Effects of Repeated Exposure to Isoproterenol in Vivo and in Vitro on Amylase Release from Rat Parotid Tissue

Hideyo OHISHIKA, Haruo TAKEMURA, Yuko SUZUKI, Michihiro KATAKURA and Mamoru TANAKA
Department of Pharmacology, Sapporo Medical College, Sapporo 060, Japan

Accepted October 3, 1983

Abstract—Effects of repeated exposure to isoproterenol (ISO), in vivo and in vitro, were investigated on amylase release from rat parotid slices responding to secretagogues. Responses to ISO and dibutyryl cyclic AMP (DBcAMP) were reduced in the tissue from ISO-treated rats (3 mg/kg, 3 times daily for 3 days), but not in the tissue from propranolol plus ISO-treated rats. Administration of inhibitors of protein synthesis with ISO resulted in a protective effect with regard to development of decreased responses to ISO, DBcAMP and carbachol. A decreased response to ISO (10^-5 M) was caused by repeated exposure to ISO in vitro and could not be prevented by pretreatment with inhibitors of protein synthesis. Although the basal level of cyclic AMP in parotid tissue was lower in ISO-pretreated rats than in control rats, after incubation with ISO, the level of cyclic AMP did not differ between ISO-pretreated and control rats. These results suggest that the decreased response (amylase release) to ISO in the parotid tissue from ISO-pretreated rats may have been due to an impairment of common secretory process(es) involving various secretagogues and that the development of the impairment may be closely connected to the de novo synthesis of proteins induced by ISO, but not the impairment of the β-adrenoceptor-adenylate cyclase system.

Since it was reported that repeated administration of isoproterenol (ISO) in rats caused an enlargement of the salivary glands (1), evidence showing ISO-induced increases of DNA and protein synthesis in these glands has accumulated (2–5). When salivation was stimulated by pilocarpine in rats, the degree of increase in the flow rate of parotid saliva and the concentration of amylase in it were lower in ISO-treated rats than in intact animals (6). Although a similar decrease in the flow rate and protein concentration of parotid saliva of treated rats was observed after an administration of ISO used as a challenge dose, no significant change was found in concentration of electrolytes in the same saliva (7).

It is reported that the number and/or affinity of binding sites for β-adrenergic agonists in the parotid tissue is decreased by the repeated administration of ISO (8, 9) and, on the other hand, that the increase in cyclic AMP in parotid acinar cells by stimulation of β-adrenoceptors may not be essential for ISO-induced amylase release from these cells (10, 11). If the effects of β-adrenergic and muscarinic agonists on parotid amylase secretion were to decrease concomitantly after repeated treatment with ISO (6, 7), the desensitization of β-adrenoceptors might be considered to be insufficient to explain the mechanism of the decreased response to a challenge dose of ISO. The present study was undertaken to clarify whether the decreased response to ISO in enlarged parotid glands is mainly due to the impairment of the β-adrenoceptor-adenylate cyclase system.

Materials and Methods

Animals and pretreatments: Male Wistar rats weighing 180–200 g were used. These animals were deprived of food 18 hr before each experiment, but were allowed water ad
libitum. Under anesthesia with pentobarbital sodium (40 mg/kg, i.p.), the parotid glands were removed.

Animals were pretreated with various drugs before use. (±)-ISO was given intraperitoneally at 3 mg/kg, 3 times daily for 3 days with or without propranolol, which was given in a dose of 5 mg/kg (i.p.) at 30 min prior to each ISO administration. In the other experiments, actinomycin D was given at a dose of 125 μg/kg daily for 3 days or cycloheximide was given at a dose of 500 μg/kg, twice daily for a total of 7 doses. The parotid glands were removed 12 hr after the last injection of these drugs. Saline was given to the control animals. In some animals, a single dose (250 μg/kg) of actinomycin D was injected at 24 hr before and a single dose (500 μg/kg) of cycloheximide was injected at 1 hr before sacrifice of the animals.

Drugs: (±)-Isoproterenol HCl (Sooner®, Kaken), (-)-isoproterenol HCl (Sigma), propranolol HCl (Inderal®, Sumitomo), N6, O2'-dibutyryladenosine 3':5'-cyclic monophosphate Na (Sigma), carbamylcholine chloride (Sigma), cycloheximide (Sigma) and actinomycin D (Cosmegen®, Banyu) were used.

Amylase release from parotid slices: Preparation and incubation of parotid slices was carried out by the procedure described previously (12). Briefly, about 20 mg of parotid tissue was incubated in a 5 ml Krebs-Ringer-Tris buffer solution (pH 7.4) bubbled with pure oxygen at 37°C. Tissue slices were then incubated for 10 min in the presence of ISO. As N6, O2'-dibutyryladenosine 3':5'-cyclic monophosphate (DBcAMP) and carbamylcholine (carbachol) caused an insufficient release of amylase during the 10 min incubation period, for the determination of the effects of these secretagogues, tissue slices were incubated for 30 min.

To measure the amount of amylase released into incubation media, 0.25 ml aliquots were taken from each incubation medium at the end of the incubation. Basal release of the enzyme was determined in the absence of secretagogues. After incubation, tissues were homogenized in 5 ml Krebs-Ringer-Tris buffer solution, and a 0.25 ml of the homogenate was used for an assay of the enzyme activity. Amylase activity in homogenates and incubation media were assayed by the method of Bernfeld (13). The amount of amylase released was expressed as the percent of the total amount of the enzyme initially contained in the tissue slices (12).

In the in vitro experiments, the slices were exposed to ISO (10^-6 M) twice for 2 min each time. The first exposure to ISO was followed by two incubation periods (10 min each) in the absence of ISO, and then this sequence for the incubation was repeated again. Finally, the slices were incubated for 10 min with or without a challenge dose of ISO (10^-5 M). In the control experiments, the slices were incubated in the absence of ISO during the same incubation periods except for the final incubation.

Cyclic AMP measurement: Cyclic AMP contents in the parotid tissues of pretreated and control rats were measured before and 2 min after incubation with 10^-6 M ISO. Cyclic AMP was measured by radiomunoassay using a commercial assay kit (Yamasa, Choshi, Japan).

Statistical analysis: Levels of significance were calculated using Student's t-test.

Results

ISO-induced amylase release in parotid tissue from ISO-pretreated rats: In the experiments using parotid tissue from intact animals, ISO increased amylase release from parotid slices with the increase in its concentrations from 10^-8 to 10^-4 M (Fig. 1). The maximal release was 32.1±0.8% of the total amylase content at 10^-4 M ISO. Pretreatment by administration of ISO (3 mg/kg, 3 times daily for 3 days) to rats caused an enlargement of the parotid and submaxillary glands. The weights of the left parotid gland in control and ISO-treated rats were 118±7 and 320±25 mg/100 g body weight, respectively. In the slices of the enlarged parotid glands, the response to ISO was markedly decreased compared with that of the intact tissues at the above-mentioned concentrations of ISO, and amylase release at 10^-4 M ISO was only 9.4±0.6% of the total content. However, the basal release of the enzyme from parotid slices for 10 min was increased by ISO-pretreatment from 0.6±0.6 to 3.4±0.5% (Fig. 1).
The response to DBcAMP on amylase release was also reduced by ISO-pretreatment (P<0.05) (Fig. 2). However, co-administration of propranolol and ISO to rats resulted in the complete disappearance of the reduced response to the secretagogues. The increased value of basal release was also reversed to the control level by propranolol pretreatment (Fig. 2).

Cyclic AMP content in parotid tissue from ISO-treated rats: The basal level of cyclic AMP in parotid tissue was significantly lower in ISO-treated rats than in control animals (P<0.05) (Table 1). The concomitant administration of propranolol and ISO to rats did not prevent the reduction of cyclic AMP. Cyclic AMP levels in the slices regardless of the kind of pretreatments in rats were elevated by the addition of ISO (10^{-5} M) into the incubation medium, and the difference in the elevated level of cyclic AMP between the ISO-treated and control rats was statistically insignificant.

**Effects of actinomycin D and cycloheximide**

---

Table 1. Cyclic AMP levels in rat parotid slices

| Pretreatment               | Cyclic AMP (pmole/mg tissue protein) |
|----------------------------|-------------------------------------|
|                            | Before stimulation                  | Isoproterenol 10^{-6} M                |
| Isoproterenol             | 4.93±0.60*                          | 99.69±24.14                            |
| Isoproterenol plus propranolol | 5.09±0.43*                         | 102.95± 8.82                           |
| Saline                    | 6.85±0.39                           | 139.72±28.96                           |

Cyclic AMP contents in parotid tissues from rats treated with isoproterenol, isoproterenol plus propranolol or saline were measured before and after incubation with isoproterenol (10^{-6} M) for 2 min. Pretreatments: isoproterenol, 3 mg/kg, 3 times daily for 3 days; propranolol, 5 mg/kg, 3 times daily for 3 days. *P<0.05 compared with saline. Values are the mean±S.E. for 5 experiments.
on the decreased response to ISO in the tissue from ISO-treated rats: To see whether the hyperplastic and/or hypertrophic effects of ISO were involved in the decreased amylase release in the enlarged parotid glands, rats were given ISO with actinomycin D or cycloheximide, and the secretory responses to various secretagogues were examined in vitro using the parotid tissues of these animals. As shown in Fig. 3, the effects of ISO (10^{-5} M), DBcAMP (10^{-3} M) and carbachol (10^{-5} M) on amylase release in the tissues from ISO-treated rats resulted in decreases in the total amylase of about 28, 9 and 3%, respectively. On the tissues from rats given ISO and actinomycin D or cycloheximide together, the effects of DBcAMP and carbachol did not differ from those in the control tissues. However, the responses to ISO (10^{-5} M) returned to almost normal values in the ISO plus cycloheximide group, but not in the actinomycin D group. The basal release of the enzyme from the tissues of rats given actinomycin D or cycloheximide alone was 5.4±1.5 and 4.1±0.4%, respectively.

**Effects of repeated exposure of parotid tissue to ISO in vitro:** After two exposures to ISO (10^{-5} M) for 2 min in vitro, ISO-induced amylase release was investigated (Fig. 4). The effect of 10^{-5} M ISO decreased from 16.9±1.2 to 10.3±1.1% after in vitro pretreatment with ISO. Pretreatments of rats with actinomycin D or cycloheximide had no protective effect on the development of the decreased response to ISO in vitro.

The cumulative amounts of released amylase during the first and second exposures to ISO followed by each two consecutive incubations without ISO were almost the same in the saline-, actinomycin D- and cycloheximide-treated groups (52–55% of

---

**Fig. 3.** Inhibitory effects of actinomycin D (ACTD) or cycloheximide (CHXM) on isoproterenol (ISO)-induced reduction of amylase release in parotid slices. Pretreatment: ISO, 3 mg/kg, 3 times daily for 3 days; ACTD, 125 μg/kg daily for 3 days; CHXM, 500 μg/kg, twice daily for a total of 7 doses. Values are the mean±S.E. for 4–5 experiments and are expressed as the percent release of total content during 30 min. *P<0.05, **P<0.01 compared with each corresponding value of the saline group. Concentrations of secretagogues: 10^{-5} M ISO, 10^{-3} M DBcAMP and 10^{-5} M carbachol (CARB).

**Fig. 4.** Effects of repeated exposure of parotid slices to isoproterenol (ISO) on amylase release induced by a challenge dose of ISO (10^{-5} M). Slices of parotid glands from rats treated with actinomycin D (ACTD), cycloheximide (CHXM) or saline were used. Pretreatments: ISO indicates that the slices were exposed twice to 10^{-5} M ISO, while NON indicates the slices were incubated without ISO. Challenge: ISO or NON indicates the final incubation (10 min) with or without 10^{-5} M ISO. *P<0.05 compared with each corresponding value in the NON-ISO group. Values are the mean±S.E. for 4–6 experiments.
Discussion

In the present study, it was ascertained that repeated administration of ISO to rats caused an enlargement of the submaxillary and parotid glands as previously reported by many investigators (1, 6–9) and that the amount of amylase released by β-adrenergic stimulation was markedly smaller in the tissue slices of the enlarged parotid glands (Fig. 1). These changes probably resulted from the repeated exposure of β-adrenoceptors to ISO since propranolol administered with ISO to rats completely prevented the development of the decreased response to ISO (Fig. 2). β-Adrenergic stimulation increases the parotid cyclic AMP which has the role of the second messenger in the regulation of amylase release (14). However, the decreased secretion of parotid amylase in ISO-treated rats (6) can not be explained as a decrease in the density of β-adrenoceptors (8, 9) because the total number of β-adrenergic binding sites in the enlarged gland may practically be the same as that in the control gland (8). Furthermore, even if a decrease in the number of β-adrenergic binding sites due to repeated administration of ISO had developed, the maximal response to the β-adrenergic agonists probably could be maintained by the remaining β-adrenoceptors (15). Our results (Table 1, Figs. 2 and 3) also suggest that the cyclic AMP level in parotid tissue may not be a major factor responsible for the decreased release of amylase in ISO-treated rats. Spearman et al. (11) showed that a measurable increase of cyclic AMP in parotid acinar cells is not necessary for the induction of amylase release by β-adrenergic stimulation. However, if the amylase release and cyclic AMP accumulation in parotid acinar cells may be regulated by two different β-adrenoceptor subtypes, β₁ and β₂ (16), the above mentioned (8) decrease of β-adrenoceptors might have to be reexamined on each subtype. At this point in time, the definite role of each β-adrenoceptor subtype has not been decided yet.

The decrease of amylase content in parotid tissue following the repeated administration of ISO (6) does not appear to be responsible for the impaired response to secretagogues on amylase release in these animals. In the present study, the concentration of amylase in parotid tissue was lower in ISO-treated rats (55.07 ± 3.91 U/mg protein) than that in control animals (70.03 ± 3.26 U/mg protein) at 24 hr after the final injection of ISO. However, with regard to the values of amylase release expressed as the percent of total amylase content, it appears that the above decrease in the concentration could not be directly attributed to the values of the decreased response to ISO (Fig. 1). The report of Robinovitch et al. (17), who showed ISO-induced morphological changes of secretory granules and the failure to obtain granules containing amylase from enlarged parotid glands, suggests alterations of membrane function and amylase distribution in the acinar cells. The increase in basal release of amylase in the parotid tissue from pretreated rats may suggest the possibility that the stored amylase in the acinar cells could be translocated from a ISO-sensitive pool to a less ISO-sensitive one by the repeated stimulation to β-adrenoceptors.

The increase in cyclic AMP in parotid tissue may be closely related to the induction of DNA and protein synthesis observed in the ISO-treated rats (4, 5), an accompanying induction of ornithine decarboxylase (18, 19), an increase in polyamines (19) and an activation of cyclic AMP-dependent protein kinase in the gland (20). It is reported that both actinomycin D and cycloheximide in a sufficient dose for inhibition of protein synthesis did not have any effect on β-adrenergic agonist-induced amylase release (21–23). These inhibitors of protein synthesis effectively blocked the development of impaired response to secretagogues in parotid tissue from ISO-administered rats (Fig. 3), but not that in parotid slices treated in vitro with ISO repeatedly (Fig. 4). These results suggest that there is a difference of mechanism between the two developments of impaired release of amylase. The fact that the repeated exposure of rat parotid slices to ISO in vitro apparently caused a reduction of ISO-induced accumulation of cyclic AMP (24) may support the above mentioned difference of the mechanism. From our
results, it may be considered that the impairment of parotid amylase release induced by repeated administration of ISO to rats was closely connected to the de novo synthesis of proteins but not the impairment of the β-adrenoceptor-adenylate cyclase system.

References
1 Selye, H., Veilleux, R. and Cantin, M.: Excessive stimulation of salivary gland growth by isoproterenol. Science 133, 44–45 (1961)
2 Barka, T.: Stimulation of protein and ribonucleic acid synthesis in rat submaxillary gland by isoproterenol. Lab. Invest. 18, 38–41 (1968)
3 Feiglin, B. and Reade, P.C.: Protein and DNA levels in the submandibular salivary glands of isoproterenol stimulated mice. Aust. J. Exp. Biol. Med. Sci. 56 (Pt. 1), 1–10 (1978)
4 Tsang, B.K., Rixon, R.H. and Whitfield, J.F.: A possible role for cyclic AMP in the initiation of DNA synthesis by isoproterenol-activated parotid gland cells. J. Cell. Physiol. 102, 19–26 (1980)
5 Guidotti, A., Weiss, B. and Costa, E.: Adenosine 3’,5’-monophosphate concentrations and isoproterenol-induced synthesis of deoxyribonucleic acid in mouse parotid gland. Mol. Pharmacol. 8, 521–530 (1972)
6 Schneyer, C.A.: Salivary gland changes after isoproterenol-induced enlargement. Am. J. Physiol. 203, 232–236 (1962)
7 Abe, K. and Dawes, C.: The secretion of protein and some electrolytes in response to α- and β-adrenergic agonists by rat parotid and submandibular salivary glands enlarged by chronic treatment with isoproterenol. J. Dent. Res. 59, 1081–1089 (1980)
8 Burke, G.T. and Barka, T.: Beta-adrenergic receptors and adenylate cyclase in hypertrophic and hyperplastic rat salivary glands. Biochim. Biophys. Acta 539, 54–61 (1978)
9 Roscher, A.A., Wiesmann, U.N. and Honegger, U.E.: Changes in beta adrenergic receptors in submaxillary glands of chronically reserpinized or isoproterenol-treated rats. J. Pharmacol. Exp. Ther. 216, 419–424 (1981)
10 Butcher, F.R., Goldman, J.A. and Nemerovski, M.: Effect of adrenergic agonists on α-amylase release and adenosine 3’5’-monophosphate accumulation in rat parotid tissue slices. Biochim. Biophys. Acta 392, 82–94 (1975)
11 Spearman, T.N., Durham, J.P. and Butcher, F.R.: The role of cyclic AMP in the regulation of exocytosis in the rat parotid gland: Evidence obtained with the isoproterenol analog PI-39. J. Cyclic Nucleotide Res. 8, 225–234 (1982)
12 Ohshika, H., Takemura, H., Endo, J., Hatta, S. and Tanaka, M.: Stimulating effect of α-adrenoceptor agonists on isoproterenol-induced amylase release in rat parotid tissue. Japan. J. Pharmacol. 31, 1021–1027 (1981)
13 Bernfeld, P.: Amylases, α and β. In Methods Enzymol., Edited by Colowick, S.P. and Kaplan, N.O., Vol. 1, p. 149–158, Academic Press, New York (1955)
14 Butcher, F.R.: Calcium and cyclic nucleotides in the regulation of secretion from the rat parotid by autonomic agonists. In Adv. Cyclic Nucleotide Res., Edited by George, W.J. and Ignarro, L.J., Vol. 9, p. 707–721, Raven Press, New York (1978)
15 Terasaki, W.L., Linden, J. and Brooker, G.: Quantitative relationship between β-adrenergic receptor number and physiologic responses as studied with a long-lasting β-adrenergic antagonist. Proc. Natl. Acad. Sci. U.S.A. 76, 6401–6405 (1979)
16 Henriksson, R.: β1- and β2-adrenoceptor agonists have different effects on rat parotid acinar cells. Am. J. Physiol. 242, G481–G485 (1982)
17 Robinovitch, M.R., Keller, P.J., Johnson, D.A., Iversen, J.M. and Kauffman, D.L.: Changes in rat parotid salivary proteins induced by chronic isoproterenol administration. J. Dent. Res. 56, 290–303 (1977)
18 Copeland, J.G., Larson, D.F., Roeske, W.R., Russell, D.H. and Womble, J.R.: β2-Adrenergic agonists regulate induction of myocardial ornithine decarboxylase in mice in vivo. Br. J. Pharmacol. 75, 479–483 (1982)
19 Inoue, H., Tanioka, H., Shiba, K., Asada, A., Kato, Y. and Takeda, Y.: Effect of isoproterenol on polyamine metabolism in mouse salivary glands. J. Biochem. 75, 679–687 (1974)
20 Spearman, T.N. and Butcher, F.R.: Rat parotid gland protein kinase activation. Relationship to enzyme secretion. Mol. Pharmacol. 21, 121–127 (1982)
21 Grand, R.J. and Gross, P.R.: Independent stimulation of secretion and protein synthesis in rat parotid gland. The influence of epinephrine and dibutyryl cyclic adenosine 3’,5’-monophosphate. J. Biol. Chem. 244, 5608–5615 (1969)
22 Grand, R.J. and Gross, P.R.: Translation-level control of amylase and protein synthesis by epinephrine. Proc. Natl. Acad. Sci. U.S.A. 65, 1081–1088 (1970)
23 Whitlock, J.P., Jr., Kaufman, R. and Baserga, R.: Changes in thymidine kinase and α-amylase
activity during isoproterenol-stimulated DNA synthesis in mouse salivary gland. Cancer Res. 28, 2211–2216 (1968)

24 Ohshika, H., Miyamoto, A. and Takemura, H.: Changes in salivary function of rat parotid tissue by repeated treatment with isoproterenol in vitro. Japan. J. Pharmacol. 32, Supp. 164P (1982)