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Induction of Salivary Polypeptides Associated With Parotid Hypertrophy by Gallotannins Administered Topically into the Mouse Mouth

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Abstract Isoproterenol-induced salivary polypeptides (IISP), a group of proline-rich proteins synthesized by mouse parotids, have been considered as markers for isoproterenol-induced parotid hypertrophy. Rodents fed diets containing high-tannin cereals (sorghum), also develop parotid hypertrophy. To test whether tannins are directly involved in provoking sialotrophic growth, we studied the effect of intraperitoneal and topical oral administrations of tannic acid (TA) on the induction of IISP polypeptides in endogamic mice (A/Snell). TA was characterized by HPLC chromatography and spectral analysis and shown to be composed solely of gallotannins, a complex family of glucose and gallic acid esters. IISP polypeptides were monitored in saliva by SDS–polyacrylamide gel electrophoresis during 36 h after ending TA stimulation. Single daily intraperitoneal administrations of TA for 3 consecutive days (0.033 mg/g bw/day), at variance of parallel administrations of isoproterenol (0.042 mg/g bw/day) failed to induce IISP polypeptides. However, repeated topical applications of TA into the mouse mouths (1.21 mg/g bw divided into three equal doses given at 4-h intervals within a single day) resulted in unequivocal induction of IISP polypeptides. That response was clearly intensified by increasing the stimulation frequency to eight equivalent doses given at 1.5-h intervals within a single day (corresponding to 3.23 mg/g bw) and even further by repeating this protocol for 3 days. Under these productive schemes of stimulations by TA, electrophoretic fractionation of parotid homogenates showed new polypeptide bands migrating in parallel to salivary IISP. These results suggest that topically administered gallotannins are effective inducers of trophic growth in mouse parotids. J. Cell. Biochem. 100: 487–498, 2007.

Key words: saliva; parotid gland; mouse; isoproterenol; proline rich-proteins; tannin; hypertrophy

Rodent salivary glands recurrently stimulated by β-adrenoceptor agonists, such as isoproterenol, have been used as a study model of hypertrophy [Schneyer, 1962; Novi and Baserga, 1971]. That cellular and glandular response has been associated with the induction, accumulation, and secretion of a group of salivary proline-rich proteins [Muenzer et al., 1979a,b; López Solís et al., 1987, 1989]. In mouse, the assessment of isoproterenol-induced salivary proline-rich polypeptides (IISPs) in saliva, rather than in salivary glands, has allowed a much earlier recognition of the trophic response of parotid acinar cells [López Solís et al., 1989, 2003a,b; López Solís and Kemmerling, 2005]. In effect, IISP polypeptides can be detected in saliva as early as 24 h after a single intraperitoneal administration of the powerful sialotrophic β-adrenoceptor agonist isoproterenol whereas protocols to induce salivary gland hypertrophy are currently based on recurrent administrations of the same agonist for several days or weeks [Muenzer

Abbreviations used: g bw, gram of body weigh; HPLC, high-pressure liquid chromatography; IISP, isoproterenol-induced salivary proline-rich polypeptides; IPR, isoproterenol; kDa, kilodalton; Mr, relative molecular mass; TA, tannic acid.

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et al., 1979a,b; Humphreys-Beher et al., 1987a; Ann et al., 1987; Li et al., 1997].

A few reports have shown that experimental salivary gland hypertrophy can be also produced by feeding rodents on cereal-based or legume-based diets containing high levels of tannin. Thus, rats fed a sorghum variety rich in condensed tannins ("high-tannin sorghum"), in contrast to rats fed either "low-tannin sorghum" or control diet of chow, showed both parotid gland enlargement and increased synthesis and accumulation of salivary proline-rich proteins [Mehansho et al., 1983, 1985, 1987; Humphreys-Beher et al., 1987b]. Similar results have been obtained in rats maintained on diets containing high levels of condensed tannins from faba beans (*Vicia faba L*) [Jansman et al., 1994]. Altogether, data from those reports highly suggest that the molecular and cellular effects produced by high-tannin diets both on the parotid salivary tissue and on saliva of rats are indistinguishable from those produced by iterative isoproterenol stimulations [Tu et al., 1993; Lin et al., 1996; Ann et al., 1997]. Despite those similarities, marked differences between isoproterenol and tannin-rich diets as sialotrophic agents may well exist. Thus, at variance of isoproterenol, whose sialotrophic effect is thought to be mediated by its direct interaction with β-adrenoceptors located on the basolateral surface of the responsive acinar cells, a mechanism linking dietary tannins to the sialotrophic response has not yet been identified [González et al., 2000]. In our view, a main difficulty in this regard is that the sialotrophic effect of cereal-based or legume-based diets rich in tannins has not yet been paralleled by reports on the corresponding effect of experimentally controlled oral administrations of diet-free tannins, the putative sialotrophic agents [Mehansho et al., 1983, 1985, 1987; Humphreys-Beher et al., 1987b; Jansman et al., 1994]. Thus, it cannot yet be discarded the possibility that other unconsidered components of the cereal or legume variants that were used in the above-mentioned feeding trials may be primarily involved in the induction of the sialotrophic response. On the other hand, salivary gland hypertrophy has been so far provoked by providing animals with permanent and unrestricted access to tannin-rich diets for several days or weeks what would hamper any temporal mapping of the molecular steps being part of mechanisms coupling the stimulus to the trophic response.

Previously, we reported that a number of synthetic structural analogs of isoproterenol that display a major sialotrophic effect in mouse also provoke the induction of a single group of IISP polypeptides in the hypertrophic glands [López Solís et al., 1990]. Later studies showed that those isoproterenol-induced salivary polypeptides (IISP) were secretory in nature and that their identification was greatly facilitated by screening whole saliva (the one collected from the mouse mouth) rather than salivary gland homogenates [López Solís et al., 1989, 2003a]. Thus, it was reasonable to assume that an eventual trophic effect of tannin extracts on mouse parotid glands may well be accompanied by changes in the polypeptide profile of saliva and particularly by the appearance of IISP polypeptides. The purpose of this study was to determine whether tannic acid (TA), a tannin extract obtained from oak gall nuts from *Quercus infectoria*, induces the appearance of IISP polypeptides in the saliva of mice when applied topically into the mouth under strict time-controlled schedules. Several administration schedules were evaluated.

MATERIALS AND METHODS

Animals

Female or male mice of the A/Snell strain, inbred in our laboratory and weighing 24 ± 3 g were used when 3–4 months old. The animals were maintained on a 12-h light and 12-h dark schedule and fed ad libitum.

HPLC Analysis of Tannic Acid (TA)

One gram of TA was dissolved in 100 ml of 20% v/v ethanol, stirred at 30°C in the dark for 2 h and concentrated in vacuo to a quarter of its original volume. The concentrate was extracted three times with 25 ml of diethyl ether, three times with 25 ml of ethyl acetate and the organic fractions were combined. The resulting organic extract was evaporated to dryness in vacuo and the residue was dissolved in 2 ml of 1:1 v/v methanol/water and 5 μl-aliquots were subjected to HPLC chromatography. To do so, reversed-phase separations of TA were carried out in triplicate at 25°C using a 300 mm × 3.9 mm i.d., 4 μm particle size Nova Pack C_{18} column. A photodiode-array detector (Waters, model 991, Milford, MA) was set at 280 nm. Two mobile

phases were used: A, water/acetic acid (98:2 v/v) and B, water/acetonitrile/acetic acid (78:20:2 v/v/v). A two-step gradient was carried out at a constant flow rate of 1.0 ml per min: 0–55 min, 100%–20% A and 55–70 min, 20%–10% A. Equilibration times of 15 min were allowed between injections. Each major peak in the HPLC chromatogram of the TA extract was characterized both by retention time and absorption spectrum in the 210–400 nm wavelength range and contrasted against an electronic library of UV/Vis spectra of standard polyphenols that has been developed in our laboratory to assist identification of grape and wine phenolic compounds by routine HPLC analysis [Peña-Neira et al., 2000].

Tannic Acid Administration

A 400 mg/ml stock solution of TA was prepared in water (no antioxidants added) immediately before its administration. Depending upon the experiment, TA was either injected intraperitoneally or administered topically into the mouse mouth. When injected, 0.2 ml of 10-fold serial dilutions of the stock solution (0.8 mg–8 μg) were administered intraperitoneally at 24-h intervals for 3 consecutive days. Saline was injected to control mice. For topical administration, 200 mg of TA were placed in an Eppendorf tube and were dissolved in 0.5 ml of water. The tube containing the TA solution was weighed in an analytical balance. Mice were hand-held by pulling the fur from the back of the neck, as for an intraperitoneal injection. During the procedure, the mouth of the mouse remains open. A narrow paintbrush was soaked into the TA solution and used for painting inside of the mouse mouth. The procedure was carried out three times at 30-s intervals (an application). Finally, the Eppendorf tube was weighed again in order to calculate the total volume used per application. In these experiments, control animals were subjected to sham treatments in which tannic acid solution was replaced by tap water.

Isoproterenol Administration

As a positive control for the induction of IISP salivary proteins, 0.2 ml of a freshly prepared aqueous solution of isoproterenol (0.16 μmol/g body weight) was administered intraperitoneally once a day at 24-h intervals for 3 days [López Solís et al., 2003a]. Control unstimulated mice consisted of animals that were injected with saline.

Salivary Tissue

Control and experimental mice were organized in groups of four mice each. In those studies in which IISP were monitored in the salivary tissue, the animals were killed by cervical dislocation and both parotid glands from each animal were dissected free of fat and lymph nodes. After vigorous homogenization in 2.5 ml of 20 mM potassium phosphate/6.7 mM sodium chloride (pH 6.9, 20°C) using five strokes in a motor-driven Teflon-glass homogenizer, the homogenates were filtered through four layers of cheese-cloth laid at the bottom of a 5 ml disposable syringe and adjusted to 2 ml with the same buffer. An aliquot from each homogenate was saved for protein quantification and the rest was distributed in multiple aliquots and stored at −84°C until the electrophoretic fractionation [López Solís et al., 1993].

Collection of Whole Saliva

A single dose of 10 μl of 4% pilocarpine was instilled directly into the mouse mouth (time zero). Once salivation became visible (at about 5 min), aspiration of the salivary fluid was initiated by means of a disposable tuberculine syringe fitted with a bent disposable tip [López Solís et al., 2001]. Saliva from every single mouse was accumulated in preweighed Eppendorf tubes while kept on ice. At the end of the collection procedure (at about 20 min), the Eppendorf tubes were weighed in order to estimate the amount of collected saliva by assuming a specific gravity of 1.00 g/ml. Routinely, over 150 μl of saliva per mouse were obtained by this procedure. A 10-μl aliquot of saliva was saved for protein quantitation and the rest of the sample was distributed into several aliquots and saved at −84°C until the electrophoretic fractionation.

Protein Content

Ten microliters of aliquots of either saliva or whole salivary gland homogenates from every single mouse were spotted onto cellulose discs and processed for spectrophotometric protein quantification as described by Bramhall et al. [1969].

Protein Electrophoresis

Aliquots of whole saliva containing 30 μg of protein or aliquots of parotid gland homogenates containing 100 μg of protein were mixed

with sample buffer and subjected to electrophoresis in SDS–polyacrylamide slab gels (12%) as specified elsewhere [Laemmli, 1970; López Solís and Miranda Wilson, 1986]. Each sample subjected to electrophoresis was obtained from a single animal. Electrophoretic fractionations were calibrated by using the following molecular weight standards: ovotransferrin (78 kDa), bovine seroalbumin (66 kDa), ovoalbumin (42.5 kDa), and carbonic anhydrase (30 kDa). After the electrophoretic separation, gels were fixed overnight in 15% isopropanol/10% acetic acid, rinsed twice in the same solution for 30 min each, stained for at least 12 h in 0.25% Coomassie blue R-250 dissolved in 45% isopropanol/10% acetic acid and rinsed exhaustively in 10% isopropanol/10% acetic acid until clear background. Finally, gels were rinsed in three 5-min changes of distilled water and scanned in an AGFA-Snapscan 1236 device. Printed reports were produced by using both TIFF and JPG image file formats.

Materials

Tannic acid (T-0125), gallic acid (G-7384), (+/−)-isoproterenol-HCl (I-5627), protein molecular weight standards for gel electrophoresis and other reagent-grade chemicals were acquired from Sigma, St. Louis, MO. Pilocarpine was obtained from pharmaceutical suppliers as a 4% ophthalmic solution (Licarpin^{TM}, Saval Laboratories, Chile). Solvents used for gel processing were purchased from Merck-Chile. Cellulose discs (Whatman grade 1; 2.5 cm diameter) were obtained from Whatman, Maidstone, England.

RESULTS

Characterization of Tannic Acid (TA)

TA are commercially available extracts obtained from a number of plant species. In this study, an extract of *Quercus infectoria* galls was used [Sigma-Aldrich, 2005]. This extract was highly soluble in water at room temperature. The absorption spectrum of freshly prepared aqueous solutions of TA showed a single peak at 275 nm. Light absorption at this wavelength is directly proportional to the concentration of TA in the range from 1 to 5 μg/ml. Under those conditions, the percent solution extinction coefficient for a water solution of TA was 2,544/[(g/100 ml) · cm]. This value was used for the routine normalization of TA solutions. Reversed-phase HPLC fractionation of TA confirmed a complex composition. At least 50 peaks eluting from 3 to 85 min after the start of the HPLC run were identified by UV detection (280 nm), being the most prominent those whose average retention times were 4.9 (gallic acid), 50.7, 60.5, and 61.5 min (Fig. 1A). Spectral analysis (wavelength range 250–600 nm) of each major peak of the chromatogram showed that, despite some minor variations, all the absorption profiles exhibited a single prominent broad peak whose maximum occurs at about 275 nm, a feature that is distinctive of gallotannins, that is, those which yield gallic acid on hydrolysis (Fig. 1B). In addition, no absorption peak whatsoever suggesting the presence of either ellagitannins or condensed tannins was observed. Thus, the TA extract appears to consist entirely of a number of variant gallotannins which would be generated by the interaction between the carboxyl group of gallic acid molecules and one or several OH groups of both glucose and other gallic acid residues (Fig. 2) [Frohlich et al., 2002].

Lack of Effect of the Intraperitoneal Administration of TA on the Salivary Polypeptide Profile

Groups of mice were injected intraperitoneally either with TA or with isoproterenol (IPR), a powerful sialotrophic catecholamine. In this study, a dose of 1 mg IPR/mouse/day was routinely used for provoking the appearance of IISP polypeptides in saliva, when injected once in a day for three successive days. By contrast, a wide range of doses of TA were assayed for their ability to induce the appearance of the sialotrophic polypeptides. Under those conditions, even the highest nontoxic dose of TA (0.8 mg/mouse/day) proved to be fully ineffective in inducing IISP polypeptides in contrast to IPR that displayed a main inductive effect (Fig. 3).

Induction of IISP Salivary Polypeptides by Topical Oral Administrations of TA

Mouse mouths were paintbrushed with freshly prepared aqueous solutions of TA. The amount of TA supplied per application was 9.7 ± 1.4 mg ($n = 60$). Once-a-day applications of TA to the mouse mouths, according to the same administration schedule used for the intraperitoneal injection of TA and IPR resulted in no effect on the salivary polypeptide profile. In contrast, when TA was administered three

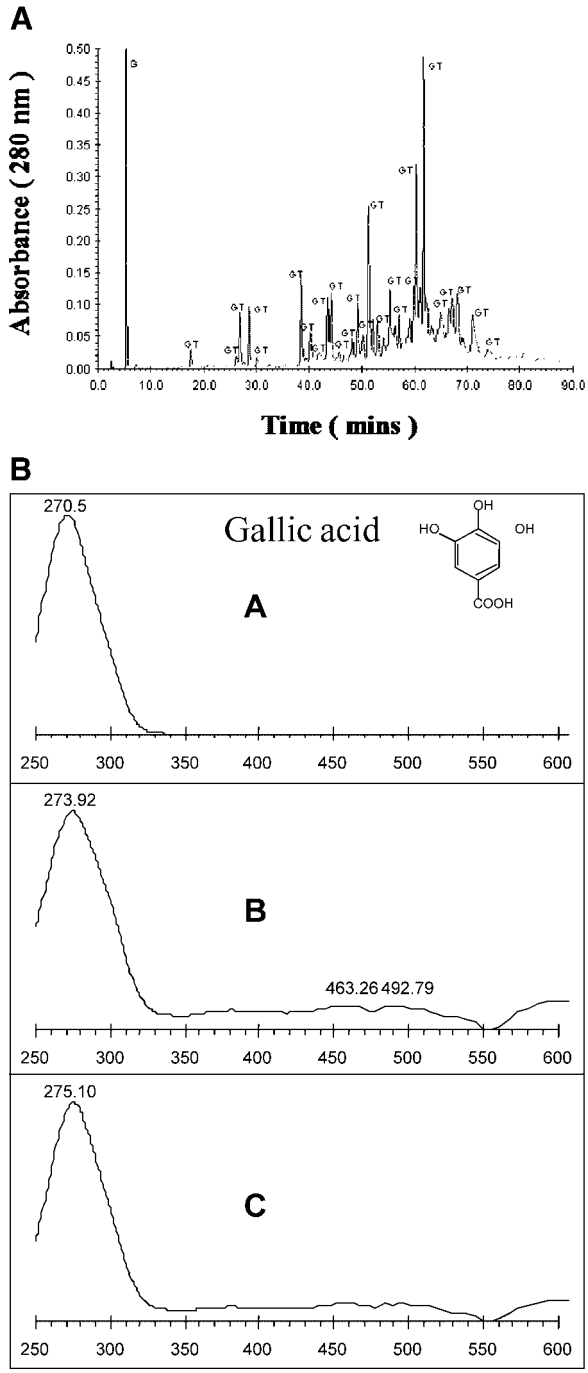

Fig. 1. HPLC chromatography of TA from *Quercus infectoria*. **A**: A methanol water solution of TA was fractionated through a Nova Pack C_{18} column according to the schedule and conditions described under Materials and Methods. Around 50 fractions were consistently identified by DAD-UV (280 nm) detection on the basis of their retention times ranging between 5 and 75 min. **B**: Each fraction displayed the characteristic absorption spectrum of gallotannins with a single peak whose maximum was between 270.5 and 275.1 nm. The panels show representative absorption spectra corresponding to three main HPLC fractions whose retention times (minutes) were 5 (gallic acid) (**panel A**), 51 (**panel B**), and 62 (**panel C**). Data are representative of three independent experiments.

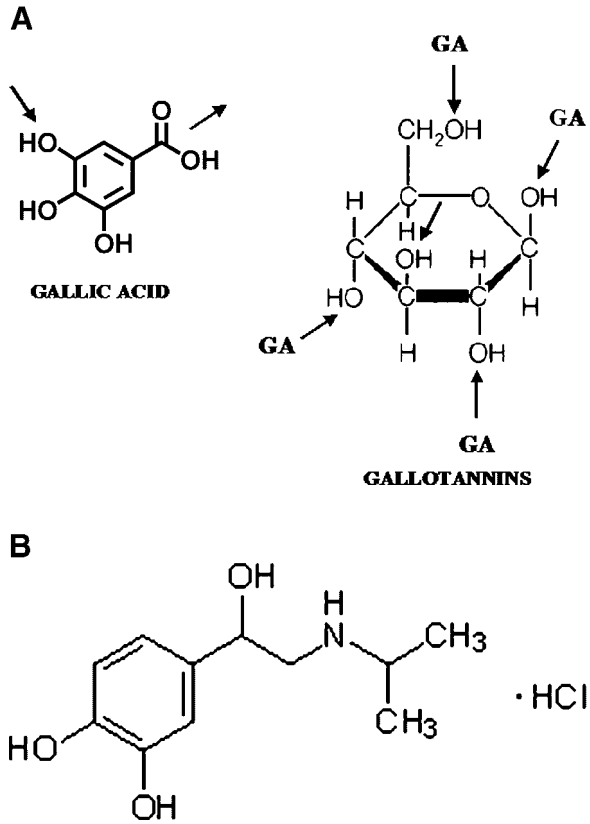

Fig. 2. Basic structure of gallotannins. **A**: A polyol core molecule, usually glucose, binds (arrows) via its OH groups to the carboxyl group of one or several gallic acid molecules (GA). In turn, the OH groups of GA may be also esterified by additional molecules of GA. TA used in this study comprises a mixture of a diversity of gallotannins. **B**: For comparison, the structure of isoproterenol, a powerful sialotrophic β-adrenergic catecholamine agonist, is shown.

times per day for 3 days, the salivary IISP polypeptides became readily visible in saliva (Fig. 4). Since the inductive effect of TA seemed to be positively influenced by the frequency of topical oral administrations, experiments were conducted in which TA was administered several times in a single day. In these experiments, animals were paintbrushed iteratively in their mouths at 1.5-h intervals for eight times (between 9 AM and 7.30 PM) in a first day and saliva was collected during the evening of the second day (7.30 PM). Under this schedule, most of the mice displayed a marked and unequivocal expression of IISP polypeptides in saliva. Interestingly, however, in these studies always a few exceptional animals exhibited no change at all in the salivary polypeptide profile (data not shown). Since in this schedule of stimulations

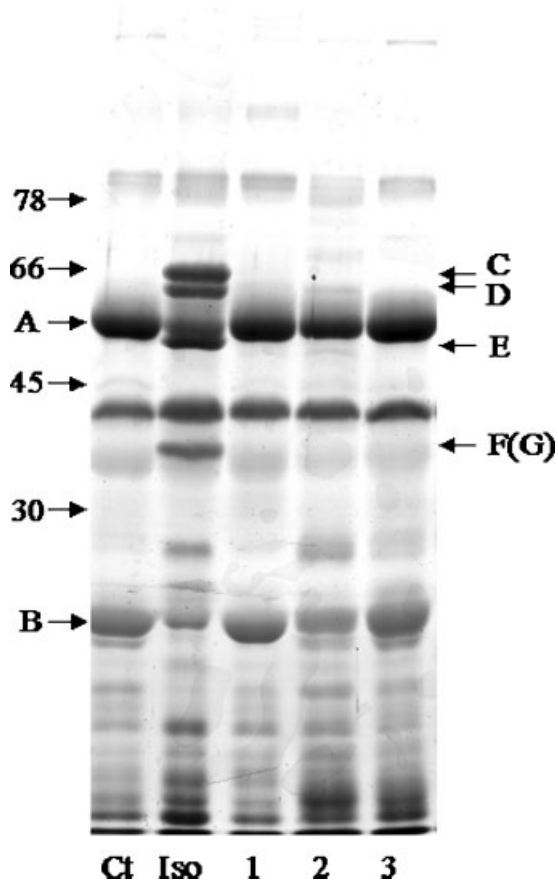

Fig. 3. Lack of effect of the intraperitoneal administration of TA on the polypeptide composition of mouse saliva. Mice were injected at 24-h intervals for 3 days either with TA (range of doses), isoproterenol (42 μg/g body weight) as positive controls or with saline as negative controls. Saliva from each experimental or control animal was taken at 24 h after the third injection. Aliquots of saliva (30 μg of protein) from individual mice were processed for gel electrophoresis. Note the marked presence of IISP polypeptides (labeled as C-G on the right of the gel) in the saliva of an isoproterenol-stimulated mouse (Iso) and their absence in the saliva of mice that received either intraperitoneal saline (Ct) or TA (doses in μg/g body weight/day: **lane 1**, 0.3; **lane 2**, 3.3; and **lane 3**, 33.3). On the left of the gel, A and B indicate normal secretory polypeptides and the numbers indicate standard molecular weights: ovotransferrine (78 kDa), bovine serum albumin (66 kDa), ovoalbumin (45 kDa), and carbonic anhydrase (30 kDa).

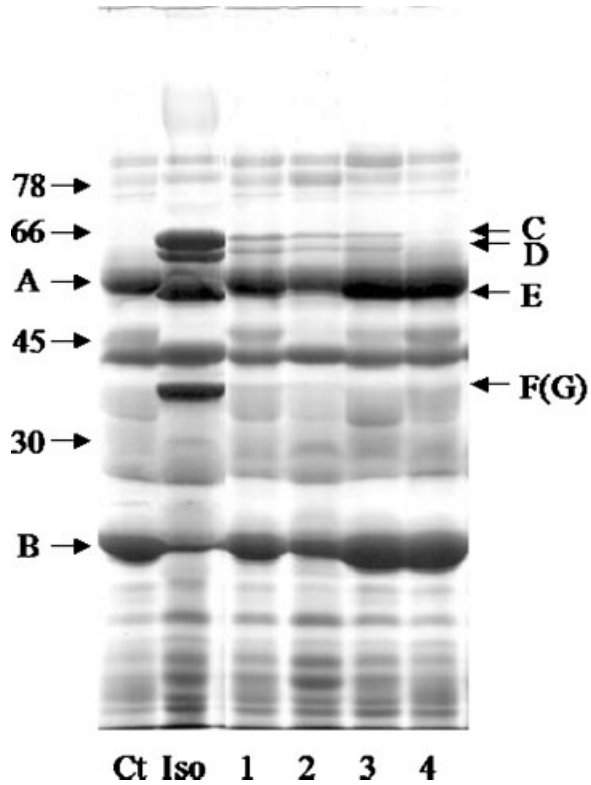

Fig. 4. Inductive effect of sparsely repeated topical oral administrations of TA on the polypeptide composition of mouse saliva. Groups of mice received three daily oral administrations of TA separated by 4-h intervals for 3 days (between 9 AM and 5 PM). Saliva from individual mice was separately collected at 24 h after the last administration of TA and processed for electrophoresis. The representative gel shows polypeptide bands C-G in three out of four TA-treated mice (**lanes 1–4**) although their intensities are clearly weaker than in saliva of an isoproterenol-injected mouse used as positive control (Iso). Ct stands for saliva of a saline-injected mouse used as negative control. Standard molecular weights and normally secretory polypeptides A and B are indicated on the left of the gel.

only 24 h elapsed between the end of the TA treatment and the collection of saliva, experiments were conducted in which saliva was collected at a later time (36 h) after the end of the TA treatment. It was expected that a longer period of rest after successive TA stimulations might contribute to a higher accumulation of the IISP polypeptides in the salivary glands. This was clearly observed among the positive control mice subjected to isoproterenol stimulations and somewhat less evidently among the TA-treated mice (Fig. 5). However, under these experimental schedules practically the whole group of TA-treated mice expressed the IISP polypeptides in saliva although some occasional unresponsive mice were still observed.

Given that the frequency of stimulations by topically administered TA into the mouse mouth appeared to be critical for the induction of IISP polypeptides and that some occasional mice exhibited a complete absence of those salivary polypeptides, a consolidated stimulation schedule was assayed. Accordingly, mice received eight topical oral applications of TA at 1.5-h intervals (between 9 AM and 7.30 PM)

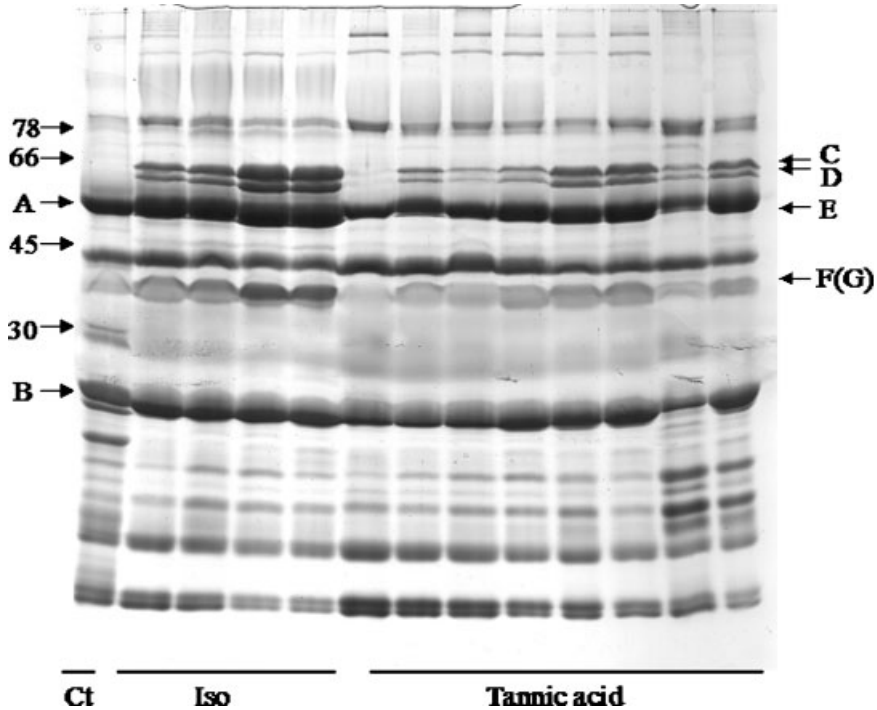

Fig. 5. Inductive effect of clustered oral administrations of TA on the polypeptide composition of mouse saliva. Groups of mice received eight oral administrations of TA separated by 1.5-h intervals in a single day (between 9 AM and 7.30 PM). Saliva from individual mice was collected either at 24 or 36 h after the last administration of TA and processed for electrophoresis. The representative gel shows the unequivocal presence of polypeptides C-G in saliva from eight TA-treated mice. The last four lanes on the right of the gel correspond to salivas collected at 36 h after the end of the TA treatment while the previous four lanes show the fractionation of salivas from TA-treated mice whose collection was performed at 24 h post-TA. For comparison, salivas from four mice injected for 3 days with isoproterenol (Iso, positive control) and collected either at 24 h (**second** and **third lanes**) or at 36 h (**fourth** and **fifth lanes**) after the third isoproterenol stimulation, as well as saliva from a saline-injected mouse (Ct, negative control), are included in the fractionation. Note the higher intensities in the polypeptide bands C-G of salivas collected at 36 h in respect to salivas collected at 24 h after the end of the stimulations by either TA or isoproterenol.

during 3 successive days and saliva was collected at 36 h after the end of the TA treatment. This time, the whole population of TA-treated mice expressed IISP polypeptides in saliva (Fig. 6).

In isoproterenol-treated mice, IISP polypeptides are synthesized and secreted by hypertrophic parotid glands [López Solís et al., 1987, 1993]. Accordingly, parotid tissue from TA-treated mice was examined for the presence of IISP polypeptides. To do so, groups of mice were paintbrushed in their mouths at 1.5-h intervals during four successive days and their parotid salivary glands were dissected at 36 h after the end of the TA-treatment, homogenized and fractionated electrophoretically. With no exception, the parotid tissue expressed IISP polypeptides whose mobilities coincided with the ones of IISP polypeptides observed in the saliva (Fig. 7).

DISCUSSION

We report here that in mouse, tannic acid is an effective IISP-inducing agent. The effect is observed when those tannins are applied topically into the oral cavity but not when administered intraperitoneally. Since the IISP salivary polypeptides are overexpressed by hypertrophic parotid acinar cells and thus are considered as markers for mouse parotid hypertrophic growth [López Solís et al., 1987, 1993, 2003a], tannic acid may be included now in a list of noncatecholamine sialotrophic agents [López Solís et al., 1990]. So far, evidence based on the induction of sialotrophic effects by providing experimental animals directly with tannin extracts rather than feeding high-tannin diets were necessary. Thus, this study lends strong support to previous observations from several laboratories that pointed to dietary tannins as

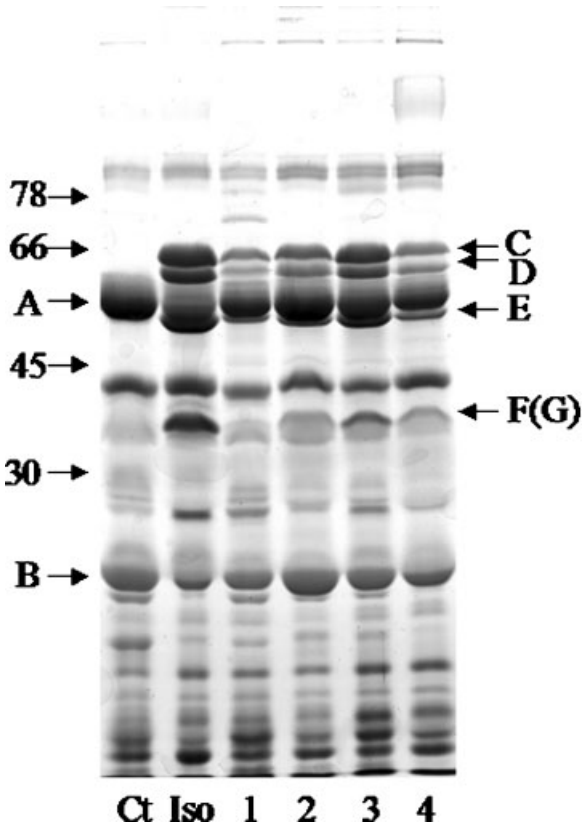

Fig. 6. Inductive effect of oral administrations of TA on the polypeptide composition of mouse saliva. Consolidated stimulation schedule. Groups of mice received eight oral administrations of TA separated by 1.5-h intervals (between 9 AM and 7.30 PM) for 3 consecutive days. Saliva from individual mice was collected at 36 h after the last administration of TA and processed for electrophoresis. The representative gel shows a marked induction of polypeptide bands C-G in all the TA-treated mice (**lanes 1–4**). For comparison, saliva from a mouse injected for 3 days with isoproterenol (Iso, positive control) and collected at 36 h after the third isoproterenol stimulation and saliva from a saline-injected mouse (Ct, negative control), are included in the fractionation. Standard molecular weights and normally secretory polypeptides A and B are indicated on the left of the gel.

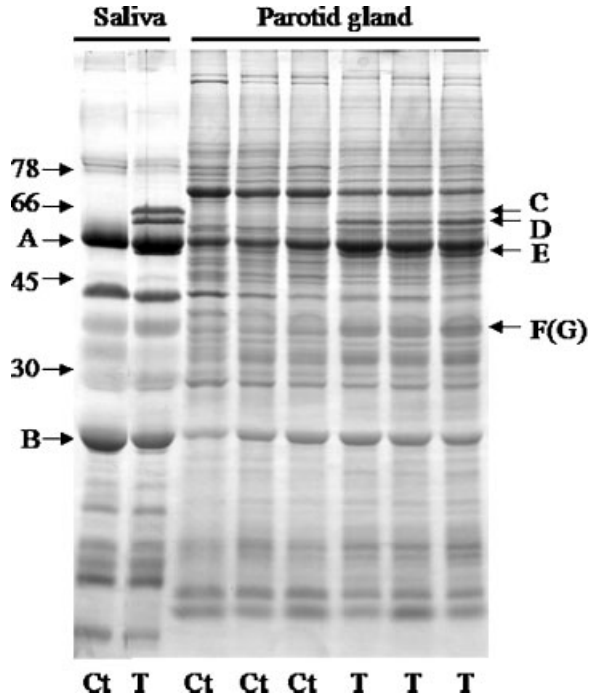

Fig. 7. Inductive effect of oral administrations of TA on the polypeptide composition of mouse parotid glands. Groups of mice received eight oral administrations of TA separated by 1.5-h intervals (between 9AM and 7.30 PM) for four consecutive days and parotid glands were dissected at 36 h after the last administration of TA and processed for gel electrophoresis. The representative gel shows the fractionation of parotid homogenates (50 μg protein/lane) prepared from three unstimulated control mice (Ct) and three TA-treated mice (T). For comparison, samples of saliva collected from a control mouse and from a TA-treated mouse are included (first two lanes). Note that the induction of polypeptides C-G can be appreciated in the glands of TA-treated mice despite the high-polypeptide background of the glands in respect to the low background observed in saliva.

the sialotrophic agents when rodent parotid glands become greatly enlarged after feeding cereal or legume variants high in tannins [Mehansho et al., 1983, 1985; Jansman et al., 1994].

Tannins include a widely diverse group of water-soluble protein precipitant polyphenols which have been grossly classified into two main categories: proanthocyanidins (or nonhydrolyzable condensed tannins) that are structured by variable numbers of CC bound units of flavan-3-ols, and hydrolysable tannins, which are complex esters whose basic units are glucose and either ellagic acid (ellagitannins) or gallic acid (gallotannins) [Haslam, 1989; Riedl et al., 1998]. In all the previous studies linking the sialotrophic response to experimental diets, only cereals or legumes containing mostly nonhydrolyzable condensed tannins were used [Mehansho et al., 1983, 1985, 1987; Humphreys-Beher et al., 1987b; Jansman et al., 1994]. In contrast, in our study the sialotrophic effect has been produced by tannic acid from *Quercus infectoria*, a complex extract in which only gallotannins were identified. Altogether, these observations suggest that the sialotrophic effect is more probably related to common functional properties of tannins, such as protein complexing and precipitation, rather than to a molecular mechanism triggered through a specific ligand (tannin)-receptor interaction.

Another main characteristic of the present study is that the expression of IISP polypeptides was assessed by analyzing saliva rather than salivary glands [Muenzer et al., 1979a; Mehansho et al., 1983, 1985, 1987; López Solís et al., 1993]. Under this approach, early expression (within 2–3 days) of a specific group of IISP polypeptides was readily defined on the basis that the polypeptide background of murine saliva is certainly much lower than the polypeptide background observed in the electrophoretic fractionation of salivary gland homogenates [López Solís et al., 2003b; López Solís and Kemmerling, 2005].

Among several stimulation schemes that were assayed in this study, those consisting in recurrent and very frequent topical oral applications of TA proved to be critical or more effective in inducing the sialotrophic response. That experimental condition resembles other effective sialotrophic schemes based on feeding trials using high-tannin diets provided *ad libitum* for several days or weeks but fully differs from those based on once-a-day or twice-a-day administrations of isoproterenol in which this β-adrenoceptor agonist becomes metabolized and eliminated from the organism within a couple of hours [Baserga, 1970; Humphreys-Beher et al., 1987a; Ann et al., 1987; Li et al., 1997]. Thus, major temporal differences are expected to occur at least at some stages in the signaling mechanisms activated by both sialotrophic agents.

A common observation in the present study was that regardless the effective topical stimulation scheme, the intensities of the TA-induced electrophoretic bands were significantly lower than those observed after daily intraperitoneal administrations of isoproterenol. However, no qualitative differences whatsoever between the IISPs induced by both types of stimuli could be appreciated. A number of previous studies regarding the effect of high-tannin diets had observed that similarity long ago [Mehansho et al., 1983]. Thus, at least some common sialotrophic mechanisms, more probably those related to salivary-specific gene expression, should be finally activated in response to both types of agonists [Lin et al., 1996; Ann et al., 1997; Zhou et al., 1997]. Despite the structural analogy between the polyphenol domains of tannins and the catechol domain of isoproterenol, that might tempt to invoke common receptor mechanisms for both sialotrophic agents on the target tissues, previous reports in reference to the relative sialotrophic power of a variety of structural analogs of isoproterenol have shown that the catechol domain is a necessary but not sufficient feature for the agonists to express a sialotrophic effect [López Solís et al., 1990]. In the present study we observed that intraperitoneal administrations of single doses of TA as low as 4 mg/mouse resulted in strong toxic effects leading frequently to a rapid death of the animal (data not shown). In contrast, we also observed that topical applications into the mouth at doses as high as 80 mg/mouse given during a time span of 8 h and repeated for 3 or 4 consecutive days were outstandingly well-tolerated with no evident toxic signs at all within the 14 days following the treatment. These observations highly suggest that gastrointestinal absorption of gallotannins in mouse would be at most a minor event. In the same direction, the oral administration of TA to rats has revealed that gallic acid, a major hydrolysis product of TA, can be detected in urine [Koss and Koransky, 1982; Zong et al., 1999]. Toxicological assays in mice have shown the full absence of adverse effects following the oral administration of acute doses of gallic acid as high as 5 mg/g body weight and subacute daily doses of 1 mg/g body weight given for a 28-day period [Rajalakshmi et al., 2001]. Contrarily, a few reports have claimed the occurrence of necrotic processes in the liver and kidney of ruminants after consumption of TA probably provoked by absorbable low-molecular weight metabolites derived from the microbial metabolism in the rumen [Austin et al., 1989; Hagerman and Robbins, 1993; Zhu and Filippich, 1995]. Thus, although any eventual gastrointestinal absorption of TA in mouse is yet to be characterized and despite the similarity in the sialotrophic response to that of isoproterenol, the sialotrophic mechanism activated by iterative topical oral administrations of TA may well differ drastically from that associated to the pharmacological agonist. While the sialotrophic response to intraperitoneal administrations of IPR would be mediated by the interaction of the agonist with adrenoceptors of the β1 subtype, that are presumably located on the basolateral surface of the target acinar cells and facing the inner milieu [González et al., 1994, 2000], most if not all the components of the TA extract would become part of oral protein–tannin complexes which

may remain unabsorbed while moving along the gastrointestinal tract as it has been observed in regard to condensed tannins [Hagerman and Butler, 1981]. Some families of salivary proteins, such as the proline-rich proteins and the histatins, are currently considered as primary candidates for being part of those complexes [Bennick, 2002]. High-affinity interactions of TA with salivary proteins would alter the protective salivary film covering the mouse mouth [Haslam, 1974; Charlton et al., 1996; Baxter et al., 1997]. That highly disturbing effect at a supramolecular level would be perceived as a tactile sensation via trigeminal nerve endings and would lead to a reflex stimulation of the salivary tissue to secrete proteins and restore the altered salivary film [Lawless, 1996]. Such a protective and adaptive response against tannins, probably mediated by physiological catecholamine neurotransmitters, would involve not only the synthesis of more proteins but the synthesis of qualitatively different sets of salivary proteins displaying a higher affinity for tannins [Hagerman and Butler, 1981; Mehansho et al., 1983; Katsukawa et al., 1999; Bennick, 2002]. That seems to be the case of the murine polypeptides IISP which are part of the major family of proline-rich proteins whose high affinity for tannins as well as their transcriptionally regulated inducibility by high-tannin diets are two of their well-known features [Hagerman and Butler, 1981; Carlson et al., 1991; Tu et al., 1993; Ann et al., 1997; Bennick, 2002]. Were it so, prolonged contact of the mouse oral mucose with high-tannin diets would result in parotid hypertrophy, as previously reported by other laboratories [Mehansho et al., 1983, 1985; Jansman et al., 1994]. In such a case, hypertrophy would be a necessary condition for parotid glands to produce new secretory macromolecules in order to neutralize the astringent effects of tannins. In agreement with that view, it has been reported that wild-herbivorous mammals feeding naturally on tannin-containing diets have become adapted by expressing in a constitutive manner high levels of proline-rich proteins in saliva [Austin et al., 1989; Hagerman and Robbins, 1993]. However, it remains to be seen whether in those species the proline-rich proteins are induced by their tannin-rich diets during the normal development of the salivary system or whether a constitutive expression of the PRP proteins allow them to feed on those diets. In humans, PRP proteins are expressed constitutively and so they serve as natural filters for the high content of tannins present in our diet [Kim et al., 1993].

Although in this study we demonstrated that gallotannins are inducers of a sialotrophic-adaptive response in mouse, it remains yet to be identified which out the many members of that subfamily of polyphenols that are present in the commercial extract of tannic acid, or all of them, have that sialotrophic potential. In humans, the intensity of the sensorial perception of astringency, which is characteristically produced by tannins present in a variety of foods, has been associated differentially to the size and degree of polymerization of those complex organic molecules [Noble, 1998; Drewnowski and Gómez-Carneros, 2000]. In the mouse model, induction of IISP salivary polypeptides by topical oral applications of tannins may well become a more objective procedure to assess the astringency of pure or complex tannin solutions.

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REFERENCES

Ann DK, Clements S, Johnstone EM, Carlson DM. 1987. Induction of tissue-specific proline-rich protein multigene families in rat and mouse parotid glands by isoproterenol. Unusual strain differences of proline-rich protein mRNAs. J Biol Chem 262:899–904.

Ann DK, Lin HH, Kousvelari E. 1997. Regulation of salivary gland specific gene expression. Crit Rev Oral Biol Med 8(3):244–252.

Austin PJ, Suchar LA, Robbins CT, Hagerman AE. 1989. Tannin-binding proteins in saliva of deer and their absence in saliva of sheep and cattle. J Chem Ecol 15: 1335–1342.

Baserga R. 1970. Induction of DNA synthesis by a purified chemical compound. Fed Proc 29:1443–1446.

Baxter NJ, Lilley TH, Haslam E, Williamson MP. 1997. Multiple interactions between polyphenols and a salivary proline-rich protein result in complexation and precipitation. Biochemistry 36:5566–5577.

Bennick A. 2002. Interaction of plant polyphenols with salivary proteins. Crit Rev Oral Biol Med 13:184–196.

Bramhall S, Noack N, Wu M, Loewenberg JR. 1969. A simple colorimetric method for determination of protein. Anal Biochem 31:146–148.

Carlson DM, Zhou J, Wright PS. 1991. Molecular structure and transcriptional regulation of the salivary gland proline-rich protein multigene families. (Review). Prog Nucl Acid Res Mol Biol 41:1–22.

Charlton AJ, Baxter NJ, Lilley TH, Haslam E, McDonald CJ, Williamson MP. 1996. Tannin interactions with a full-length human salivary proline-rich protein display a stronger affinity than with single proline-rich repeats. FEBS Lett 18:289–292.

Drewnowski A, Gómez-Carneros C. 2000. Bitter taste, phytonutrients and the consumer: A review. Am J Clin Nutr 72:1424–1435.

Frohlich B, Niemetz R, Gross GG. 2002. Gallotannin biosynthesis: Two new galloyltransferases from Rhus typhina leaves preferentially acylating hexa- and hepta-galloylglucoses. Planta 216:168–172.

González MJ, López Solís RO, Brañes C, Alliende C. 1994. Plasma membrane desialylation and DNA synthesis are induced via adrenergic receptors of the β1 subtype in mouse parotid glands. Biochim Biophys Acta 1222:208–214.

González MJ, Peña y Lillo S, Alliende C, López Solís RO. 2000. Cell-enlargement related polypeptides are induced via β1-adrenoceptors in mouse parotids. Exptl Mol Pathol 69:91–101.

Hagerman AE, Butler LG. 1981. The specificity of proanthocyanidin-protein interactions. J Biol Chem 256:4494–4497.

Hagerman AE, Robbins CT. 1993. Specificity of tannin-binding salivary proteins relative to diet selection by mammals. Canadian J Zool 71:628–633.

Haslam E. 1974. Polyphenol-protein interactions. Biochem J 139:285–288.

Haslam E. 1989. Plant polyphenols: Vegetable tannins revisited. Cambridge, UK: Cambridge University Press. pp 1–13.

Humphreys-Beher MG, Schneyer CA, Kidd VJ, Marchase RB. 1987a. Isoproterenol-mediated parotid gland hypertrophy is inhibited by effectors of 4β-galactosyltransferase. J Biol Chem 262:11706–11713.

Humphreys-Beher MG, Robinson GD, Al-Zahid S, Schneyer CA. 1987b. Neural and dietary modulation of proline-rich protein and 4β-galactosyltransferase biosynthesis in rat parotid glands. Archs Oral Biol 32: 573–578.

Jansman AJ, Frohlich AA, Marquardt RR. 1994. Production of proline-rich proteins by the parotid glands of rats is enhanced by feeding diets containing tannins from faba beans (*Vicia faba L*). J Nutr 124:249–258.

Katsukawa H, Imoto T, Ninomiya Y. 1999. Induction of salivary gurmarin-binding proteins in rats fed gymnema-containing diets. Chem Senses 24:387–392.

Kim HS, Lyons KM, Saitoh E, Azen EA, Smithies O, Maeda N. 1993. The structure and evolution of the human salivary proline-rich protein gene family. Mamm Genome 4:3–14.

Koss G, Koransky W. 1982. Enteral absorption and biotransformation of food additive octylgallate in the rat. Food Chem Toxicol 20:591–594.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Lawless HT. 1996. Flavor. In: Friedman MP, Carterette EC, editors. Cognitive ecology. Chapter 8. San Diego, CA: Academic Press. pp 325–380.

Li D, Lin HH, McMahon M, Ma H, Ann DK. 1997 Oncogenic Raf-1 induces the expression of non-histone chromosomal architectural protein HMGI-C via a p44/p42 mitogen-activated protein kinase-dependent pathway in salivary epithelial cells. J Biol Chem 272:25062–25070.

Lin HH, Tu ZJ, Ann DK. 1996. Involvement of nuclear orphan receptor NGFI-B in transcription activation of salivary-specific R-15 gene by cAMP. J Biol Chem 271: 27637–27644.

López Solís RO, Kemmerling U. 2005. Codominant expression of genes coding for different sets of inducible salivary polypeptides associated with parotid hypertrophy in two inbred mouse strains. J Cell Biochem 95:99–107.

López Solís RO, Miranda Wilson DM. 1986. Changes in the polypeptide composition of mouse parotid glands after stimulation of secretion and DNA synthesis by isoproterenol. Biochim Biophys Acta 881:422–429.

López Solís RO, Alliende C, González J, Miranda D. 1987. Changes in the polypeptide composition related to the growth response in chronically-isoproterenol-stimulated mouse parotid glands. Biochim Biophys Acta 930:463–469.

López Solís RO, Miranda D, Alliende C, González J, Díaz N, Díaz H, Ruiz F. 1989. Secretory character of a group of isoproterenol-induced polypeptides in mouse parotid glands. J Cell Physiol 141:660–666.

López Solís RO, Miranda Wilson D, González Burgos J. 1990. Effect of isoproterenol analogs on the polypeptide composition of mouse parotid glands: Relationship to enhanced growth. Biochim Biophys Acta 1054:311–316.

López Solís RO, González MJ, Castillo L, Peña y Lillo S, Alliende C. 1993. Transcriptional and post-transcriptional control in the synthesis of growth-marker polypeptides in mouse parotids. Am J Physiol 265:G514–G520.

López Solís RO, Puente M, Wenk C, Morales I, Durán V. 2001. Characterization of mouse salivary polypeptide secretion after oral administration of pilocarpine. Rev Chil Hist Nat 74:193–203.

López Solís RO, Puente Díaz M, Morales Bozo I, Kemmerling Weis U, Díaz F. 2003a. Early detection in saliva of polypeptides associated to isoproterenol-induced mouse parotid hypertrophy. Biochim Biophys Acta 1621:41–47.

López Solís RO, Kemmerling U, Ramos A, Hoecker G. 2003b. Differential expression of isoproterenol-induced salivary polypeptides in two mouse strains that are congenic for the H-2 histocompatibility gene complex. J Cell Biochem 90:945–956.

Mehansho H, Hagerman A, Clements S, Butler L, Rogler J, Carlson DM. 1983. Modulation of proline-rich protein biosynthesis in rat parotid glands by sorghums with high tannin levels. Proc Nat Acad Sci USA 80:3948–3952.

Mehansho H, Clements S, Sheares BT, Smith S, Carlson DM. 1985. Induction of proline-rich glycoprotein synthesis in mouse salivary glands by isoproterenol and by tannins. J Biol Chem 260:4418–4423.

Mehansho H, Ann DK, Butler LG, Rogler J, Carlson DM. 1987. Induction of proline-rich proteins in hamster salivary glands by isoproterenol treatment and an unusual growth inhibition by tannins. J Biol Chem 262: 12344–12350.

Muenzer J, Bildstein C, Gleason M, Carlson DM. 1979a. Purification of proline-rich proteins from parotid glands of isoproterenol-treated rats. J Biol Chem 254:5623–5628.

Muenzer J, Bildstein C, Gleason M, Carlson DM. 1979b. Properties of proline-rich proteins from parotid glands of isoproterenol-treated rats. J Biol Chem 254:5629–5634.

Noble AC. 1998. Why do wines taste bitter and feel astringent? In: Waterhouse AL, Ebeler S, editors. Chemistry of wine flavor. Washington DC: American Chem. Society (ACS Symposium series 714) pp 156–165.

Novi AM, Baserga R. 1971. Association of hypertrophy and DNA synthesis in mouse salivary glands after chronic administration of isoproterenol. Am J Pathol 62:295–308.

Peña-Neira A, Hernández T, García-Vallejo C, Estrella J, Suárez JA. 2000. A survey of phenolic compounds in Spanish wines of different geographical origin. Eur Food Res Technol 210:445–448.

Rajalakshmi K, Devaraj H, Niranjali-Devaraj S. 2001. Assessment of the no-observed-adverse-effect level (NOAEL) of gallic acid in mice. Food Chem Toxicol 39:919–922.

Riedl KM, Jones GA, Sovik KN, Ritchard NT, Hartzfeld PW, Riechel TL. 1998. High molecular eight plant polyphenolics (Tannins) as biological antioxidants. J Agric Food Chem 46:1887–1892.

Schneyer CA. 1962. Salivary gland changes after isoproterenol-induced enlargement. Am J. Physiol 203:232–236.

Sigma-Aldrich Co. 2005. Tannic acid. Product Information: T0125.

Tu ZJ, Lazowsky KW, Ehlenfeldt RG, Wu G, Lin HH, Kousvelari E, Ann DK. 1993. Isoproterenol/tannin-dependent R15 expression in transgenic mice is mediated by an upstream parotid control region. Gene Expr 3:289–305.

Zhou J, Wright PS, Wong E, Jessen K, Morand JN, Carlson DM. 1997. Cyclic AMP regulation of mouse proline-rich protein gene expression: Isoproterenol induction of AP-1 transcription factors in parotid glands. Arch Biochem Biophys 338:97–103.

Zhu J, Filippich LJ. 1995. Acute intra-abomasal toxicity of tannic acid in sheep. Vet Hum Toxicol 37:50–54.

Zong L, Inoue M, Nose M, Kojima K, Sakaguchi N, Isuzugawa K, Takeda T, Ogihara Y. 1999. Metabolic fate of gallic acid orally administered to rats. Biol Pharmaceutical Bull 22:326–329.