CYPROHEPTADINE-INDUCED DEPLETION OF INSULIN IN THE RAT\textsuperscript{1,2}

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ABSTRACT


The pancreatic toxicity of cyproheptadine (CPH) in rats was characterized through measurement of proinsulin and insulin levels in the pancreas as well as plasma immunoreactive insulin (IRI) and glucose levels at various times during and after drug treatment. Daily oral doses of CPH (45 mg/kg) depleted pancreatic IRI to 25% of control within 3 days. Pancreatic IRI levels showed no further decrease during the rest of a 2-week treatment period and returned to normal levels 2 days after withdrawal of the drug. Pancreatic proinsulin levels were not significantly changed by CPH treatment, and the decrease in pancreatic IRI can be ascribed primarily to a decrease in insulin. Nonfasting hyperglycemia was evident after two doses of CPH and persisted throughout the treatment period. Plasma IRI was not significantly altered compared to control and was inappropriately low for the hyperglycemia that was produced by CPH. Daily doses of CPH given i.p. (45 mg/kg) caused a transient decrease in pancreatic IRI, but hyperglycemia did not occur. Lower i.p. doses (22.5 mg/kg/day) caused a sustained decrease in pancreatic IRI content to 45% of control after 2 weeks and hyperglycemia was evident throughout the treatment period. No changes in plasma IRI were observed as a result of drug treatment even though blood glucose was elevated. These results suggest that biochemical changes affecting insulin synthesis and/or storage are associated with previously reported CPH-induced morphologic alterations in the rat \( \beta \)-cell.

Previous reports from this laboratory described the morphologic changes involved in a species specific and unique type of pancreatic \( \beta \)-cell toxicity in rats produced by high doses of cyproheptadine (CPH) and some other structurally related compounds (Wold et al., 1971; Longnecker et al., 1972; Fischer et al., 1973). Given orally CPH (45 mg/kg) produced progressive degranulation of the pancreatic \( \beta \)-cell followed by vesiculation of the endoplasmic reticulum. These vesicles appeared to coalesce to form large cytoplasmic vacuoles by the end of a 14-day course of CPH treatment (Longnecker et al., 1972). Identical i.p. doses of CPH produced less severe pancreatic islet cell damage as determined by light microscopy. Rats which received as few as two daily oral doses of CPH...
Male, Wistar rats (Simonsen Laboratories, Gilroy, Calif.) weighing 250 to 300 g were housed in suspended stainless steel cages and had free access to water. The light cycle was controlled (light on from 0600-1800 hours). Control animals in all experiments were pair-fed with the respective experimental group; controls received the same amount of food (Wayne Lab Blox, Allied Mills, Chicago, Ill.) which was eaten by the experimental group on the previous day.

Cyproheptadine [4-5H-dibenzo[a,d]cyclohepten-5-ylidine]-1-methyipiperidine] as the hydrochloride monohydrate was provided by Dr. C.A. Stone, (Merck Institute for Therapeutic Research, West Point, Pa.). The drug was administered orally (45 mg/kg) or i.p. (45 or 22.5 mg/kg) once a day as an aqueous solution. Control animals received water (1.5 ml/100 g) by the appropriate route. When CPH was administered orally, groups of control and treated animals were killed by decapitation at 12 or 24 hours after a single dose, and 24 hours after the 2nd, 8th and 14th daily dose. Additional groups were killed during a recovery period on the 2nd, 3rd and 8th day following the 14th daily dose. Animals receiving CPH i.p. were killed 24 hours after a single dose and 24 hours following the 2nd, 8th and 14th daily dose. In all experiments, the animals were killed between 0800 and 1000 hours in order to minimize the effects of diurnal variations.

At the time of killing, blood was collected in heparin-treated tubes and the plasma was separated and saved for measurement of IRI and glucose. The pancreas was quickly removed and homogenized in 6 volumes of ice-cold acid-ethanol, and insulin was extracted according to the procedure of Davoren (1962). The insulin-containing precipitate obtained upon addition of ether was dissolved in 3 ml of 3 M acetic acid and stored at -80°C until use. Estimation of IRI in the extracts was accomplished after diluting the extract 1:2500 in 0.05 M phosphate buffer, pH 7.4. Aliquots were then removed for radio-immunoassay using a Sephadex-bound antibody (Phadebas Insulin Test, Pharmaeia Laboratories, Piscataway, N.J.). Plasma IRI was measured without extraction in undiluted samples. Units for IRI are immunological micro-unit equivalents of standard porcine insulin. Dilutions of pancreatic extracts from control and CPH-treated rats yielded curves parallel to each other and to dilutions of standard porcine insulin throughout the useful range of the assay. This indicated that porcine insulin was an adequate standard for these experiments. IRI was calculated as micro-units per milligram wet weight of tissue and the values from CPH-treated animals were expressed as a percentage of the appropriate control animals. Plasma glucose was estimated using a glucose oxidase method (Thompson, 1966).

Separation of proinsulin and insulin in the pancreatic extracts was accomplished by exclusion chromatography (Sando et al., 1972). Bio-Gel P-30 was equilibrated with 3 M acetic acid and packed to a height of 50 cm in a 0.9 cm (inside diameter) column. Appropriate dilutions of the pancreatic extracts were made so that 400 to 800 μU equivalents of IRI could be applied in a volume of 100 μl. The extract was then eluted with 3 M acetic acid and fractions of 0.5 to 0.8 ml were collected. The separation of proinsulin and insulin was verified using bovine insulin and a sample of bovine proinsulin supplied by Dr. D. F. Steiner. Aliquots (0.1 ml) of the fractions were dried in vacuo and redissolved in 50 μl of 0.05 M phosphate buffer, pH 7.4, and the radioimmunoassay was performed. All glassware which came into contact with insulin-containing solutions was coated with Siliclad (Clay-Adams, Parsippany, N.J.). Pipettes were rinsed with an albumin solution prior to use (Sando et al., 1972).

Tissue for electron microscopy was fixed in 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3. The tissue was postfixed in buffered 2% osmium tetroxide, dehydrated in methanol solutions and embedded in Epon 812. Sections were cut with a diamond knife on an LKB III ultramicrotome, stained with 0.4% uranyl acetate and 0.4% lead citrate and examined on a Siemens ELMISKOP 101 at 60 kV. Statistically significant differences were detected
using Student's $t$ test when appropriate (Steel and Torrie, 1960). Plasma glucose levels in treated rats displayed variances which were statistically different from control by Bartlett's test of homogeneity of variance (Steel and Torrie, 1960) in several cases. This data was evaluated for statistical significance using the Mann-Whitney $U$ test (Goldstein, 1964). Differences where $P < .05$ were judged to be statistically significant.

**Results**

The effects of oral CPH administration (45 mg/kg) on pancreatic IRI, plasma glucose and plasma IRI are shown in figure 1. Pancreatic

![Graph showing changes in pancreatic IRI, nonfasting plasma glucose, and plasma IRI over time with CPH treatment.](image)

**Fig. 1.** Pancreatic IRI (top), nonfasting plasma glucose (middle) and plasma IRI (bottom) in rats during a course of oral administration of CPH (45 mg/kg). Each point is the mean value ($\pm$ S.E.M.) of three to six animals. ●—●, control; ○—○, CPH-treated animals. CPH was administered during the time indicated by the bar (14 days). An asterisk (*) indicates a significant difference ($P < .05$) from control.
IRI declined rapidly on initiation of CPH treatment reaching a nadir after the second daily dose of the drug. Levels remained low throughout the rest of the treatment period. Upon cessation of CPH treatment, levels of IRI in the pancreas rapidly returned to normal and increased to above normal levels after 8 days of recovery. Nonfasting plasma glucose levels became elevated and highly variable after two daily oral doses of CPH. Hyperglycemia persisted after withdrawal of CPH, although there was a trend toward normoglycemia. Mean plasma IRI levels appeared to deviate from control on initiation and cessation of CPH treatment, but no statistically significant differences occurred. Plasma IRI levels were inappropriately low considering the marked hyperglycemia which occurred during the course of CPH treatment.

Electron micrographs of $\beta$-cells from treated and control animals were obtained for correlation of morphologic and biochemical changes caused by CPH. Figure 2 shows a normal rat pancreatic $\beta$-cell (fig. 2A) compared to a $\beta$-cell from a rat which had received 14 daily oral doses (45 mg/kg) of CPH (fig. 2B). At the end of the treatment period, large cytoplasmic vacuoles were present, and electron dense secretion granules were nearly absent. Large numbers of secretion granules were apparent after an 8-day recovery period (fig. 3), but some large cytoplasmic vacuoles remained.

Rats which received i.p. doses of CPH (45 mg/kg) also exhibited a decrease in pancreatic IRI (fig. 4). As with oral treatment, pancreatic IRI was depleted to low levels after 2 days, but continuation of daily CPH treatment by the i.p. route did not result in continued suppression of pancreatic IRI. Rather, levels of pancreatic IRI rose to nearly 75% of control after 14 days of treatment. Nonfasting plasma glucose and plasma IRI levels in these rats remained normal throughout the course of treatment. Electron micrographs obtained after 14 daily i.p. doses of CPH (45 mg/kg) (not shown) revealed neither large cytoplasmic vacuoles nor a readily apparent decrease in dense-cored secretion granules.

Treatment of rats i.p. with CPH (45 mg/kg/day) resulted in a 22% weight loss associated with a 50% reduction in food intake over a 14-day period. These effects were not observed after identical oral doses of the drug. Reducing the i.p. dose to 22.5 mg/kg/day allowed the animals to maintain normal weight. The effects of the 22.5 mg/kg i.p. dose of pancreatic IRI and plasma glucose and IRI are shown in figure 5. Pancreatic IRI was depleted rapidly during the first 2 days of treatment, but the depletion was not as severe as that produced by the higher i.p. dose. Thereafter, pancreatic IRI levels declined more slowly, reaching 45% of control after 14 days of treatment. The animals displayed nonfasting hyperglycemia throughout the course of treatment. Plasma IRI levels were not significantly different from control and were inappropriately low for the hyperglycemic state of CPH-treated rats.

The changes in $\beta$-cell ultrastructure which occurred during 14 days of i.p. treatment with 22.5 mg/kg of CPH were much less severe than those which occurred when CPH was given orally at the higher dose. The number of insulin secretory granules was apparently decreased after 14 days of treatment, but large cytoplasmic vacuoles did not develop (fig. 6).

In order to test whether decreased food consumption could be responsible for the decreased pancreotoxicity of intraperitoneally administered CPH (45 mg/kg), a group of three rats was given CPH orally and was pair-fed with a similar group receiving the same dose i.p. Examination of the pancreatic tissue of both groups by light microscopy as previously described by Wold et al. (1971) showed that reducing food intake of orally treated rats had no effect on the severity of CPH-induced pancreotoxicity. All orally treated animals exhibited severe cytoplasmic vacuolization of islet cells while no vacuolization occurred in i.p. treated rats.

Since pancreatic IRI is composed of both insulin and proinsulin, it was necessary to determine whether CPH treatment altered one or both of these components. Table 1 shows the number of microunit equivalents of proinsulin and insulin per milligram of rat pancreatic tissue after various treatment and recovery periods. There were no statistically significant differences in the amount of pancreatic proinsulin found in treated and control animals. Lower amounts of pancreatic insulin were found after 1 and 14 days of oral treatment with CPH. Pancreatic insulin remained low the 1st day after with-
Fig. 2. Electron micrographs of pancreatic β-cell from control (A) and CPH-treated (B) (45 mg/kg for 14 days) rats. Magnification ×9000. Arrows indicate several of the numerous secretion granules in control rat β-cell. N, nucleus; V, cytoplasmic vacuole due to CPH treatment.
nal of CPH but was not different from control by the 8th day of recovery.

The amount of proinsulin found in the pancreas of rats which did not receive water vehicle or drug was 3.7 ± 0.7% of the extractable IRI. This is in good agreement with the data of others (Steiner et al., 1969). Handling of rats during treatment with water vehicle increased this percentage somewhat (table 1). In several cases, the increase was statistically significant demonstrating the importance of proper controls in studies such as this.

Discussion

Morphologic changes observed in β-cells of rats treated orally with CPH in this study confirmed the results of Longnecker et al. (1972), and micrographs obtained at all time points have not been presented here in the interest of brevity. Pancreatic IRI levels corresponded to the time course of degranulation, reaching their lowest point after two daily oral doses of CPH (45 mg/kg). Electron micrographs obtained after two doses of the drug in this study and that of Longnecker et al. (1972) revealed that few secretion granules were present. During the rest of a 14-day course of oral treatment, pancreatic IRI levels remained low while electron microscopy showed that the endoplasmic reticulum of the β-cell underwent vesiculation and subsequent coalescence of the vesicles into large cytoplasmic vacuoles (Longnecker et al., 1972). Withdrawal of CPH produced a rapid regranulation of the β-cell and an equally rapid return of pancreatic IRI levels to control values. Some cytoplasmic vacuoles remained, however, as long as 8 days after the last dose of CPH when pancreatic IRI levels were 150% of control. Elevated nonfasting plasma glucose levels which occurred at this time indicate that regranulation of the β-cell and normal levels of pancreatic IRI are not in themselves sufficient for recovery of rats from CPH-induced hyperglycemia. The hyperglycemia after 8 days of recovery may be due to effects peripheral to the pancreas or perhaps to altered insulin secretion caused by resid-
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Fig. 4. Pancreatic IRI (top) and nonfasting plasma glucose (middle) and plasma IRI (bottom) in rats during a course of i.p. administration of CPH (45 mg/kg). Each point is the mean value (± S.E.M.) of three to six animals. Legend as in figure 1.

Fig. 5. Pancreatic IRI (top), nonfasting plasma glucose (middle) and plasma IRI (bottom) in rats during a course of i.p. administration of CPH (22.5 mg/kg). Each point is the mean value (± S.E.M.) of three to six animals. Legend as in figure 1.

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CPH produces a different type of β-cell toxicity than that caused by alloxan or streptozotocin. Longnecker et al. (1972) have discussed differences in the morphologic changes produced by CPH and other diabetogenic agents. The data presented here show that CPH reduces pancreatic IRI levels without necrosis and to a lesser degree than either alloxan or streptozotocin (Rerup, 1970). The presence of large vacuoles along with insulin secretory granules in β-cells recovering from CPH damage suggests that repletion of IRI after CPH treatment may not depend upon formation of new β-cells as is required after alloxan or streptozotocin (Rerup, 1970). This may be the reason that recovery of pancreatic IRI after severe CPH-induced islet cell damage is apparently more rapid and predictable than after diabetogenic doses of the two more commonly used β-cytotoxic agents.

The pancreatic β-cell dysfunction caused by high i.p. doses of CPH (45 mg/kg) was less than that caused by the same daily dose given orally. This correlates with previously reported morphologic findings (Wold et al., 1971). Al-
though orally administered CPH produces β-cell vacuolization in a dose-dependent manner (Wold et al., 1971), data presented here suggest that β-cell dysfunction, as measured by pancreatic IRI levels, is greater after low i.p. doses of CPH (22.5 mg/kg) than after higher i.p. doses of the drug (45 mg/kg). Reasons for the inability of high i.p. doses of CPH (45 mg/kg/day) to produce a sustained reduction of pancreatic IRI equivalent to that caused by the same dose given orally or that produced by lower i.p. doses (22.5 mg/kg/day) are unclear. An explanation which was considered concerned the difference between the nutritional state of animals receiving the drug i.p. and those receiving it orally. Rats given daily high (45 mg/kg) i.p. doses of CPH consume about half the amount of food eaten by animals which receive the low dose (22.5 mg/kg) i.p. or the high dose orally. Since control animals in this study were pair-fed with treated animals, the control group for animals receiving CPH (45 mg/kg) i.p. were partially starved. These control rats had a pancreatic IRI content which averaged 105 ± 12% that of another group of untreated animals fed *ad libitum*. The return of pancreatic IRI levels toward normal in animals receiving CPH (45 mg/kg) i.p., therefore, was not an artifact due to a steady decline in the pancreatic IRI levels of the partially starved control animals. Pair-feeding of orally treated animals with i.p. treated animals indicated that reduced food intake alone was not sufficient to protect rats from the pancreotoxicity of orally administered CPH. Studies are in progress to elucidate the mechanism for the intriguing differences in CPH pancreotoxicity when given by the oral and i.p. routes.

The scheme of insulin synthesis and release as presently proposed (Kempler et al., 1972) contains several points at which CPH or a metabolite could act to yield decreased pancreatic insulin levels. For instance, a lack of the precursor, proinsulin, may limit the amount of insulin which can be formed. The fact that proinsulin levels in the pancreas of CPH-treated rats are not dramatically altered casts suspicion on that hypothesis. The data presented here are more in accord with a defect in the conversion
of CPH and some structurally related corn-electron micrographs and Mr. William Raun agents commonly known to alter the function of insulin. Experiments following the incorporation of proinsulin to insulin on in the storage of insulin. Proinsulin and insulin are needed to help clarify the effects of CPH treatment on the endocrine pancreas (Fischer, 1964).

Finally, it is worth noting that the effects of CPH and some structurally related compounds appear to be different from any of the agents commonly known to alter the function of the endocrine pancreas (Fischer et al., 1973). Studies on the mechanism by which these agents alter the rat pancreas may provide new insight into the normal and abnormal function of the β-cell.

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References


