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# Effects of *p*-Chlorophenylalanine on the Synthesis of Pancreatic Amylase in Rats

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Previously, we have reported that *p*-chlorophenylalanine (PCPA), a serotonin depletor, profoundly increased pancreatic fluid and bicarbonate secretion but remarkably inhibited pancreatic amylase secretion in anesthetized rats. The present study was performed to verify the detailed effects of PCPA on pancreatic amylase synthesis that is directly related to amylase exocrine secretion. PCPA significantly decreased pancreatic RNA and protein contents as well as the amylase activity. However, pancreatic DNA content, trypsin and chymotrypsin activities were not influenced by the treatment of PCPA. The rate of pancreatic amylase synthesis, which was assessed by the amount of incorporated [$^{35}$S]-methionine into amylase for 1 h, was also significantly decreased by 44% in PCPA-treated rats. In order to determine whether the PCPA-induced decrease of amylase synthesis resulted from change in the level of amylase mRNA, Northern blot analysis was performed. The mRNA expression level of amylase was also decreased by 48% in the PCPA-treated rats, indicating that the inhibitory effect of PCPA on the synthesis of pancreatic amylase was mainly regulated at a step prior to translation. It was also revealed in SDS-polyacrylamide gel electrophoresis that the qualitative change of amylase was induced by PCPA. The 54 KDa amylase band seems to be degraded into small molecular weight protein bands in PCPA-treated rats, suggesting that the PCPA-induced decrease of amylase may be partly attributed to the degradation of synthesized amylase.

Key Words: Pancreatic amylase, PCPA, Protein synthesis, mRNA, Pancreas

## INTRODUCTION

The serotonin synthesis inhibitor *p*-chlorophenylalanine (PCPA) has been originally known to deplete brain serotonin by binding irreversibly to tryptophan hydroxylase (Koe & Weissman, 1966; Koyuncuoglu et al, 1975; Datla & Curzon, 1996). Therefore, PCPA has been principally used to investigate the functions of serotonin in the brain (Steinman et al, 1987; Tohyama et al, 1988). However, the reports of several studies indicated that PCPA also exerts some pharmacological effects, which are unrelated to tryptophan hydroxylase inhibition (Marley & Whelan, 1976; Aumuller et al, 1981; Park et al, 1992; Arias & Bendayan, 1993). Especially, PCPA induces dramatic changes of the function and the morphology of pancreas. PCPA is known to disrupt the regulated secretory pathway in rat pancreatic acinar cells, including the aggregation and crystallization of secretory proteins within the lumen of the rough endoplasmic reticulum (RER, Forssmann & Metz, 1976; Arias & Bendayan, 1993; Arias et al, 1994; Chen et al, 1996). It has been shown that pancreatic amylase release is also decreased concomitantly with morphological changes induced by PCPA in rats and chickens (Forssmann & Metz, 1976; Furuse et al, 1993). However, the precise mechanism by which the PCPA affects the secretory process in acinar cells remains unclear. We reported previously that PCPA profoundly increased pancreatic fluid, bicarbonate and chloride secretion but remarkably inhibited pancreatic amylase secretion in anesthetized rats (Park et al, 1992). Neither neuroblockers such as atropine and phentolamine nor a serotonin

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receptor antagonist, cyproheptadine modified these actions of PCPA. Although PCPA has been shown to perturb protein secretion in the lumen of the RER and decrease amylase exocrine secretion, the effect of PCPA on pancreatic protein synthesis has not yet been examined. Thus, the present study was performed applying biochemical and Northern blot analysis to verify the detailed effects of PCPA on pancreatic amylase synthesis in rats.

## METHODS

### *Materials*

Male Sprague-Dawley rats weighing 200 to 250 g were supplied from the experimental animal center at Hallym University. *p*-Chlorophenylalanine (PCPA), ascorbic acid, Triton X-100, diphenylalanine, orcinol, dinitrosalysilic acid, shell fish glycogen, N-benzoyl-L-tyrosine ethyl ester, N-toluenesulfonyl-L-arginine methyl ester and enterokinase were obtained from Sigma (St. Louis, USA). Acrylamide, N,N'-methylene-bis-acrylamide and tetramethylenediamine were purchased from Bio-Rad (Richmond, USA). Agarose was from Duchefa (Haarlem, Netherland) and TRIzol reagent was from GibcoBRL (Frederick, USA). Reagents used for Digoxigenin (Dig)-nonradioactive Northern blotting were purchased from Boehringer Mannheim (Mannheim, Germany). Dig-labelled antisense oligonucleotide for the exon (1,427-1,456) sequences of rat amylase mRNA was synthesized from Operon (Alameda, USA). [$^{35}$S]-Methionine (1,437 Ci/mmol) was purchased from Amersham (Buckinghamshire, UK). All other chemicals were of the highest analytical grade available.

### *Experimental protocol*

The rats received a single intraperitoneal injection of 30 mg/100 g body weight PCPA dissolved in 0.1% ascorbic acid as described previously (Park et al, 1992). Fasted rats for 24 h were anesthetized with a single intraperitoneal injection of 25% urethane at a dose of 0.5 ml/100 g of body weight. Pancreata were removed and quickly trimmed free of fat. One fragment was homogenized in 25 mM tris (hydroxymethyl)-aminomethane (Tris), pH 8.0, 0.1% Triton X-100 for subsequent determination of DNA, RNA, protein and digestive enzyme contents (Rick & Stegbauer, 1972; Rick, 1972a; Ricks, 1972b; Richards, 1974; Bradford, 1976) and for determination of the rate of protein synthesis. The rest of pancreatic tissues were rapidly frozen in liquid nitrogen and processed for Northern blot analysis.

### *Determination of protein and amylase synthesis*

For determination of the relative rate of protein synthesis, 10 $\mu$Ci/100 g body weight of [$^{35}$S]-methionine was injected into the jugular vein 60 min before the sacrifice. An aliquot of the pancreatic homogenate was treated with cold trichloroacetic acid (TCA) (10% w/v) to precipitate proteins by procedure previously described (Ponnappa et al, 1988). TCA-insoluble precipitates were washed twice with cold 10% TCA and solubilized by overnight incubation with 0.1 M NaOH at 37°C. Aliquots of the TCA-soluble and TCA-insoluble samples were used for the determination of radioactivity in a Beckman liquid scintillation counter, model LS5801 (Beckman Instruments, Fullerton, USA) using Beckman Ready Safe as the scintillation cocktail. The relative rate of pancreatic protein synthesis was expressed as TCA-precipitated radioactivity, cpm/mg DNA. The [$^{35}$S]-methionine incorporated amylase was separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (1970). The individual protein bands were stained by Coomassie blue R-250. For quantification of radioactivity, amylase band was sliced and solubilized in 30% hydrogen peroxide as described by Wicker et al (1984). Radioactivity in the solubilized gel was determined in a liquid scintillation counter.

### *Analysis of purified amylase on SDS-PAGE*

To investigate the effect of PCPA on the degradation of amylase, we purified amylase from control and PCPA-treated pancreatic homogenates by a simple precipitation method of Schramm & Loyter (1966). For quantitative purpose, an identical amount of homogenate protein was subjected to purification. The purified amylase was separated by 10% SDS-PAGE and stained by Coomassie blue R-250.

### *Analysis of amylase mRNA*

Total RNA was extracted from rat pancreas using TRIzol reagent in accordance with the manufacturer's protocol. The quality of pancreatic RNA preparations

was verified by running aliquots on agarose-formaldehyde gel and controlling the relative amounts of 18 S and 28 S rRNAs. Amylase mRNA was analyzed by Dig-nonradioactive Northern blotting in accordance with the Boehringer Mannheim user's guide. Twenty micrograms of total RNA was size-fractioned on a 1% agarose gel containing 0.66 M formaldehyde and blotted to Hybond-$N^+$ membrane (Amersham, UK). And then, the membrane was cross-linked with UV light (UV Cross-Linker, Stratagene, La Jolla, USA). The prehybridization was performed for at least 1 h at 59°C in 5×SSC (0.15 M NaCl, 0.015 M sodium citrate), 50% (v/v) deionized formamide, 50 mM sodium phosphate, 0.1% (w/v) sodium lauryl sarcosine, 7% SDS, and 2% (w/v) blocking reagent. The hybridization at 59°C for at least 3h was carried out with the Dig-labeled antisense oligonucleotide for rat amylase mRNA. After hybridization, the blot was washed twice for 15 min in 1×SSC containing 0.1% (w/v) SDS and twice for 15 min in 0.1×SSC containing 0.1% (w/v) SDS. After exposing the blot to a blocking solution for 1h, the anti-Dig antibody was added to the solution and incubated for 30 min. The blot was washed twice for 15 min in 0.1 M maleic acid, pH 7.5, 0.15 M NaCl, and incubated with 1 : 100 diluted CSPD (Mannheim, Germany) in 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 25 mM $MgCl_2$. After incubation for 10 min at 37°C, the labeled bolts were exposed to X-ray film. The relative magnitude of each band was determined by densitometry equipped with PC.

*Statistical analysis*

All data were illustrated as means±SE. The statistical analysis was evaluated by the Student's t-test. The difference was considered significant when $p<0.05$.

## RESULTS

*Effects of PCPA on the contents of pancreatic DNA, RNA, protein and enzyme activities*

As illustrated in Fig. 1, the mean DNA content per

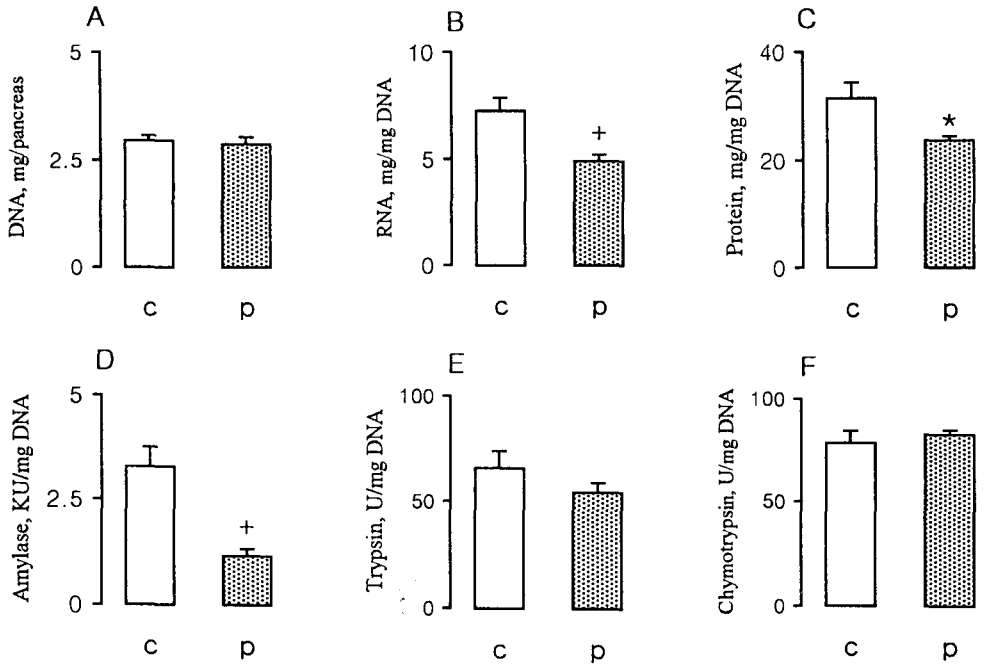


**Fig. 1.** Effects of PCPA on the contents of pancreatic DNA (A), RNA (B), protein (C), amylase activity (D), trypsin activity (E), and chymotrypsin activity (F). The open bars represent the values of control rats (c) and the dotted bars represent the values of PCPA-treated rats (p). Each bar represents mean±SE obtained from six rats. An asterisk and crosses denote statistical significance at $p<0.05$ and $p<0.01$, respectively.

pancreas was 2.72±0.21 mg in control rats and did not change significantly in PCPA-treated rats. By contrast, pancreatic RNA (7.24±0.54 mg/mg DNA) and protein content (28.3±33.34 mg/mg DNA) in control rats were significantly decreased by 33% and 25% in PCPA- treated rats, respectively. Among the pancreatic digestive enzymes examined, only the content of amylase activity was markedly decreased by 65% in PCPA-treated rats (from 3.26±0.48 KU/mg DNA in control rats to 1.15±0.17 KU/mg DNA in PCPA-treated rats). The contents of pancreatic trypsin and chymotrypsin activities were not influenced significantly by the treatment of PCPA.

PCPA also induced a qualitative change in amylase. Fig. 2 shows SDS-PAGE of amylase purified from control and PCPA-treated pancreatic homogenates by the precipitation method of amylase-glycogen complex. The 54 KDa pancreatic amylase band was weaker in PCPA-treated rats compared with control rats, whereas several new small molecular weight bands including 39 KDa band, 30~33 KDa bands and 16 KDa band appeared in the pancreas of PCPA-treated rats.

*Effects of PCPA on the synthesis of pancreatic protein and amylase*

The incorporation of [$^{35}$S]-methionine into TCA-insoluble pancreatic protein is shown in Fig. 3. As indicated, the rate of incorporation of [$^{35}$S]-methionine into total pancreatic protein for 1 h was 2.55×

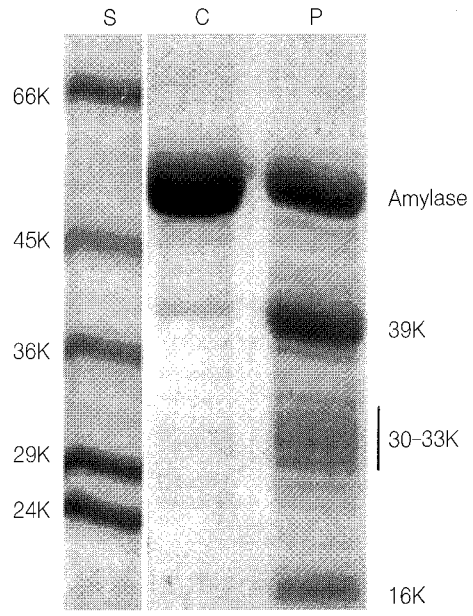


**Fig. 2.** SDS-PAGE of amylase purified from rat pancreas by the precipitation method of amylase-glycogen complex. Data is representative of three experiments. Protein bands were stained with Coomassie Blue R-250. S, molecular weight standard; C, control rats; P, PCPA-treated rats.

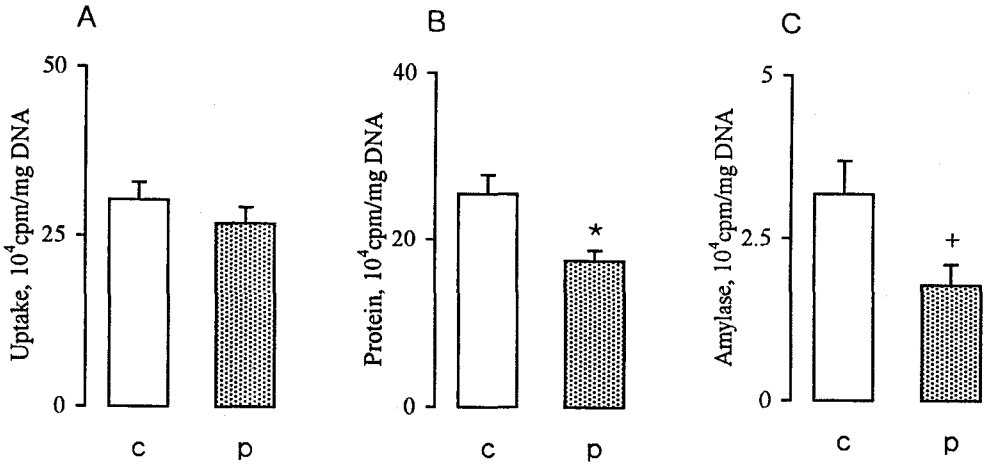


**Fig. 3.** Effects of PCPA on the [$^{35}$S]-methionine uptake into the pancreas (A), the rate of [$^{35}$S]-methionine incorporation into pancreatic protein (B), and the rate of [$^{35}$S]-methionine incorporation into pancreatic amylase (C). The open bars represent the values of control rats (c) and the dotted bars represent the values of PCPA-treated rats (p). Each bar represents mean±SE obtained from six rats. An asterisk and cross denote statistical significance at $p<0.05$ and $p<0.01$, respectively.

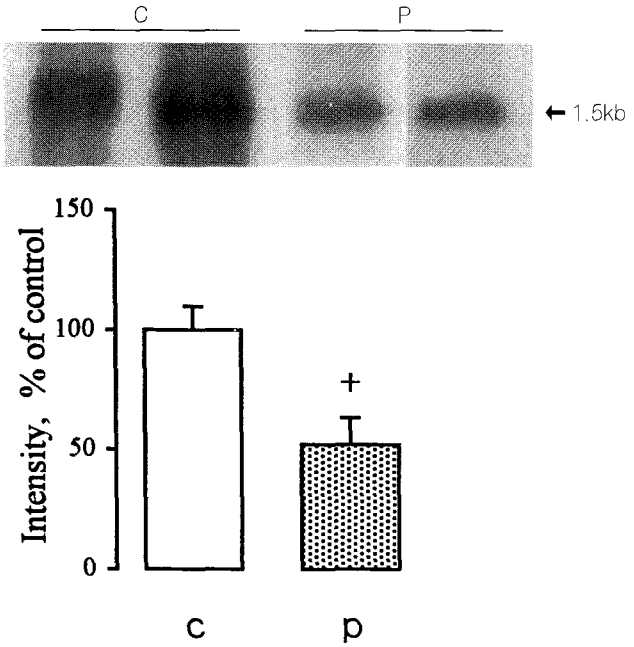


**Fig. 4.** Effect of PCPA on the level of pancreatic amylase mRNA. C, control rats; P, PCPA-treated rats. Lower panel represents the relative quantification of amylase mRNA blots. Each bar represents mean ± SE obtained from four amylase mRNA blots. A cross denotes statistical significance at $p < 0.01$.

$10^5$ cpm/mg DNA in control rats. This protein synthesis rate was significantly decreased to $1.75 \times 10^5$ cpm/mg DNA by the treatment of PCPA. Liquid scintillation counting of radiolabeled amylase extracted from gel slice revealed that pancreatic amylase synthesis was also decreased from $3.18 \times 10^4$ cpm/mg DNA in control rats to $1.78 \times 10^4$ cpm/mg DNA in PCPA-treated rats. On the other hand, [$^{35}$S]-methionine uptake into pancreatic tissue was also slightly reduced in PCPA-treated rats. Therefore, decrease in the rate of [$^{35}$S]-methionine incorporation into protein could be partly due to a reduction of amino acid uptake into the pancreas.

*Effect of PCPA on the level of pancreatic amylase mRNA*

In order to determine whether the PCPA-induced decrease of amylase synthesis resulted from change in the level of their mRNA, Northern blot analysis was performed by probing identical amount of pancreatic total RNA with a antisense oligonucleotide for rat amylase mRNA. As shown in Fig. 4, mRNA expression level of pancreatic amylase was notably decreased in the PCPA-treated rats as compared with control rats. The relative magnitude of amylase mRNA in PCPA-treated rats was 52% of that of control rats.

## DISCUSSION

PCPA have been reported to cause disturbances in the structural and functional properties of the pancreas, including protein secretion, crystal formation and aberrant aggregation of secretory protein within the lumen of the RER (Forssmann & Metz, 1976; Arias & Bendayan, 1993; Chen et al, 1996). In our previous study focused on pancreatic exocrine secretion in vivo, we found that PCPA profoundly increased pancreatic fluid and bicarbonate secretion but remarkably inhibited pancreatic amylase secretion in anesthetized rats (Park et al, 1992). To provide a more complete description of the decreased amylase secretion resulting from PCPA, we monitored PCPA-induced changes in the content of amylase activity, rate of amylase synthesis, and amylase mRNA expression level in rat pancreas.

In the present study, we found that 1) PCPA selectively decreased the content of pancreatic amylase activity but did not alter trypsin and chymotrypsin activities, 2) PCPA decreased the rate of pancreatic amylase synthesis as well as the cellular level of amylase mRNA, 3) PCPA induced degradation of synthesized amylase.

The present study showed that PCPA did not alter the mean DNA content per pancreas but decreased pancreatic RNA and protein content, indicating that PCPA generally exerts inhibitory influences on pancreatic gene expression. However, these actions of PCPA were not shown in all of the digestive enzymes. Among the digestive enzymes examined, only amylase activity was markedly decreased but trypsin and chymotrypsin activities were not altered in PCPA-treated rat pancreas (Fig. 1). Arias and Bendayan demonstrated that amylase accumulation in the intracisternal crystals of RER was found to be about 10 times higher than that of chymotrypsinogen in PCPA-treated pancreatic acinar cells (Arias & Bendayan, 1993). In the biosynthetic pathway of pancreas, newly synthesized secretory proteins are inserted into the RER, where they are efficiently sorted and after maturation reach their eventual exact location in the cells (Lynn & Kelly, 1987; Pfeffer & Rothman, 1987; Pryer et al, 1992). Some posttranslational modifica-

tions including specific proteolysis, disulfide bond formation, amidation and glycosylation play important roles in pancreatic protein sorting (Scheele, 1986; Helenius et al, 1992; Pryer et al, 1992). The defects in the normal pattern of protein sorting could account for the selective aggregation of proteins into intracisternal crystals (Arias & Bendayan, 1993). It is well known that pancreatic amylase possesses carbohydrate moiety and disulfide bond, which are essential for the full activity of amylase (Stiefel & Keller, 1973; Rinderknecht, 1986). Therefore, it is possible to speculate that PCPA decrease the content of amylase activity through accelerating the accumulation of immature amylase into intracisternal crystals. However, at this stage, we could not exclude the possibility of PCPA-induced disturbance of posttranslational modifications (i.e. glycosylation, disulfide bond formation) of amylase.

In this study, we also observed that PCPA induced a qualitative change of amylase. The 54 KDa band of pancreatic amylase in SDS-PAGE was weaker in PCPA-treated rats compared with control rats, whereas the small molecular weight bands including 39 KDa band, 30~33 KDa bands and 16 KDa band newly appeared in the pancreas of PCPA-treated rats (Fig. 2). Since the all bands on the gel could be formed by making complex with glycogen, these small molecular weight bands might be regarded as a degradation product of amylase. Therefore, it is likely that PCPA-induced decrease of amylase may be partly attributed to the PCPA-induced degradation of synthesized amylase. The precise nature of these small proteins remains to be established in the future study.

In order to determine if the content of amylase activity was correlated with the alteration of amylase synthesis rate, [$^{35}$S]-methionine incorporation into the amylase was measured. The rate of incorporation of [$^{35}$S]-methionine into amylase in PCPA-treated rats was also decreased by 44% (Fig. 3). Although both amylase activity and synthesis rate were decreased in PCPA-treated rats, amylase activity was decreased to a greater extent (65%) than did amylase synthesis rate. It is indicating that synthesized amylase was further hampered by the unidentified posttranslational processes such as degradation and some inadequate modifications (Scheele, 1986; Wong et al, 1991; Helenius et al, 1992). Measurement of mRNA level for amylase was made to determine if the change in enzyme synthesis was due to change in mRNA transcription or to posttranscriptional mechanism. The cellular content of amylase mRNA in PCPA-treated pancreas was decreased by 48% (Fig. 4), which value is similar to that of protein synthesis rate (44%). This positive correlation between the relative synthetic rate of the amylase and mRNA expression level may suggest that synthesis was predominantly regulated at steps prior to translation (Perkins et al, 1995). Conceivably, it seems that PCPA may change the cellular content of amylase mRNA by altering the rate(s) of several processes including transcription, mRNA processing, nuclear transport of mRNA.

Taken together, the present experimental results demonstrate that the inhibitory effect of PCPA on the synthesis of pancreatic amylase was mainly regulated at a step prior to translation. The results of this study furthermore suggest that the decreased content of pancreatic amylase in PCPA-treated rats may be partly attributed to posttranslational degradation of synthesized amylase. However, further detailed studies at the intracellular level are needed to establish a direct interaction between PCPA and pancreatic enzyme synthesis.

## ACKNOWLEDGMENT

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