Morphological Changes in Salivary Glands Induced by Theophylline in Rats

Satoru Kajikawa1, Ayano Takeuchi1, Aisuke Nii1, Hiroyuki Nakayama2, and Kunio Doi2

1Safety Research Laboratories, Yamanouchi Pharmaceutical Co., Ltd., 1–8, Azusawa 1-chome, Itabashi-ku, Tokyo 174–8511, Japan
2Department of Veterinary Pathology, Graduate School of Agricultural and Life Science, The University of Tokyo, 1–1, Yayoi 1-chome, Bunkyo-ku, Tokyo 113–8657, Japan

Abstract: Theophylline, a phosphodiesterase inhibitor, is known to induce enlargement of the salivary glands. This enlargement has been thought to be associated with enhanced cellular levels of cyclic AMP as a result of inhibition of phosphodiesterase. In the present study, to clarify the sequential changes in salivary glands following the administration of theophylline, a four-day repeated dose study and a single dose study were carried out in male rats, and the parotid and submaxillary glands were examined histologically. The results from the glands were almost identical. In the repeated dose study, the increased organ weight just before the last administration was reduced at 4 hr and then restored at 8 hr after the last administration. In the single dose study, a slight but significant increase in organ weight was observed at 24 hr after administration, following a transient reduction. Changes in acinus histology were consistent with these organ weight changes. Acinar cells were loaded with excessive secretory granules just before the last administration in the repeated dose study. After the last administration, granules were reduced at 4 hr, and then reaccumulated at 8 hr. Such time-course variability in the number of secretory granules corresponded well to clinically observed salivation. These results suggest that inhibition of phosphodiesterase is primarily involved in the discharge of secretory granules, and that the salivary hypertrophy previously reported is an adaptive response caused by the repeated stimulation of saliva secretion. (J Toxicol Pathol 2003; 16: 215–221)

Key words: parotid gland, submaxillary gland, hypertrophy, salivation, adaptation

Introduction

Theophylline (1,3-dimethylxanthine), a phosphodiesterase inhibitor, is a methylated xanthine closely resembling caffeine. The most prominent use of theophylline is in the treatment of a variety of respiratory disorders, such as bronchial asthma and emphysema. It is also used as a cardiac stimulant for certain acute cardiovascular conditions, as a diuretic, and as a smooth muscle relaxant. These pharmacological effects are thought to be associated with the increased levels of cellular cyclic AMP as a result of inhibition of phosphodiesterase.

In the field of toxicology, it is known that repeated administration of theophylline produces salivary gland hypertrophy1–3. Recently it was revealed that other phosphodiesterase inhibitors, namely ICI 153,110 and rolipram, also induce salivary gland hypertrophy4,5.

Muir et al.6 showed that theophylline increases intracellular levels of cyclic AMP in salivary glands. This elevation of cyclic AMP level, however, was a transient phenomenon. On this basis it has been postulated that cyclic AMP does not directly control acinar cell hypertrophy in the salivary glands but may be a ‘trigger’ for a series of events leading to acinar cell hypertrophy.

It is also known that clinical signs such as salivation are produced when theophylline is administered to rats. In the present study, to clarify the process of theophylline-induced acinar cell hypertrophy of salivary glands, we sequentially examined the histology of the parotid and submaxillary glands in relation to salivation after single and repeated administration to male Fischer rats.

Materials and Methods

Animals

Eighty male Fischer rats (F344/DuCrj) were purchased from Charles River Japan (Kanagawa, Japan) and acclimated until used. They were housed in stainless steel cages in an
animal room under controlled conditions (23 ± 3°C with a relative humidity of 55 ± 5%, ventilation of 10 times/hr and a 13-hr light/11-hr dark cycle). At the start of dosing, the animals were 9–10 weeks old and weighed 185–220 g. They were given a commercially available diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum. The experiments were conducted in accordance with in-house guidelines for laboratory animal experimentation.

**Dosing procedures**

Theophylline (Wako Pure Chemical Industries, Osaka, Japan) was dissolved at 5 mg/mL in physiological saline. A total of 10 mL/kg was given intraperitoneally using a 26 gauge needle. As vehicle controls, physiological saline was given in the same fashion.

**Experimental designs**

Forty rats each were assigned to the single dose and repeated dose studies. In the single dose study, 20 rats each were given vehicle or theophylline at 50 mg/kg, and 5 rats of each group were sacrificed under ether anesthesia by exsanguination at 1, 4, 8, and 24 hrs after administration, respectively. In the repeated dose study, 20 rats each were given vehicle or theophylline at the same dose twice a day (at 9 a.m. and 5 p.m.) for four days. Necropsy was carried out on 5 rats of each group just before the last administration, and 1, 4, and 8 hrs after the last administration under the same procedures, respectively. All animals were weighed on the first day of administration and the day of necropsy. At necropsy, the major salivary glands, the parotid gland and submaxillary gland with the sublingual gland, were excised.

In addition, 6 rats were used for electron microscopic examination. Namely, 3 rats each were given vehicle or theophylline twice a day for four days in the same manner, sacrificed at 1 hr after the last dosing, and then the submaxillary glands were excised.

**Histopathology**

The salivary glands were fixed in 10% phosphate-buffered formalin. Organ weight of both sides was measured following removal of adjacent fat and connective tissues and relative organ-to-body weight (g%) was calculated. Four-micrometer paraffin sections were stained with hematoxylin and eosin (H&E) for histopathological examination. In addition, to examine the histological changes in myoepithelium, immunohistochemistry for α-smooth muscle actin (SMA) was performed on paraffin sections obtained from rats of the repeated dose study by the labeled streptavidin biotin technique (Streptavidin peroxidase; 1:2500; Zymed laboratories, San Francisco, CA) using anti α-SMA antibody (1:100; DAKO Cytomation, Glostrup, Denmark) as the primary antibody and biotin-labeled anti-mouse Ig’s antibody (1:100; Biosource International, Camarillo, CA) as the secondary antibody.

**Electron microscopy**

Small pieces of the submaxillary gland were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde, postfixed in 1% osmiumtetroxide, and embedded in Epon 812. Ultrathin sections were double-stained with uranyl acetate and lead citrate, and examined under an electron microscope (JEM-1210, JEOL, Tokyo, Japan).

**Statistics**

Differences in the relative organ weights of the parotid and submaxillary glands between the control and theophylline-treated groups in the single dose study were analyzed by Student’s t-test. In the repeated dose study, differences in relative organ weight between just before the last administration and at each time point were analyzed by Dunnett’s test.

**Results**

**Clinical signs and body weight**

Theophylline-related clinical signs such as preening, grooming, and excessive saliva in the oral cavity were observed following each administration. These signs started within several minutes and lasted for about 4 hr after administration. Especially, these signs were frequently observed from 30 min to 2 hr, but were no longer observed at the next administration. In the repeated dose study, body weight gain in the theophylline-treated group was slightly depressed (data not shown).

**Organ weight**

In the single dose study, as compared with the control group, the relative weight of the parotid gland in the theophylline-treated group was slightly but significantly lower at 4 hr after administration, but became significantly higher at 24 hr (Fig. 1A). In the repeated dose study, it was 1.3-fold higher just before the last administration, gradually decreased to the control level at 4 hr, and then increased again at 8 hr after the last administration (Fig. 2A).

The time-course changes in the relative weight of the submaxillary gland were almost identical to those in the parotid gland in both the single dose (Fig. 1B) and repeated dose study (Fig. 2B).

**Histopathology**

In the single dose study, the time-course changes in the histology of both salivary glands were similar to those in the repeated dose study (data not shown).

The sequential changes in histology of the parotid and submaxillary glands in the repeated dose study are shown in Figs. 3 and 4. In the parotid gland of the theophylline-treated group, acinar cells were enlarged with numerous zymogen granules just before the last administration (Fig. 3A). At 1 hr after the last administration, the number of zymogen granules reduced slightly, and at 4 hr the size of cells was considerably decreased due to a prominent decrease in the number of zymogen granules (Fig. 3B). At 8 hr, zymogen granules drastically reaccumulated and the morphology of the acinus was restored to that just before the last
administration (Fig. 3C). Positive reactivity for α-SMA antibody was found in myoepithelial cells around the intercalated ducts of the control parotid gland. Such reactivity for α-SMA antibody in the parotid gland was not affected by the administration of theophylline (Figs. 3D and E). Additionally, positive reactivity in myoepithelial cells around the acinar units of the control parotid gland was less evident than in those of the control submaxillary gland.

In the submaxillary gland of the theophylline-treated group, the sequential changes in acinar histology were similar to those in the parotid gland. Acinar cells were enlarged with an accumulation of secretory granules just before the last administration. Secretory granules were decreased in their number at 4 hr (Fig. 4A), but thereafter granules were drastically restored to the level just before the last administration at 8 hr (Fig. 4B). Alpha-SMA-positive myoepithelial cells were distributed around the acinar units as well as the intercalated ducts. The number and distribution of α-SMA-positive myoepithelial cells were little affected by the administration of theophylline (Figs. 4C and D).

Furthermore, vacuolation was most frequently observed in the epithelial cells of the intercalated ducts in the submaxillary gland at 1 hr after the last administration when excessive salivation was observed clinically (Fig. 5). This change was not observed in the parotid gland. To analyze the morphologic characteristics of vacuolation in greater detail, electron microscopic examination was performed. In the intercalated duct of the control group, there were junctional complexes at the luminal side and well-developed interdigitation at the basal side between epithelial cells (Fig. 6A). In the theophylline-treated group, in contrast, vacuoles which were distinctly enclosed by membranes were observed between the duct epithelial cells (Fig. 6B), and contained small granules and flocculent materials. These contents were similar to those in the ductal lumen. Vacuoles
were connected with the basement membrane and ductal lumen via narrow intercellular spaces. There were no discernible alterations in other glandular compartments, including granules in the granular duct.

**Discussion**

Repeated administration of theophylline has been shown to induce hypertrophy of rat salivary glands. In the present study, we focused on salivation following administration of theophylline and chronologically investigated the histology of the salivary glands during...
Fig. 4. Histology of the submaxillary gland after the last administration of theophylline in the repeated dose study. A and B, H&E stain; C and D, immunostaining for α-SMA, bar = 50 µm. A: Considerable decrease in the number of contained granules at 4 hr. B: Secretory granules reaccumulate and the acini are restored to the level just before the last administration at 8 hr. C: Myoepithelial cells distributed around the acinus units as well as the intercalated duct just before the last administration. D: There are no apparent changes in the number and distribution of myoepithelial cells during salivation at 4 hr.

Fig. 5. Histology of the submaxillary gland at 1 hr after the last administration in the repeated dose study. Vacuolation (arrows) is observed on the basal side of the epithelium in the intercalated duct. H&E stain, bar = 50 µm.
salivation. Consequently, our results revealed that theophylline induces transient shrinkage in accordance with salivation and that hypertrophy occurs from 8 hr following the last administration. In this regard, note that previous studies on the effect of theophylline on the histology of the salivary glands were done on the day following the day of administration. Our study revealed two interesting results. Firstly, we showed a transient reduction in secretory granules in the salivary glands following administration of theophylline. The maximum decrease in weight and number of contained granules clearly coincided with the termination of salivation. Amsterdam et al. showed that isoprenaline, a catecholamine, induces a significant decrease in zymogen granules as well as amylase content in acinar cells of rat parotid glands. This chronological histology is similar to our present results. Both catecholamine and theophylline are also known to initiate an increase in intracellular cyclic AMP. Taken together, these results suggest that the elevation in intracellular cyclic AMP primarily leads to the discharge of secretory granules in salivary acini.

Secondly, we showed that the weight of salivary glands at 8 hr after the last administration was eventually restored to the level just before the last administration in the repeated dose study. In the single dose study, the weight of salivary glands showed a slight but significant increase at 24 hr after administration. Since a pharmacokinetic study on theophylline reported that the elimination half-life in intravenously treated rats was 3.4 hr, the restoration or enlargement observed from 8 hr after administration of theophylline may not be caused by the primary effect of theophylline as mediated by enhanced cyclic AMP. We therefore concluded that each single administration of theophylline evokes a slight salivary enlargement, and that repetition of this mild stimulation eventually results in salivary hypertrophy which is characterized by excessive accumulation of secretory granules in acini. Westwood et al. reported that the salivary hyper trophy induced by a phosphodiesterase inhibitor, ICI 153,110, recovered following a drug withdrawal period. This result also suggests that the salivary hypertrophy is an adaptive response.

It is well known that myoepithelial cells are located over the outer surface of the acinus in a basket-like configuration. This geometry and its ultrastructural features suggest a role in expelling the primary secretion. There are, however, no reports on the histological relationship between intracytoplasmic granule discharge by acinar cells and the contraction of myoepithelial cells. Because immunohistochemistry for α-SMA in the present study was not able to show significant changes in the morphology, number, and distribution of myoepithelial cells during the course of secretion, other approaches were needed to demonstrate the histological changes in myoepithelial cells. In addition, we found only a few myoepithelial cells in the acinus unit of the parotid gland. The difference in the distribution of myoepithelium among salivary glands was thought to be associated with the property of saliva from each gland.

Microscopically, vacuolation was frequently observed in the epithelium of the intercalated duct in the submaxillary glands at 1 hr after the last administration of theophylline. Electronmicroscopic examination demonstrated that this vacuole was made up by an extension of intercellular space, because vacuoles were connected with the basement membrane and interdigitation. To our knowledge, the structures with such characteristics have not been previously reported. Since vacuolation is frequently encountered during secretion of saliva, vacuolation might be involved in water transportation during salivation. Primary saliva, which is discharged from secretory granules in acinar cells, is known to be modified by the ductal system. Ductal
epithelium is known to play a role in water secretion and sodium reabsorption\textsuperscript{10,11}. The histological significance of such function, however, remained unclear in the present study.

Although salivation is sometimes observed in regular toxicity studies, there is little knowledge of the histological changes in the salivary glands during salivation. The present results indicate that the salivary glands undergo considerable changes in weight and morphology during the secretory cycle of saliva.

In conclusion, the results of this study showed that the salivary glands shrink transiently after the administration of theophylline and subsequent excessive salivation, and suggest that the hypertrophy of the salivary glands induced by theophylline may be an adaptive response.

Acknowledgement: The authors would like to thank Satoru Sasaki for the administration of test articles, Minoru Inoue, Kenji Nakano, Shuji Ishikawa, and Eiichi Nukui for preparation of numerous specimens, and Hisaki Miura for electron microscopy.

References


