Tannin-Mediated Induction of Proline-Rich Protein Synthesis

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Feeding high-tannin sorghum to rats causes dramatic changes in gene expression of parotid glands similar to that induced by isoproterenol treatment. Within 3 days parotid glands enlarge about 3-fold and a series of proline-rich proteins (PRPs) increase about 12-fold. These morphological and biochemical changes have now been demonstrated to occur in response to the ingestion of purified tannins. Addition of either dimer or polymer condensed tannins or tannic acid to diets resulted in both the hypertrophic effect and induction of PRPs. Addition of propranolol, a β-antagonist, to the high-tannin diet blocked both the induction of PRPs and the hypertrophic effects. In vitro translations of total RNA from parotid glands showed dramatically reduced levels of PRP mRNAs, indicating that propranolol may have inhibited transcription. The inhibiting effect of propranolol suggests a major role for β-receptors in the tannin induction of PRP biosynthesis and parotid gland hypertrophy.

INTRODUCTION

Proline-rich proteins (PRPs), which contain 25–45% proline, have been isolated from the salivary glands and salivary secretions of various species (Bennick, 1982). Two families of either acidic or basic PRPs with variations in glycoslylation or phosphorylation are commonly found (Mehansho et al., 1987a; Muenzer et al., 1979a). In rats (Muenzer et al., 1979b), mice (Mehansho et al., 1985), and hamsters (Carlson et al., 1985), the synthesis of these proteins is considerably enhanced by isoproterenol treatment. PRPs account for over 50% of the total soluble proteins of parotid glands from rats treated with isoproterenol (Muenzer et al., 1979b). Northern blot analysis showed that the increase in protein synthesis resulted from striking changes in the levels of PRP mRNAs (Ziemer et al., 1984; Mehansho et al., 1985). Isoproterenol treatment also causes hypertrophy and hyperplasia of the salivary glands (Selye et al., 1961; Muenzer et al., 1979b; Mehansho et al., 1985).

Previously we reported the induction of PRP synthesis and parotid gland hypertrophy in rats (Mehansho et al., 1983) and mice (Mehansho et al., 1985) fed high-tannin sorghum. However, whether tannins are the sole cause of the observed phenotypic changes had not been determined. Tannins are polyphenolic polymers that can strongly bind proteins. Salivary PRPs are among those proteins having the highest binding affinity for tannins (Hagerman and Butler, 1981). As measured by competitive binding assays, PRPs have at least a 1000-fold higher affinity for tannin than do other proteins such as lysozyme. These observations may be significant with respect to human nutrition because tannins are commonly found in our diets (i.e., fruits, wine, tea, and sorghum) (Mehansho et al., 1987a; Salunkhe et al., 1990). Sorghum is of particular concern since millions of people in the semiarid tropics rely on it for calories and protein. The growth rates of rats and chicks are substantially reduced (Rostagno et al., 1973; Jambunathan and Mertz, 1973) when they are fed diets containing high-tannin sorghum, even when the grain was cooked (Price et al., 1980). More recent studies suggest that high-tannin diets can be acutely toxic to hamsters (Mehansho et al., 1987b), rats (Mole et al., 1990), and deer (Robbins et al., 1987). In addition, tannins have been implicated with some human cancers (Kirby, 1960; Morton, 1980). A prominant role of salivary PRPs may be to minimize these harmful effects by forming tannin–PRP complexes.

This study reports the enhancement of PRP synthesis and parotid gland hypertrophy by purified tannins when added to tannin-free diets. These effects of tannins were specifically blocked by β-antagonists and mimicked by β-agonists.

METHODS

Materials. All materials were of the highest purity available and were purchased from commercial sources unless otherwise indicated. The following materials were purchased from the respective companies: L-[3,4(*n*)-^{3}H]-proline (100 Ci/mmol) from ICN; *dl*-isoproterenol hydrochloride and *dl*-alprenolol *d*-tartrate salt from Sigma; phentolamine from Ciba-Geigy; EN^{3}HANCE from New England Nuclear; and crude quebracho powder from Trask Chemical Corp. Reticulocyte lysate was a gift from Dr. D. Kuhn, Department of Biochemistry, Purdue University. The high-tannin sorghum (Savanna) and low-tannin sorghum (RS-610) were grown at the Purdue University Agronomy Farm under the direction of Dr. J. Axtell.

Feeding Trials. Sprague-Dawley male rats (70–80 g) were used. Sorghum grain was ground and incorporated into diets as described elsewhere (Mehansho et al., 1983). Feed and water were provided ad libitum. Detoxification of sorghum tannin by ammoniation was carried out as described by Price et al. (1979). A kilogram of whole grain was treated with 500 mL of 0.1 N NH_4OH for 12 h, and the samples were dried at room temperature before grinding and incorporation into diets. Gelatin was added at a level of 8% in place of cellulose. Tannins were added at a level of 2% in place of cellulose.

Administration of β- and α-Blockers. Where indicated, *dl*-propranolol, *dl*-alprenolol, and phentolamine were added to diets at levels shown in Table II. These compounds were added daily to the diets. From the amount of high-tannin diet consumed, about 3 mg of either propranolol or alprenolol was ingested daily by each animal.

Proline-Rich Protein Isolation and Characterization. Rats were euthanized as described (Muenzer et al., 1979a). The parotid glands were removed, stripped of connective tissues, and weighed. PRPs were isolated from pooled glands by using a trichloroacetic acid extraction procedure (Mehansho and Carl-

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Table I. Effects of Tannins, Tannic Acid, Gelatin, and Ammoniation on Rat Salivary Glands

diet[a]	gland wt,[b] mg/g of body wt		PRPs,[c] mg/g of body wt	
	parotid	submandibular	parotid	submandibular
RS-610	3.3	1.8	0.03	0.03
RS-610 + gelatin	3.1	3.0	0.03	0.02
RS-610 + NH_4OH	3.2	3.4	0.03	0.02
Savanna	7.9	2.7	0.31	0.04
Savanna + gelatin	3.7	3.2	0.02	0.03
Savanna + NH_4OH	4.9	3.4	0.07	0.03
RS-610 + tannin dimer	9.6	3.0	0.27	0.05
RS-610 + tannin polymer	6.5	2.9	0.22	0.05
RS-610 + catechin	2.3	2.8	0.03	0.04
RS-610 + tannic acid	11.4	2.8	0.37	0.05

[a] Animals were fed the respective diets for 3 days. [b] Averages from four rats. [c] Glands from each treatment were pooled, homogenized in 10% TCA, and assayed for PRP levels as described under Methods.

son, 1983). Proteins were quantitated by absorbance at 230 nm, because they have negligible amounts of aromatic amino acids (Mehansho and Carlson, 1983). The calculated extinction coefficient for nonglycosylated PRPs ($A^{1\%}_{1cm}$) is 25.

Isolation and Cell-Free Translation of mRNAs. Total RNA was isolated according to the method of Cathala et al (1983). Cell-free translations were performed with [^{3}H]proline in the rabbit reticulocyte lysate system as previously described (Ziemer et al., 1982). PRPs were precipitated by using 10% trichloroacetic acid containing 1% phosphotungstic acid.

SDS–Polyacrylamide Gel Electrophoresis. Gel electrophoresis was performed as described by Laemmli (1970). Proteins were precipitated with acetone, dissolved in sample buffer, and heated in boiling water bath for 5 min. Electrophoresis was done on 12% polyacrylamide gels unless otherwise specified. Fixing and staining of proteins were accomplished as described by Steck et al. (1980) with slight modification (Mehansho and Carlson, 1983). Radioactivity on gels was identified by fluorography using EN^{3}HANCE (Laskey and Mills, 1975).

Tannin Assays. Sorghum grains and purified quebracho tannins were assayed for total phenols (Price and Butler, 1977), relative tannin chain length (Butler et al., 1982), and protein precipitating activity (Hagerman and Butler, 1978). Savanna contained 1.4% tannin and 10% total phenolics (w/w), whereas RS-610 had no detectable tannin and 0.6% total phenolics (w/w). Low molecular weight (dimers) and high molecular weight (polymers) condensed tannins were purified from crude quebracho powder (Asquith and Butler, 1985). Dimers were obtained from the 30% acetone/water wash and polymers from the 60% acetone/water wash off of the LH-20 column.

RESULTS

Effects of Ammonia Treatment and Addition of Gelatin. Table I lists the average weights and PRP content of parotid and submandibular glands from rats fed control and experimental diets. As previously demonstrated (Mehansho et al., 1983), high-tannin sorghum induced parotid gland hypertrophy and PRP synthesis. However, the size and PRP content of the submandibular glands were unaffected. Earlier studies have demonstrated that treatment of high-tannin sorghum grain with dilute ammonium hydroxide decreased assayable tannins and increased the weight gains of animals fed the treated grain (Price et al., 1979). We now observe that the induction of PRPs and hypertrophy of parotid glands in rats fed high-tannin sorghum were greatly reduced by ammonia treatment (Table I). However, no changes were observed in the amount of PRPs and size of the parotid glands of animals fed ammoniated low-tannin sorghum.

The dramatic induction of PRP synthesis and hypertrophy of parotid glands observed in animals fed high-tannin sorghum was also prevented by including calf skin gelatin in the diet (Table I). Furthermore, gelatin supplementation dramatically improved growth of rats fed the high-tannin diet (7-day gains of 22.2 and 4.5 g for RS-610 and Savanna, respectively, without dietary gelatin and 25.7 and 19.2 g with dietary gelatin). A similar effect of dietary gelatin alleviating the growth depression of high-tannin sorghum was observed with hamsters (Mehansho et al., 1987b). Since gelatin binds very tightly to tannins (Hagerman and Butler, 1981), its action is likely due to interaction with the dietary tannins. It is important to note that gelatin had no effect on the animals fed the low-tannin sorghum diet.

The effects of ammoniation and gelatin addition on PRP induction were analyzed by SDS–PAGE (Figure 1A). Parotid glands of rats fed ammoniated high-tannin sorghum contained much lower amounts of PRPs than did the glands of animals fed the same sorghum without the ammonia treatment. However, ammoniation did not completely inhibit PRP increase, whereas addition of gelatin to the high-tannin sorghum diet completely blocked its effect on PRP synthesis. These results strongly suggest that tannins present in the high-tannin sorghum diet are the primary effectors for the various responses observed in rat parotid glands.

Studies on the Effects of Purified Tannins. Direct evidence for tannin induction of PRP synthesis and parotid gland hypertrophy was obtained by adding purified condensed tannins or tannic acid to the low-tannin diet (Table I). Addition of condensed tannin, either dimer or polymer, or tannic acid resulted in parotid gland hypertrophy and PRP induction similar to that observed in rats fed the high-tannin diet. Feeding of catechin failed to induce the changes so noticeable from the polymers. Changes in PRP levels induced by purified tannins were analyzed by SDS–PAGE (Figure 1B). The intensities, patterns, and electrophoretic mobilities of the PRPs induced by the condensed tannins and tannic acid were identical to those obtained from parotid glands of rats fed high-tannin sorghum.

Effect of β- and α-Blockers. The mechanism by which tannins cause hypertrophy of the parotid glands and PRP induction are not known. Because feeding tannins mimics the effects of isoproterenol, a β-agonist, we asked whether propranolol, a β-antagonist, would block the tannin-induced changes in the rat parotid glands. As shown in Table II, addition of propranolol to the high-tannin sorghum diet inhibited PRP induction and parotid gland hypertrophy. Figure 2 shows that propranolol inhibition of PRP induction is concentration dependent. The apparent discrepancy in PRP content between the spectrophotometric measurements (Table II) and gel patterns (Figure 2) is probably related to the much higher sensitivity of autoradiography. The gel data are consistent with the gland weight values (Table II, column 1). Like propranolol, the β-antagonist alprenolol prevented PRP induction by tannins; in contrast, the α-antagonist phentolamine had no effect (Figure 3), indicating that the effects of

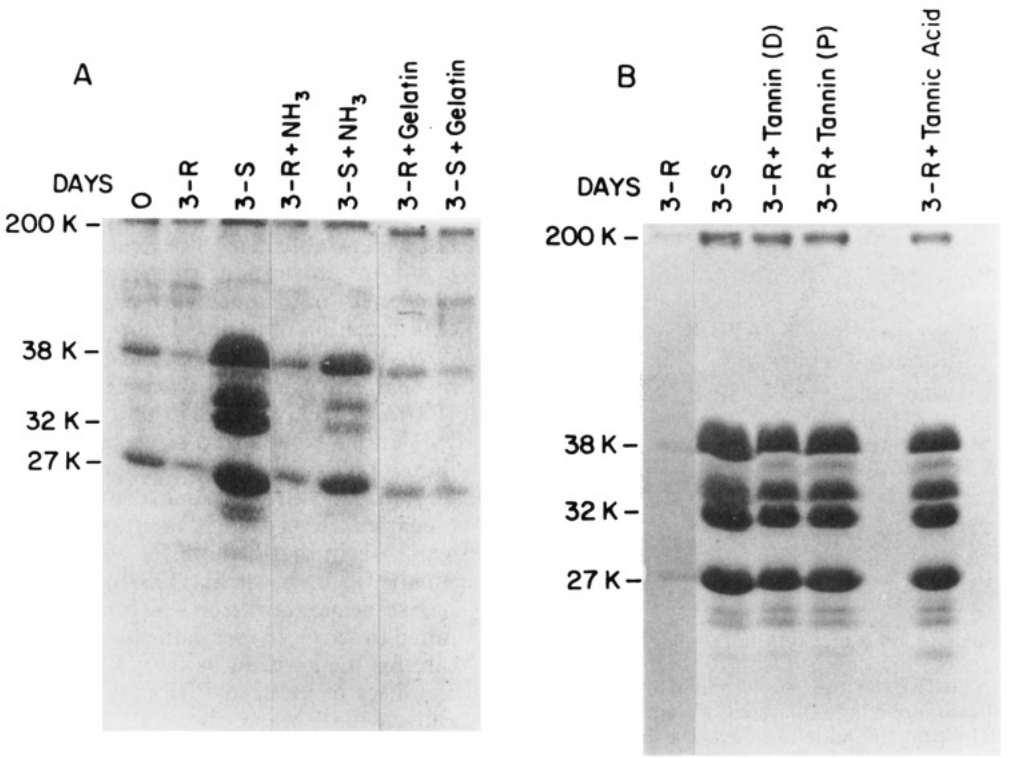

Figure 1. SDS–polyacrylamide gel electrophoresis of trichloroacetic acid soluble fractions from parotid glands. (A) Effects of ammoniation and addition of gelatin; (B) effects of purified tannins; (R) rats fed RS-610 sorghum; (S) rats fed Savanna sorghum; (D) rats fed condensed tannin dimer; (P) rats fed condensed tannin polymer. Acid-soluble protein equivalent to 2 mg of tissue was applied to each lane.

Table II. Effects of Propranolol, Alprenolol, and Phentolamine on Rat Salivary Glands

diet[a]	gland wt,[b] mg/g of body wt.		PRPs,[c] mg/g of body wt	
	parotid	submandibular	parotid	submandibular
RS-610	3.2	2.0	0.03	0.03
Savanna	5.6	2.2	0.21	0.02
Savanna + propranolol (0.13%)	2.8	2.2	0.04	0.03
Savanna + propranolol (0.10%)	3.7	1.9	0.03	0.03
Savanna + propranolol (0.06%)	4.4	1.8	0.05	0.01
Savanna + propranolol (0.03%)	4.7	1.9	0.05	0.02
Savanna + alprenolol (0.13%)	2.6	2.2	0.04	0.02
Savanna + phentolamine (0.13%)	5.3	2.1	0.19	0.02

[a] Animals were fed the respective diets for 3 days. [b] Averages from four rats. [c] Glands from each treatment were pooled, homogenized in 10% TCA, and assayed for PRP levels as described under Methods.

dietary tannins are mediated via the β-receptors but not through the α-receptors.

Cell-Free Translations. Total RNA from animals fed rat chow, low-tannin sorghum, or high-tannin sorghum ± propranolol was translated in a reticulocyte lysate system. Tritiated proline was included in these translations to facilitate detection of PRPs. SDS–PAGE analyses of the translation products labeled with [^{3}H]proline are shown in Figure 4. As previously shown (Mehansho et al., 1983) feeding high-tannin sorghum greatly increased the in vitro translation of PRPs with RNA from rat parotid glands. The increase was completely prevented by addition of propranolol to the high-tannin diet. The results obtained from the cell-free translations demonstrate that the propranolol-dependent inhibition of PRP synthesis likely occurs during transcription of PRP mRNA.

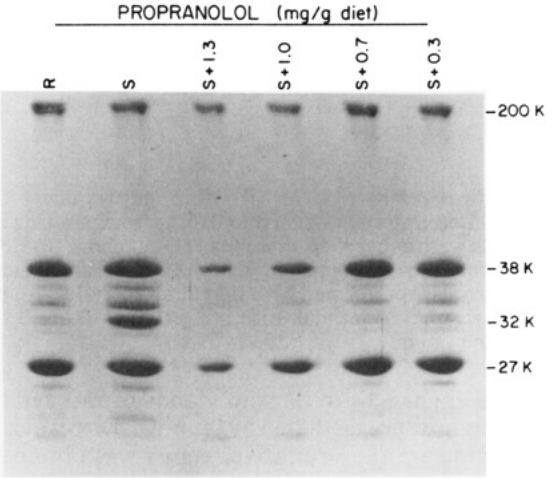

Figure 2. Inhibition of proline-rich protein synthesis by propranolol. TCA-soluble fractions equivalent to 3 mg of tissue were applied to each lane. (R) Rats fed RS-610 sorghum; (S) rats fed Savanna sorghum.

DISCUSSION

Previously we reported the induction of PRP synthesis and hypertrophy of parotid glands in rats (Mehansho et al., 1983) and mice (Mehansho et al., 1985) fed sorghum high in tannins. Within 3 days of feeding high-tannin sorghum, rat parotid glands were enlarged about 3-fold and the level of PRPs increased by 12-fold. The results presented here demonstrate that these morphological and

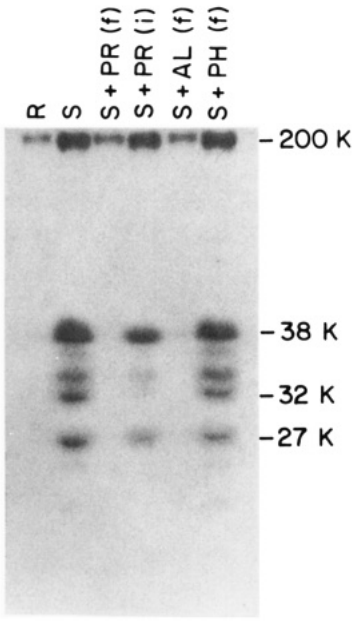

Figure 3. Effects of β- and α-blockers on rat glands in animals fed Savanna sorghum. (R) Rats fed RS-610 sorghum; (S) rats fed Savanna sorghum; [PR (f)] propranolol added to the Savanna diet; [PR (i)] propranolol administered by intraperitoneal injection; [AL (f)] alprenolol added to Savanna diet; [PH (f)] phentolamine added to the Savanna diet.

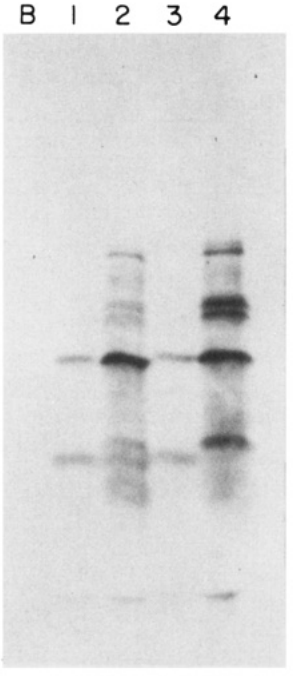

Figure 4. Cell-free translations of mRNAs from parotid glands. Total RNA was prepared from rats fed (1) rat chow, (2) RS-610, (3) Savanna plus propranolol, and (4) Savanna. Cell-free translations were performed with the rabbit reticulocyte system as described under Methods with [^{3}H]proline. Lane B shows translation system without added RNA.

biochemical changes in parotid glands of rats fed high-tannin sorghum are mediated by tannins. Direct evidence that tannins are the primary effectors was obtained by adding condensed tannin (dimer or polymer) or tannic acid to low-tannin sorghum diets. This resulted in both hypertrophy of the parotid glands and striking increases in PRP syntheses. Ammoniation of the high-tannin grain, which drastically lowers levels of extractable tannins and improves weight gains in chicks (Price et al., 1979), markedly reduced the changes observed with the high-tannin sorghum diet. In these experiments, addition of gelatin to the diet also eliminated the effects induced by high-tannin sorghum.

PRPs comprise about 70% of the proteins in human saliva (Bennick, 1982). These unusual proteins are also the predominant components of parotid glands in rats (Mehansho et al., 1983) and mice (Mehansho et al., 1985) fed high-tannin diets. The biological function of PRPs is not well established. The acidic PRPs have been implicated in prevention of excess deposition of calcium phosphate onto tooth surfaces and in maintaining the concentration of ionic calcium in saliva (Bennick, 1982).

We have suggested that basic PRPs serve as a defense mechanism against dietary tannins (Mehansho et al., 1987a). PRP synthesis in rats is dramatically enhanced by tannins (Mehansho et al., 1983), and blocking their induction causes a severe reduction in the conversion of digested food into body matter (Mole et al., 1990). Further, hamsters are very sensitive to tannin toxicity because PRP synthesis in these animals is not induced by dietary tannins (Mehansho et al., 1987b). Unlike rats and mice, hamsters maintained on a diet containing 2% (w/w) tannin failed to grow. In fact, hamsters fed a diet containing 4% tannins died within as few as 3 days. Lastly, the high affinities of induced PRPs for tannins (Hagerman and Butler, 1981; Mehansho et al., 1983; Asquith and Butler, 1986) strongly argue that they have a specific biological function to bind tannin.

The mechanism by which tannins cause parotid gland hypertrophy and PRP induction is unknown. They probably bind to a carrier protein or receptor in the digestive tract which in some fashion triggers PRP induction. Protein precipitation is not the key factor for PRP induction because the dimer and polymer fractions had equivalent biological activities. Short phenolic polymers tend to form soluble complexes with proteins (McManus et al., 1985), whereas longer polymers will precipitate them. Therefore, the biological activity of dietary phenols is probably related more to direct physiological effects than to precipitation of dietary proteins or digestive enzymes (Mole et al., 1990).

Tannin-induced changes could be mediated via the β-receptors since they mimic those induced by a β-agonist, isoproterenol (Mehansho et al., 1983, 1985). Addition of either propranolol or alprenolol, β-antagonists, to the high-tannin diet prevented the tannin-mediated parotid hypertrophy and induction of PRP synthesis. In contrast, feeding phentolamine, an α-antagonist, had no effect on the tannin-mediated responses. These results show that tannins likely induce PRP synthesis and cause hypertrophy of parotid glands via the β-receptors.

It was not known whether parotid hypertrophy and PRP induction are defensive responses by the animal, a toxic effect from the plant or a coincidence. Our data suggest that it is a defensive response by the animal to a dietary toxin.

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