Potentiation of the Hepatic Action of Insulin by Chlorpropamide

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SUMMARY

In perfused livers of fed rats, chlorpropamide inhibits glucagon-stimulated glucose production by augmenting the action of insulin. This effect is associated with a decrease in cyclic AMP accumulation in liver and perfusate.

Alterations in glucose production appear to correlate more closely with changes in the amount of cyclic AMP in the perfusate than with changes in intrahepatic concentration of nucleotide.

Potentiation by chlorpropamide of the hepatic action of insulin does not require administration of the drug prior to perfusion. Further, it is demonstrable at concentrations of insulin and glucagon (10^{-11}M) that approximate the normal plasma levels of these hormones. DIABETES *26*:485-89, May, 1977.

A substantial body of evidence supports the belief that the sulfonylurea drugs reduce plasma glucose concentrations, at least in part, through extrapancreatic effects.[1] In particular, experimental data indicate that these compounds inhibit hepatic glucose production and potentiate insulin-mediated glucose transport into skeletal muscle.[2,3]

Clinical observations are consistent with these data. It is well known, for example, that in a diabetic patient whose plasma glucose falls in response to chronic treatment with a sulfonylurea drug, plasma insulin concentration frequently decreases also. Yet, if these agents acted *solely* to stimulate pancreatic insulin secretion, any hypoglycemic effect that could be ascribed to them would require higher, rather than lower, plasma insulin levels.[4,5]

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The experiments to be described in this report were undertaken to reëvaluate the effects of a widely used sulfonylurea drug, chlorpropamide, on hepatic carbohydrate metabolism. Although the sulfonylureas have achieved only modest success in the treatment of maturity-onset diabetes mellitus, increased understanding of their mode of action in the perfused rat liver might suggest more effective methods of restraining the inappropriately high rates of hepatic glucose production that characterize the diabetic state.

MATERIALS AND METHODS

Livers of fed male 150-300-gm. Sprague-Dawley rats were perfused with an oxygenated recirculating medium by the technique of Exton and Park.[6] This medium was prepared by making two additions to Krebs bicarbonate buffer: (1) enough bovine serum albumin to produce a concentration of 3 per cent, and (2) enough outdated human red blood cells to yield a hematocrit of 18-24 per cent. In numerous experiments perfusate pH values and oxygen tensions remained stable over a period of two hours (7.2-7.4 and 300-400 mm. Hg, respectively).

Each perfusion experiment followed the same design and lasted 125 minutes. During the first hour the liver was perfused with oxygenated medium alone. Livers were biopsied at 60 and 125 minutes for determination of intracellular concentrations of glycogen and cyclic AMP. As the experimental protocol required, a bolus injection of 300 μg. of porcine glucagon,* 0.5 U. of regular insulin (Lilly U-100

*Generously supplied by Lilly Research Laboratories, Eli Lilly & Co., Indianapolis, Indiana.

†Generously supplied by Pfizer, Inc., Brooklyn, New York.

Regular Iletin), or 100 mg. of chlorpropamide† was given at 60 minutes and was followed immediately by infusion of the same quantity of hormone or drug at a constant rate over the next 65 minutes. Perfusate samples were obtained at 65 minutes and at 125 minutes. Perfusate volumes ranged from 70 to 100 cc. In the tables that follow, estimations of glycogenolysis and cyclic AMP accumulation *in liver* relate to the last 65 minutes of perfusion, i.e., from 60 to 125 minutes; data concerning glucose and cyclic-AMP accumulation *in perfusate* refer to the last 60 minutes of perfusion.

Glucose concentrations in filtrates of perfusion medium were determined with the Beckman 670800 Glucose Analyzer (Beckman Instruments, Fullerton, Calif.). For the measurement of tissue glycogen levels, samples of liver were digested in 30 per cent potassium hydroxide in tubes immersed in a boiling-water bath. Glycogen was twice precipitated with 95 per cent ethanol and then heated in 2N sulfuric acid. Excess acid was neutralized with 2N sodium hydroxide, and the glucose liberated by acid hydrolysis was determined with the Beckman Analyzer. Glycogen was expressed as milligrams of glucose per gram wet weight of liver.

For measurement of tissue cyclic AMP levels, specimens of liver were pulverized in a stainless-steel mortar and pestle at dry-ice temperature. The powdered tissue was extracted with 10 per cent perchloric acid (4 cc. per gram of tissue) and centrifuged to separate the precipitate. The supernatant fluid was then added directly to a Dowex column and eluted with 0.1 N hydrochloric acid. Eluate was collected, freeze-dried, and taken up in Tris/EDTA buffer. Percentage recovery of cyclic AMP was estimated by putting a known amount of labeled nucleotide through a Dowex column. Cyclic AMP was determined by competitive protein-binding assay (cyclic AMP Assay Kit, Amersham-Searle, Arlington, Ill.). Samples of perfusate were prepared for analysis of cAMP by adding 4.2 cc. of 6 per cent perchloric acid per gram of perfusate, centrifuging to remove the precipitate, and titrating the supernatant back to pH 3-5 with 5 M K_2CO_3. Aliquots of supernatant fluid were then analyzed directly for cyclic AMP with the Amersham-Searle Assay Kit.

RESULTS

(A) Experiments Using Higher Concentrations of Glucagon and Insulin

Table 1 demonstrates that perfusion with glucagon (300 μg. by bolus plus 300 μg. by infusion over 65 minutes) caused an almost sixfold increase in hepatic glucose output over control perfusions. In perfused livers from larger rats (250-300 gm.), increased glycogen breakdown accounted for most of the glucagon-induced increment in glucose output. Thus, in 16 livers from larger rats, 87.4 per cent of total glucose formation could be ascribed to glycogenolysis. In contrast, in livers from smaller rats (150-200 gm.), glucagon stimulated glucose production not only from glycogen but also from other substrates. Thus in six smaller livers, glycogenolysis accounted for only 42.1 per cent of total glucose formed in response to glucagon.

TABLE 1

Lack of effect of various methods of chlorpropamide administration on glucagon-stimulated glucose production* by perfused livers of fed rats. (All glucose values that differ significantly from those in the glucagon-treated group are marked with a double dagger.)

Experimental group	Number of experiments	Glucose production (mg./gm. wet wt. liver/60 min. ± S.E.)
Control†	17	3.52 ± 0.39‡
Glucagon	23	18.20 ± 0.71
Chlorpropamide pretreatment (3 days) + glucagon	11	15.47 ± 1.25
Chlorpropamide pretreatment (3 days) + Chlorpropamide in perfusate + glucagon	4	22.01 ± 2.93
Chlorpropamide pretreatment (10 days) + glucagon	6	15.95 ± 2.45

*No exogenous substrate used. Glucose production is determined by subtracting the 65-minute perfusate glucose from the 125-minute value and dividing by wet weight of liver.

†No hormonal or drug additions to perfusate. No exposure to chlorpropamide prior to perfusion.

Table 1 also shows that pretreatment of rats with chlorpropamide for three days (20 mg. of drug injected intraperitoneally per 100 gm. body weight per rat per day) did not affect glucagon-stimulated hepatic glucose production even when the drug was *also* administered during the perfusion. Ten days of chlorpropamide pretreatment similarly failed to inhibit the action of glucagon.

Table 2 records the results of perfusion of livers with glucagon and with glucagon plus insulin. When

TABLE 2

Relation of glucose production to cyclic AMP accumulation* in perfused livers of fed rats. (All glucose and cyclic AMP values that differ significantly from the corresponding values in the glucagon-treated group are marked with a dagger.)

Experimental group	Glucose production (mg./gm. wet wt. liver/ 60 min. ± S.E.)	Cyclic AMP accumulation in perfusate (nmol/gm. wet wt. liver/ 60 min. ± S.E.)	Cyclic AMP accumulation in liver (nmol/gm. wet wt. liver/ 60 min. ± S.E.)
Control	3.52 ± 0.39† (N = 17)	0† (N = 4)	0† (N = 4)
Glucagon	18.20 ± 0.71 (N = 23)	12.77 ± 1.66 (N = 8)	5.60 ± 0.82 (N = 6)
Glucagon + insulin	17.57 ± 1.09 (N = 11)	19.98 ± 4.08 (N = 8)	6.12 ± 1.07 (N = 5)
Glucagon + insulin + chlorpropamide pretreatment (3 days)	9.90 ± 1.40† (N = 11)	3.60 ± 0.78† (N = 10)	1.84 ± 0.68† (N = 9)
Glucagon + insulin + chlorpropamide in perfusate	11.28 ± 2.14† (N = 6)	5.72 ± 0.76† (N = 6)	10.38 ± 2.18 (N = 6)

**Intrahepatic* cyclic AMP accumulation is calculated as cyclic AMP in liver at 125 minutes minus the corresponding value at 60 minutes divided by wet weight of liver. *Perfusate* cyclic AMP accumulation is determined by subtracting the 65-minute perfusate cyclic AMP value from the 125-minute value and dividing by wet weight of liver.

given together, the hormones were infused in a molar ratio of 0.45:1 (insulin-to-glucagon) because this proportion closely approximates the molar ratio of insulin to glucagon observed by Unger in normal subjects after a 48-72–hour fast.[7] Administration of this hormonal combination was therefore considered likely to provide an experimental model in which the influence of insulin would be minimal and hepatic glucose production would be regulated predominantly by glucagon. It can be seen that under these conditions insulin did not alter glucagon-stimulated glucose output. Maximum initial perfusate concentrations in these studies were about 3×10^{-7} M for glucagon and 1.3×10^{-7} M for insulin.

Table 2 shows, further, that pretreatment of rats with chlorpropamide for three days followed by simultaneous perfusion with insulin and glucagon caused a significant reduction of glucagon-mediated glucose output. Moreover, in livers of rats not previously exposed to chlorpropamide, perfusion with chlorpropamide, insulin, and glucagon also significantly decreased glucagon-stimulated glucose output.

Table 2 also correlates the changes in hepatic glucose output with concomitant changes in cyclic AMP accumulation in liver and perfusate. In the absence of hormone or drug (control perfusions), there was no detectable increment in cyclic AMP in liver or perfusate. Perfusion with glucagon caused a sixfold increase in glucose output as well as substantial accumulations of cyclic AMP in liver and perfusate. Simultaneous perfusion with insulin and glucagon gave results that were not statistically different from those obtained with glucagon alone. On the other hand, pretreatment of rats with chlorpropamide for three days followed by perfusion with insulin and glucagon caused a 50 per cent decrease in glucagon-stimulated glucose output as well as a significant reduction in cyclic AMP accumulation in liver and perfusate. Perfusion of livers with chlorpropamide, insulin, and glucagon—in the absence of chlorpropamide pretreatment—did not interfere with intrahepatic cyclic AMP accumulation but did decrease both hepatic glucose output and accumulation of cyclic AMP in the perfusate. Thus, in this group of livers the decline in glucagon-induced glucose output was associated with diminished cyclic AMP accumulation in perfusate but no longer paralleled the changes in intrahepatic levels of cyclic AMP.

(B) Experiments Using Lower Concentrations of Glucagon and Insulin

In the studies described above, pharmacologic amounts of glucagon and insulin were used (maximum initial perfusate concentrations of 10^{-7} M). Hence questions could properly be raised concerning the relevance of these studies to normal biologic processes. However, in the experiments to be described the maximum initial perfusate concentrations of glucagon and insulin were about 10^{-11} M, a value that is well within the range of physiologic variation. The molar ratio of the hormones was maintained at 0.45:1 (insulin-to-glucagon). At the same time, initial perfusate concentrations of chlorpropamide were of the

order of 40 mg. per cent (which can be compared with plasma levels of 15-25 mg. per cent reported in patients on maintenance doses of chlorpropamide).[8]

As table 3 shows, when smaller doses of drug and hormones were employed, neither chlorpropamide nor insulin alone significantly decreased glucagon-stimulated hepatic glucose output. However, the data do suggest a limited action of insulin to reduce the accumulation of glucose in the perfusate. Simultaneous administration of insulin and chlorpropamide caused a significant inhibition of glucagon-mediated glucose output, thus reproducing the findings of the earlier studies.

TABLE 3

Glucose production by perfused livers of fed rats when exposed to "physiologic" perfusate concentrations of insulin and glucagon (see text). (All glucose values that differ significantly from those in glucagon-treated group are marked with an asterisk.)

Experimental group	Number of experiments	Glucose production (mg./gm. wet weight liver/60 min. ± S.E.)
Glucagon	7	14.15 ± 1.69
Glucagon + chlorpropamide	2	13.38
Glucagon + insulin	6	9.84 ± 2.04
Glucagon + insulin + chlorpropamide	5	6.47 ± 1.48*

DISCUSSION

It is well established that insulin inhibits glucagon-stimulated hepatic glucose production.[9-11] Under the experimental conditions described in this report, neither chlorpropamide nor insulin alone effectively opposed the action of glucagon (although there was some indication of an antagonistic action of insulin). Nevertheless, the *combination* of chlorpropamide and insulin significantly diminished the glucose output elicited by glucagon. Thus, chlorpropamide appears to potentiate the hepatic action of insulin. Of additional interest is the observation that this effect can be obtained without administering the drug prior to perfusion.

There have been reports that tolbutamide, another sulfonylurea drug, inhibits phosphodiesterase activity in rat liver.[12] These reports cannot be readily reconciled with our own finding that rats given chlorpropamide for three days before perfusion with insulin showed significant blunting of glucagon-induced cyclic AMP accumulation in liver and perfusate (see table 2).

The observation that, in rats unexposed to chlorpropamide, the perfusion of livers with chlorpropamide and insulin reduced glucagon-stimulated glucose production and cyclic AMP accumulation in the perfusate without lowering intrahepatic nucleotide concentrations (table 2) deserves some comment. It would appear that alterations in the amount of biologically active nucleotide may be more accurately reflected by changes in the quantity of cyclic AMP in the perfusate than by changes in tissue levels of cyclic AMP. Conceivably, insulin and chlorpropamide decrease the size of one pool of cyclic AMP that is closely linked to the regulation of carbohydrate metabolism within the liver cell. If this pool were small relative to total cyclic AMP, a reduction in pool size might be reflected by diminished nucleotide accumulation in the perfusate but not necessarily by a measurable decrement in intrahepatic cyclic AMP concentration.

Table 3 shows that chlorpropamide augmented the action of insulin on the perfused rat liver at "physiologic" concentrations of insulin and glucagon. This tends to confirm previous observations[13-15] and suggests that chlorpropamide (and other sulfonylureas) diminish plasma glucose levels in man, at least in part, by potentiating the hepatic effects of insulin. Chlorpropamide has also been shown to augment the renal action of antidiuretic hormone[16] and to blunt parathormone-mediated increases in renal cortical cyclic AMP concentrations.[17] It seems clear that greater efforts should be made to identify other drug-hormonal interactions that might be exploited clinically—particularly in the management of maturity-onset diabetes mellitus. Use of the perfused rat liver preparation should facilitate the development of more effective methods of enhancing the peripheral action of insulin.

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