Isoprenaline-induced changes in rat parotid and submandibular glands are age- and dosage-dependent

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(Received 12 October 1983/Accepted 14 March 1984)

Neonatal rats treated with chronic injections of isoprenaline (isoproterenol) for 10 days revealed differential induction of proline-rich proteins and glycoprotein synthesis between the parotid and submandibular glands. Biosynthesis of proline-rich proteins ($M_r$ 17000–35000) and a $M_r$-220000 glycoprotein were detectable by solubilization in 10% trichloroacetic acid extracts from parotid glands 14 days after birth. The enzyme lactose synthase (UDP-galactose:2-acetamido-2-deoxy-D-glucosamine 4β-galactosyltransferase) (EC 2.4.1.22) is also induced 4–7-fold in specific activity compared with control neonatal rats, but again only after 14 days post partum, with isoprenaline treatment. This is in accord with the ability of the parotid gland to respond to β-receptor stimulation and subsequent increases in intracellular cyclic AMP necessary for induction of protein synthesis [Grand, Chong & Ryan (1975) Am. J. Physiol. 228, 608–612]. Induction of the proline-rich proteins and a $M_r$-190000 glycoprotein in the soluble fraction from the submandibular gland were not detected until 49 days after birth under identical conditions in the same animal. Cyclic AMP in the submandibular gland undergoes increases on β-receptor stimulation similar to those achieved in the adult animal, 1 day after birth (Grand et al., 1975). This same differential induction between parotid and submandibular gland was obtained with a range of isoprenaline dosages in adult animals. Trichloroacetic acid-soluble proline-rich proteins were isolated from parotid glands at a dosage of 4.0mg of isoprenaline/kg body wt., but 7.0mg/kg was required to induce also biosynthesis of these proteins in the submandibular gland. Gland hypertrophy showed the same differential dosage kinetics, based on gland weight, between the two glands; however, hypertrophy could be accomplished at a lower dosage of isoprenaline than that used to induce proline-rich-protein biosynthesis.

Administration of the catecholamine isoprenaline (isoproterenol) causes a variety of morphological and cytochemical changes in the parotid and submandibular glands in rats. Initially, isoprenaline causes glandular secretion through interaction with the cell-surface β-receptors, which result in raising the concentrations of cyclic AMP (Schneyer & Hall, 1969; Ekfors et al., 1972; Schramm & Selinger, 1974). Single injections of isoprenaline result in increased synthesis of DNA, RNA and protein (Schneyer, 1972). Continued treatment of rats with isoprenaline results in hypertrophy and hyperplasia of both the parotid and the submandibular glands (Selye et al., 1961; Brown-Grant, 1961; Schneyer, 1962). Biochemical studies on the soluble fraction of cell lysates from both glands after treatment with isoprenaline have shown the induced synthesis of a unique series of basic (pl > 11) proteins rich in the amino acid proline and which are soluble in 10% trichloroacetic acid (Muenzer et al., 1979a,b; Mehancho & Carlson, 1983). The parotid gland normally produces a single glycoprotein of $M_r$ 200000, but upon chronic injection of isoprenaline a second glycoprotein, of $M_r$ 220000, is induced (Humphreys-Beher et al., 1982). The submandibular gland normally produces two glycoproteins, of $M_r$ 180000 and 200000 (Humphreys-Beher et al., 1982). After injection of isoprenaline, the submandibular gland produces a new glycoprotein, of $M_r$ 158000 (Mehancho & Carlson, 1983). The proline-rich proteins charac-

Abbreviations used: SDS, sodium dodecyl sulphate; Mes, 4-morpholine-ethanesulphonic acid.
teristically have roughly 60% of their amino acid composition as proline, glycine and glutamic acid (Muenzer et al., 1979a,b). The parotid gland additionally shows a 6-10-fold increase in the Golgi enzyme marker lactose synthase (UDP-galactose:N-acetylglucosamine 4β-galactosyltransferase, EC 2.4.1.22) after chronic treatment with isoprenaline (Zinn et al., 1972; Humphreys-Beher, 1983).

In the neonatal rat, the salivary glands are not fully differentiated and undergo maturation during the first 4-5 weeks post partum (Schneyer & Hall, 1969; Redman & Sreebney, 1970; Bressler, 1972). Treatment with isoprenaline at this stage of development leads to precocious morphological development of the parotid and submandibular glands (Barka & van der Noen, 1976; Schneyer, 1978; Sheetz & Menaker, 1979). The ability of the parotid gland to undergo complete morphological and biochemical maturation in response to isoprenaline occurs only after day 14 post partum (Grand et al., 1975; Grand & Schay, 1978). However, the ability of the submandibular gland to respond to isoprenaline stimulation by advanced biochemical and morphological development is the same 1 day after birth as it is in the adult (Bressler, 1972; Grand et al., 1975). This effect has been observed not only in terms of accelerated morphological development, but in overall protein composition and response of adenylate cyclase in increasing cyclic AMP (Ekfors et al., 1972; Bressler, 1972; Grand et al., 1975; Grand & Schay, 1978; Lumford & Talamos, 1980). Although the synthesis of the proline-rich proteins is induced in adult animals on treatment with isoprenaline, they are not present at any time during development post partum in either the parotid or the submandibular gland (Humphreys-Beher et al., 1982). This suggests these proteins are not under developmental control, although there has been no investigation of the synthesis of these proteins in utero.

In the present paper, evidence is presented for the differential induction of the proline-rich proteins and glycoproteins synthesized by the parotid and submandibular glands in response to chronic isoprenaline treatment as a consequence of age. Induced changes in the proline-rich proteins in the parotid gland demonstrate the same kinetics of induction as that of cyclic AMP in response to stimulation of the cell-surface β-receptor. However, the submandibular gland shows no such similarity in the increases in cyclic AMP generated by isoprenaline treatment and the ability to induce proline-rich proteins or glycoproteins. Synthesis of these proteins did not occur until 49 days after birth in the submandibular gland, but they were inducible by day 14 in the parotid gland. Furthermore, the effect of isoprenaline on rat salivary glands is shown to be dosage-dependent in the adult animal, with the parotid gland again showing more sensitivity to treatment than the submandibular gland at lower dosages of the drug.

Materials and methods

Chemicals

(+)-Isoprenaline and trichloroacetic acid were purchased from Sigma. All reagents for polyacrylamide-gel electrophoresis were of ultra-pure quality, obtained from Bio-Rad. UDP-[1-14C]galactose (sp. radioactivity 300Ci/mmol) was purchased from New England Nuclear. All other chemicals were of analytical-grade quality and obtained from commercial sources.

Isoprenaline treatment

Male Wistar rats (7-35 days old) received 0.025mg of isoprenaline/g body wt. for 10 days. Adult male rats weighing 200-250g received intraperitoneal injections of 5mg of isoprenaline daily and were fed ad libitum unless otherwise stated in the Results and discussion section.

Tissue preparation

Parotid and submandibular glands were identified by gross morphology. The glands were removed from animals that had been anaesthetized with sodium pentobarbital and killed by exsanguination, and soluble and insoluble membrane fractions were prepared by homogenization in 10mm-Tris/HCl buffer, pH 8.0, with a Dounce apparatus. The slurry was then centrifuged at 100000g for 1h. Protein assays were performed by a modification of the Lowry method with bovine serum albumin as standard (Schacterle & Pollack, 1973). For purification of the salivary-gland glycoproteins and proline-rich proteins, the soluble fraction was diluted with an equal volume of 20% trichloroacetic acid. The proline-rich proteins and parotid and submandibular glycoproteins remain soluble in 10% trichloroacetic acid (Muenzer et al., 1979a,b). Insoluble proteins were removed by centrifugation at 15000g for 15 min, and trichloroacetic acid was removed by repeated extraction with diethyl ether.

Polyacrylamide-gel electrophoresis and protein staining

Protein samples were electrophoresed in an 8% or 12.5%-polyacrylamide gel by using a modification of the Laemmli Tris/glycine system (Pugsley & Schnaitman, 1979). All gels were fixed and stained by a modification of the method of Fairbanks et al. (1971) for staining total protein with Coomassie Blue R-250, or by staining with periodic acid/ Schiff reagent (Fairbanks et al., 1971) for identifica-
tiation of glycoproteins. In staining polyacrylamide gels containing proline-rich proteins, staining time was shortened to 30 min with Fairbanks' staining solution no. 1 only and destaining was performed in several changes of 10% acetic acid over 24 h. Samples for gels were made up to 1 mg of protein/ml of sample buffer. Lane 3 of Figs. 2(a) and 3(a) has 40 μl of sample added to enhance the background proteins present before induction of proline-rich proteins by isoprenaline.

*Galactosyltransferase assay*

The activities of UDP-galactose:N-acetylgalactosamine 4β-galactosyltransferase and UDP-galactose:N-acetylgalactosaminyl-protein 3β-galactosyltransferase were measured by a modification of the assay used by Carlson et al. (1973). Membrane fractions were obtained as described above and resuspended in 10 mm-Tris/HCl buffer, pH 8.0, to give a final protein concentration of 20 mg/ml. The assay mixture (total volume 50 μl) contained 0.1 M-Mes, pH 6.3, 25 mm-MnCl₂, 0.5% Triton X-100, 1 mm-UDP-[1-14C]galactose (6 x 10⁶ c.p.m./μmol) and either 10 mm-N-acetylgalactosamine or asialo-(sheep submaxillary mucin) and 0–0.5 mg of the enzyme preparation. The asialo-mucin was sheep submaxillary mucin from which sialic acid residues had been removed by sialidase treatment or by acid hydrolysis (Carlson et al., 1973). After incubation at 37°C for 40 min, the reaction was stopped by adding 10 μl of 10 mm-EDTA in 7.5% (w/v) sodium tetraborate. High-voltage paper electrophoresis was performed on Whatman 3 MM paper for 45 min at 1700 V as described by Carlson et al. (1973).

*Protein purification*

Trichloroacetic acid-soluble glycoproteins were isolated from the proline-rich proteins by chromatography on a Sephadex G-100 column (1 cm x 90 cm). The protein was eluted with 10 mm-Tris/HCl buffer, pH 7.5, and 2 ml fractions were collected. Protein elution was monitored by A₂₅₀ (Me honesto & Carlson, 1983). Since the parotid-gland glycoproteins after isoprenaline treatment could not be separated by this method, the two proteins were co-purified for subsequent analysis.

*Amino acid analysis*

Purified glycoproteins were subsequently dialysed to remove Tris buffer and freeze-dried. A 0.2 mg sample of protein was analysed for amino acid composition after hydrolysis in 6 M-HCl for 18 h, as described by Muenzer et al. (1979b). Amino acid analysis was performed on a Beckman 119 analyser, by using single-column methodology.

### Results and discussion

Beginning at 7 days after birth, neonatal animals were given daily injections of 0.025 mg of (±)-isoprenaline/g body wt. for 10 days. After this time the parotid and submandibular glands had undergone gross morphological changes in appearance, resembling those glands isolated from mature animals, as well as showing increased hypertrophy. The soluble fraction isolated from cell lysates also gave patterns on SDS/polyacrylamide-gel electrophoresis resembling those of the proteins produced by adult animals (Fig. 1). Although not fully differentiated, the protein pattern at 7 days *post partum* (treated animal) bears a similarity to the protein profile for the adult (Fig. 1, lanes 2 and 5 respectively). Only in the 14-day-old animal can the changes in parotid-gland glycoprotein (Mₑ 220000) be seen, whereas these changes do not occur in the parotid gland of the 7-day newborn (control or isoprenaline-treated) or the adult (Fig. 1). Treatment of neonatal-rat submandibular gland in the same manner resulted in precocious changes in the soluble cell proteins typical of the gel profile found

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**Fig. 1. Electrophoresis (8% polyacrylamide gel) of the 100000 g soluble fraction of parotid-gland cells, stained for total protein by Coomassie Brilliant Blue**

Lane 1, 7-day newborn, untreated; lane 2, 7-day newborn, treated for 10 days with isoprenaline; lane 3, 14-day newborn, untreated; lane 4, 14-day gland treated with isoprenaline; lane 5, adult untreated soluble fraction. Dots indicate the Mₑ 200000 and 220000 glycoproteins. Mₑ markers were myosin (200000), β-galactosidase (116000), phosphorylase (94000), serum albumin (68000) and ovalbumin (43000).
in this analysis, the soluble protein fraction of the parotid and submandibular gland was extracted with trichloroacetic acid to detect better the induced expression of proline-rich proteins and glycoproteins. As shown in Figs. 2(a) and 3(a), the soluble cell extracts from parotid glands of neonatal rats were able to respond to chronic isoprenaline treatment with the induction of synthesis of proline-rich proteins and the Mr-220000 glycoprotein after day 14 after birth (Fig. 2, lane 5; Fig. 3, lane 4). In comparison, lanes 8 and 9 (Figs. 2a) refer to parotid glands from control and isoprenaline-treated adult animals respectively. Lanes 9 and 10 of Fig. 3(a) show the periodate–Schiff-stained glycoproteins isolated from control and isoprenaline-treated parotid glands. The neonatal animals beyond 14 days of age are able to respond to treatment in the same manner as the adult animals in terms of the proteins induced.

The UDP-galactose: N-acetylgalactosamine 4β-galactosyltransferase activity could be stimulated from 0.27 (untreated rat newborns) to 1.38–1.87 nmol/min per mg of protein after 14 days from birth on treatment with isoprenaline (Table 1). This was not the case for enzyme activity assayed in animals 7, 9 or 11 days post partum which received injections of isoprenaline for 10 days (Table 1). Enzyme activity for UDP-galactose: N-acetylgalactosamine 3β-galactosyltransferase (no EC number) remained unchanged for both treated and untreated animals over the developmental period studied (results not shown).

![Fig. 2. Electrophoresis (12.5%polyacrylamide gels) of 10%trichloroacetic acid-soluble proteins isolated from (a) parotid glands and (b) submandibular glands of rats treated for 10 days with isoprenaline](image)

The gels are stained with Coomassie Brilliant Blue R-250 for total protein present. Lanes in both parts: 1, Mr standards; 2, 7-day neonatal rat; 3, 9-day neonatal rat; 4, 11-day neonatal rat; 5, 14-day neonatal rat; 6, 28-day neonatal rat; 7, 35-day neonatal rat; 8, adult control; 9, adult rat injected for 10 days with isoprenaline. Dots represent migration of proline-rich proteins. Each lane contains 20 μg of total protein, except lane 3, which was run with 40 μg to show differences in the acid-soluble proteins between induced and non-induced states.

![Fig. 3. Electrophoresis (8%polyacrylamide gels) of trichloroacetic acid-soluble proteins stained with periodic-acid/Schiff reagent for glycoproteins from the parotid (a) and submandibular (b) glands of rats treated with isoprenaline](image)

Lane 1, untreated 7-day newborn. Lanes 2–8, 7–, 9–, 11–, 14–, 21–, 28– and 35-day neonatal rats treated with isoprenaline. Lane 9, 49-day-old untreated adult. Lane 10, adult animal (49 days), chronic isoprenaline treatment.

<table>
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<tr>
<th>Age (days)</th>
<th>Control</th>
<th>Isoprenaline-treated</th>
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<tbody>
<tr>
<td>7</td>
<td>0.27</td>
<td>0.31</td>
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<tr>
<td>9</td>
<td>0.31</td>
<td>0.41</td>
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<tr>
<td>11</td>
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<td>21</td>
<td>0.35</td>
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<tr>
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<tr>
<td>49</td>
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When the submandibular glands from the same animals were examined for the induction of the trichloroacetic acid-soluble proteins, no evidence was found for their synthesis before 49 days after birth (Figs. 2b and 3b). The control submandibular gland from adult and neonatal animals has been previously reported (Humphreys-Beher et al., 1982) to produce two glycoproteins, of Mr 180000 and 200000 (Fig. 3b), which appear to be present in trichloroacetic acid extracts from neonatal rats treated with isoprenaline. The acid-soluble glycoproteins from a 7-day newborn (untreated control) are shown in Fig. 3(b), lane 1. The Mr-190000 glycoprotein is unique to the submandibular-gland trichloroacetic acid extracts of adults (Fig. 3b, lane 10) and could be the Mr-158000 protein reported by Mehando & Carlson (1983), but demonstrating a slightly higher Mr, owing to the differences in gel systems used.

To test the relatedness of Mr-190000 to the Mr-158000 glycoprotein (gp 158), these proteins were purified by trichloroacetic acid extraction and subsequent chromatography on a Sephadex G-100 column eluted with 10mM-Tris/Cl buffer, pH7.5 (results not shown). The glycoprotein isolated from the isoprenaline-treated submandibular gland was analysed for a comparison of its amino acid composition with that of the composition of gp158 (Mehando & Carlson, 1983). The overall amino acid composition suggests that gp158 and the Mr-190000 glycoprotein are different proteins of the proline-rich type.

In an attempt to understand further this phenomenon of differential induction of biosynthesis of glycoprotein and proline-rich proteins, adult rats were given various doses of isoprenaline for 10 days. Syntheses of the proline-rich proteins were induced with as little as 1 mg of isoprenaline/200g animal in the parotid gland, whereas a greater dosage (1.75 mg/animal) was necessary to induce synthesis fully in the submandibular gland (Figs 4a and 4b). The biosynthesis of the Mr-190000 submandibular glycoprotein occurs at a concentration of 1.5 mg/animal (Fig. 5b). Because of the gel concentration (12.5%), the induction of the Mr-220000 glycoprotein from the parotid is not evident in Fig. 4(a), but it is indeed induced at 1.0 mg of isoprenaline/animal (results not shown). Finally, gland hypertrophy was examined after the 10 days with various dosages of isoprenaline. This response was found to be more sensitive to isoprenaline treatment than was induction of proline-rich-

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**Fig. 4. Electrophoresis (12.5% polyacrylamide gels) of trichloroacetic acid-soluble proteins isolated from adult rat parotid (a) and submandibular (b) glands**

The concentrations of isoprenaline shown at the tops of the gels were given each day for 10 days to 200g animals. The gels are stained with Coomassie Brilliant Blue. STD, Mr, markers given in (b): phosphorylase b (94000); bovine serum albumin (68000); ovalbumin (43000); carbonic anhydrase (30000); soya-bean trypsin inhibitor (21000); lysozyme (14000).

**Fig. 5. Gland hypertrophy after 10 days treatment with various concentrations of isoprenaline**

- Parotid gland; ○, submandibular gland.
protein biosynthesis. As shown in Fig. 5, at a concentration of 0.1 mg of isoprenaline/animal the parotid gland has increased in wet weight. However, the submandibular gland again requires a 10-fold higher concentration of isoprenaline before hypertrophy becomes evident, and requires an even greater dosage of isoprenaline to induce subsequent synthesis of the proline-rich proteins.

Isoprenaline stimulates parotid and submandibular-gland activity by interaction with the cell-surface β-receptors. The interactions leads to a rise in cyclic AMP through activation of adenylate cyclase. In the parotid gland the ability of isoprenaline to cause an increase in cyclic AMP is not evident until 14 days post partum (Grand et al., 1975; Grand & Schay, 1978; Lumford & Talamos, 1980) and has been suggested to result from a low content of guanine-nucleotide-binding regulatory protein, required for adenylate cyclase activity (Lumford & Talamos, 1983). However, this does not explain the inability of the submandibular gland to synthesize the proline-rich proteins and Mr 190,000 glycoprotein in the neonatal animal or at corresponding doses of isoprenaline in the adult animal. It may be that induction of these proteins in the submandibular glands requires the participation of other factors induced by isoprenaline in other glands, such as hormones, or it may be that the submandibular gland is more refractory to isoprenaline stimulation, owing to its lower content of cell-surface β-receptors compared with the parotid (Grand et al., 1975). Injections of both thyroxine and cortisol have been shown to cause precocious development of neonatal-mouse parotid glands (Kumigawa et al., 1977; Takuma et al., 1978; Imai et al., 1982). Clearly, further investigation is required to determine the exact nature of this difference in isoprenaline sensitivity.

This work was supported in part by National Institutes of Health grant AM-0734021 and National Institute of Dental Research Public Health Service grant 1 RO3 DOE 6356-01 to me. I further thank Ms. Dae Lynn Hollis and Mr. David Wells for technical assistance.

References


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