND	ND
K. C.	ND	ND	ND	ND	ND	ND	ND
D. W.	ND	ND	ND	ND	ND	ND	ND
C. A.	3.0§	3.0	3.1	3.2	3.1	3.1	4.2
R. S.	ND	ND	ND	ND	1.3	ND	ND
Diabetic receiving saline							
M. P.	ND	ND	ND	ND	ND	ND	ND

* Glucagon infusion (50 ng/kg/min) begun at zero time.

‡ ND signifies nondetectable.

§ May represent proinsulin bound to endogenous antibody; does indicate that volunteer C. A. does have residual endogenous insulin secretion.

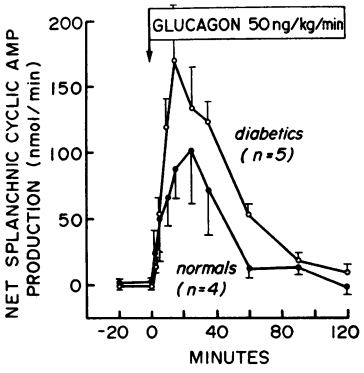

FIGURE 3 Effect of glucagon infusion on NScAMPP in normal and diabetic men. Mean values ± SE are shown.

diabetics the mean hepatic vein and brachial artery cAMP concentrations were essentially equal. In the normals, however, the hepatic vein cAMP concentrations were consistently higher than the arterial, thus producing a positive NScAMPP. Whereas the mean hepatic vein concentrations in the two groups did not differ significantly, the arterial levels were significantly lower in the normals. These intriguing observations are as yet unexplained. Under the conditions of this study, the diabetics could not be shown to release increased amounts of hepatic cAMP under basal conditions.

The plasma cAMP production rate, as determined by a constant infusion of labeled cAMP, has been estimated to be 9–17 nmol/min (26). The normal subjects in the present study had a basal net splanchnic cAMP release of 1.7±0.6 nmol/min. Since the liver is a site of cAMP degradation (27) as well as synthesis, this net value probably underestimates the actual hepatic release of newly synthesized cAMP. These results are in contrast to the dog where no arterial-hepatic venous cAMP difference was noted (28). It appears, therefore, that at least in normal basal man, the liver is a source of circulating plasma cAMP.

TABLE III
Basal Hepatic Vein and Brachial Artery cAMP Concentrations and NScAMPP in Normal and Diabetic Man

	Basal hepatic venous cAMP concentration	Basal brachial artery cAMP concentration	Basal NScAMPP
	nM	*nM*	*nmol/min*
Normals (n = 19)	23.6±1.1	21.9±0.9	1.7±0.6
Diabetics (n = 8)	24.7±1.1	25.0±0.6	−0.2±0.6
P	NS	<0.01	<0.05

Glucagon, insulin, and net splanchnic glucose production. Studies in the isolated perfused rat liver have demonstrated antagonism between the actions of glucagon and insulin in the regulation of several metabolic processes including glycogenolysis. Several laboratories have observed that the glycogenolytic effect of physiologic levels of glucagon (3×10^{-10}, 1×10^{-10}, 4×10^{-10}, and 4×10^{-10} M) could be inhibited by insulin (5, 29–31). These same groups also noted that the glycogenolytic activity of higher doses of glucagon (6×10^{-9}, 1×10^{-8}, 1×10^{-8}, and 2×10^{-9} M) could not be inhibited by insulin even when extremely high insulin levels (2.5×10^{-8} M; 3,600 μU/ml) were employed. We wanted to know, therefore, if such an inhibitory effect of insulin on glucagon-stimulated hepatic glucose release could be demonstrated under the conditions of this study.

The 50 ng/kg/min glucagon infusion raised arterial glucagon concentrations (Fig. 4) to a plateau at approximately 4,400 pg/ml (1.2×10^{-9} M) which was achieved by 15 min into the infusion and was maintained for the duration of the study. Approximately 17% of the glucagon presented to the liver was extracted. In both groups, this glucagon infusion resulted in an immediate rise in net splanchnic glucose production (NSGP) which was noted in all subjects by 5 min into the infusion (Table IV; graph of this data can be found in accompanying paper [32]). The NSGP of the diabetics and normals remained elevated throughout the glucagon infusion and did not differ significantly. The glucagon infusion with its accompanying rise in arterial glucose concentration strongly stimulated endogenous insulin secretion in the normals. Arterial IRI (Fig. 2) rose to a peak of 225 μU/ml (1.6×10^{-9} M). This concentration of insulin, which was undoubtedly higher in the hepatic vein, could not inhibit appreciably the glucagon-induced hepatic glucose or cAMP release. This statement is based on the observation that the results in the two groups did not differ significantly in regard to hepatic glucose and cAMP release. There may be two reasons

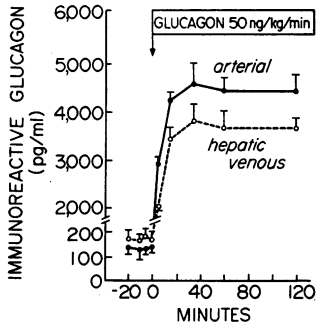

FIGURE 4 Effect of glucagon infusion on brachial artery and hepatic vein plasma glucagon concentration in five diabetic men. Mean values ± SE are shown.

TABLE IV

Effect of Glucagon on NSGP in Normal and Diabetic Men

Subjects	Time, *min*	−20	−10	0*	2.5	5	10	15	25	35	45	60	75	90	105	120
		NSGP														
		mg/min														
Normals (n = 4) + glucagon																
Mean		173	150	142	155	438	422	462	481	435	404	253	278	322	328	348
SE		18	31	21	27	20	51	47	101	54	73	45	55	57	16	45
Normal + saline																
		131	185	84	243	213	185	176	168	176	130	126	138	154	235	148
Diabetics (n = 5) + glucagon																
Mean		132	139	132	214	338	352	379	345	357	313	282	284	283	234	270
SE		46	26	25	36	41	96	102	63	60	66	61	71	89	37	63
Diabetic + saline																
		50	50	80	40	80	70	40	40	50	50	60	100	90	50	50

for the failure of insulin to inhibit these two glucagon-stimulated processes. First, the insulin:glucagon molar ratio ($1.6 \times 10^{-9}/1.2 \times 10^{-9}$) may not have been high enough as proposed by Unger (33) and, secondly, the glucagon concentration of 1.2×10^{-9} M may simply have been in the range of those concentrations of glucagon which in the in vitro studies mentioned above could not be inhibited even by extremely high insulin concentrations (31).

Glucagon and catecholamines. Very high doses of glucagon are known to cause the release of adrenal catecholamines (34–36) which also stimulate hepatocellular cAMP formation (2). To determine if the glucagon-mediated rise in plasma cAMP could be attributed to secondary catecholamine release, Broadus et al. compared a 100 ng/kg/min glucagon infusion with a 450 pmol/kg/min epinephrine infusion (13). The epinephrine infusion caused significant increases in pulse, blood pressure, lactate, and urinary catecholamines with only a minimal rise in plasma cAMP. The 100 ng/kg/min glucagon infusion (twice the dose used in the present study) caused no changes in these parameters, except for a profound increase in plasma cAMP. This suggests that the rise in NScAMPP reported in this study is not mediated through a rise in circulating catecholamines.

An unexpected finding in this study was the significantly lower estimated hepatic plasma flow in the diabetics ($P = < 0.01$) (Table I). Previous studies using bromsulphalein as an indicator reported no difference between normals and diabetics (37, 38). No explanation for our results is currently available. Glucagon has been shown to increase splanchnic blood flow when administered in very large doses (39). In the present study employing only moderate doses of glucagon, splanchnic blood flow did not increase.

In conclusion, glucagon appears to stimulate the release of cAMP from the human liver in a spike-decline pattern. The sharp decline in this rate of cAMP release is not due to the concomitant rise in circulating insulin. Diabetic men do not release increased amounts of hepatic cAMP under basal conditions, nor do they release significantly more cAMP than normal men during glucagon stimulation.

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