Androgen-Induced Myogenesis and Chondrogenesis in the Larynx of Xenopus laevis

DAVID SASSOON, NEIL SEGIL, AND DARCY KELLEY

Department of Biological Sciences, Columbia University, New York, New York, 10027

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We investigated a possible role for testosterone-induced cell proliferation in the development of sexual dimorphism in the larynx of South African clawed frogs, Xenopus laevis. Androgen-induced cell proliferation was studied using \[^{3}H\]thymidine autoradiography. Nuclei of cartilage, perichondrium, and muscle were labeled in the larynx of sexually immature frogs of both sexes but not in adults. Cell proliferation did not occur with estradiol treatment nor was it seen in nonlaryngeal muscle or cartilage. Electron microscopic/autoradiographic studies of laryngeal muscle indicate that testosterone stimulates satellite cell division which later results in formation of myonuclei. We conclude that testosterone induces both chondrogenesis and myogenesis in juvenile larynx and that this process may contribute to the pronounced sexual dimorphism of the adult vocal organ.

INTRODUCTION

Steroid hormones are known to direct the dimorphic development of secondary sexual characteristics in vertebrates (Jost, 1972; Wilson et al., 1981). These dimorphic characteristics include certain specialized muscles which participate in male-specific reproductive behaviors. Two possible mechanisms have been suggested to explain hormonally regulated sex differences in muscle mass: prevention of ontogenetic cell death and steroid-induced hypertrophy. A developmental mechanism involving hormone-mediated cell survival has been suggested in the sexual differentiation of the rat levator ani, a muscle present in both sexes at birth that involutes in females unless exogenous androgens are supplied perinatally (Cihak et al., 1970; Breedlove and Arnold, 1983a). Both the levator ani and the sexually dimorphic bird syrinx respond to fluctuations in circulating levels of male sex hormones by an increase or decrease in mass (Venable, 1966; Luine et al., 1980). Thus, a second mechanism that may contribute to dimorphic muscle development is androgen-induced hypertrophy.

An additional mechanism by which steroids influence the growth of nonmuscle target tissues is through stimulation of cell proliferation (Wilson et al., 1981). We investigated a possible role for testosterone-induced cell proliferation in the development of sexual dimorphism in the larynx of South African clawed frogs. In Xenopus laevis, the larynx of adult males is 3-4 times larger in cartilage and muscle mass than in females (Ridewood, 1897; Nieuwkoop and Faber, 1956). Our ongoing studies indicate that this dimorphism is established during the first year following metamorphosis, and that laryngeal size can be increased by administering exogenous testosterone.

In the present experiments we administered testosterone to juvenile and adult X. laevis and used \[^{3}H\]thymidine autoradiography to assess any stimulated cell division. We report that testosterone, but not estradiol, induces myogenesis and chondrogenesis in the larynx of juvenile frogs of both sexes. This response includes the proliferation of a myogenic precursor, the satellite cell, which otherwise can be induced to proliferate only in response to muscle or nerve damage (Mauro, 1979; Murray and Robbins, 1982a, b). We do not observe testosterone-induced cell division in adults of either sex although adult female larynx does increase in size after prolonged testosterone treatment.

MATERIALS AND METHODS

All surgical procedures and injections (including sham procedures) were performed under cold narcosis. Frogs were obtained from Nasco (Ft. Atkinson, Wis.). Juveniles from the same ovulation group were studied 3 weeks after metamorphosis. Crystalline testosterone propionate or \(\beta\)-estradiol (Sigma) was compressed into 3-mg pellets and implanted through a small incision into the dorsal lymph sac. Sham-operated, non-hormone-treated controls received an incision in the dorsal lymph sac. Four days later, \[^{3}H\]thymidine (New England Nuclear, NEN-027, aqueous, 6.7 mCi/mmol) was injected intraperitoneally at a dose of 1 \(\mu\)Ci/mg (dose/body wt). Juveniles were sacrificed 1 day after thymidine injection and whole body or laryngeal autoradiograms prepared from formalin-fixed, paraffin-sectioned (10 \(\mu\)m in the horizontal plane) material. Kodak NTB-2 emulsion coated sections were exposed for 3 weeks, developed with Kodak D-19, and counterstained with hematoxylin and eosin. Sex was determined by visual inspection or his-
RESULTS AND DISCUSSION

Many heavily labeled cells were found in muscle, cartilage, and perichondrium of laryngeal tissue from testosterone-treated juveniles (Fig. 1). In both males and females the labeling index in testosterone-treated juveniles was approximately 10 times that of controls (Table 1). From the whole-body autoradiograms we observed that other muscle and cartilage in testosterone-treated juveniles showed a virtual absence of labeled cells indicating that androgen-induced proliferation is tissue specific. The labeling indices from other non-hormone-treated larynges were somewhat greater than indices from non-hormone-treated other muscle (larynx: 4% vs thigh: <1%) suggesting active myogenesis in the larynx at this time in development. Estradiol-treated juveniles did not show proliferation in laryngeal tissue (labeling index <4%; n = 2 males, n = 3 females), indicating that steroid induced proliferation is hormone specific. As noted, the effects of testosterone were not confined to laryngeal muscle; labeled cells were numerous within laryngeal perichondrium and included mature chondrocytes within the cartilagenous matrix, thus indicating chondrogenesis (Fig. 2). In response to androgen treatment, the perichondrial zone underwent marked expansion (Fig. 1) and is one of the most heavily labeled regions in testosterone-stimulated larynx.

If testosterone is stimulating myogenesis in juvenile larynx, we would expect to observe proliferation of myogenic cells followed by the presence of labeled myonuclei. Muscle is a heterogeneous tissue type that includes myofibers, fibroblasts, pericytes, and satellite cells. It is not possible to determine the cellular identity of labeled nuclei in autoradiograms prepared for light microscopy (see Fig 1). Therefore, tissue obtained from juveniles treated with testosterone and [3H]thymidine was prepared for electron microscopy. Satellite cells were identified by their unique location under the basal lamina of a differentiated muscle fiber, a relatively heterochro-

**Table 1**

**Labeling Index of Testosterone-Treated and Control Juvenile Frogs in Laryngeal Muscle**

<table>
<thead>
<tr>
<th>T-treated</th>
<th>Sham-operated non-hormone-treated controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Labeled nuclei</td>
<td>(Total nuc. counted)</td>
</tr>
<tr>
<td>Male</td>
<td></td>
</tr>
<tr>
<td>30 ± 10</td>
<td>(504)</td>
</tr>
<tr>
<td>32 ± 5</td>
<td>(594)</td>
</tr>
<tr>
<td>33 ± 4</td>
<td>(412)</td>
</tr>
<tr>
<td>Female</td>
<td></td>
</tr>
<tr>
<td>37 ± 12</td>
<td>(403)</td>
</tr>
<tr>
<td>38 ± 6</td>
<td>(439)</td>
</tr>
<tr>
<td>44 ± 4</td>
<td>(398)</td>
</tr>
</tbody>
</table>

Notes. T-treated > controls; P < 0.001* (Mann–Whitney U test). Mean (±SEM) percent labeled nuclei (labeling index) after [3H]thymidine injection of testosterone-treated and untreated (control) individual juvenile frogs. Mean percent labeled nuclei was determined from six fields per larynx. Testosterone treatment was for 4 days. Both sham-operated non-hormone-treated controls and T-treated juveniles received a single injection of [3H]thymidine on Day 4 and were sacrificed 24 hr following nucleotide injection.
FIG. 1. Photomicrographs of laryngeal autoradiograms. (a) The first panel shows the three tissue types in a sham operated, non-hormone-treated juvenile larynx (control). No labeled cells are present in the cartilage (C) or perichondrium (P). Two labeled cells (arrows) can be seen in the muscle (M). Control juveniles were sacrificed 24 hr after a single injection of \(^{3}\)Hthyridine. (b) In larynx treated 4 days with androgen and then injected with \(^{3}\)Hthyridine, both perichondrium and muscle show heavy labeling. Note that the perichondrial layer has also undergone marked expansion as indicated by the white bracket. Photomicrograph was obtained from the same section level as the control (Fig. 1a) for comparison. As with the controls, the androgen-treated juveniles were sacrificed 24 hr after injection of the radiolabeled nucleotide. Bar: 10 μm.

FIG. 2. Autoradiogram of testosterone-treated laryngeal cartilage showing numerous chondrocytes. (Juvenile treated as in Fig. 1b). Many cells deep within the cartilaginous matrix accumulate silver grains (e.g., arrows) suggesting that in situ chondrogenesis is stimulated by androgen administration. Bar: 5 μm.
FIG. 3. Identification of proliferating cell types in laryngeal muscle after 4 days of testosterone stimulation. As in Fig. 1b, a single injection of [3H]thymidine was given and tissue processed 24 hr later. Plastic sections (0.5 μm) of muscle were prepared for tritiated thymidine autoradiography and exposed for at least 3 weeks. Heavy labeling assisted identification of [3H]thymidine-labeled cells. Immediately adjacent thin sections were examined at low magnification under the electron microscope (X100) to locate labeled cells. The identity of the labeled cell nucleus was then determined at higher magnification (X100,000). (a) Autoradiogram of laryngeal muscle obtained after 4 days of testosterone treatment; plane of focus is between tissue and silver grains. Arrow indicates labeled cell nucleus. Bar: 2.5 μm. (b) Adjacent low magnification electron micrograph. The three most common nuclei types found in skeletal muscle (myonuclei, mn; satellite cell nuclei, SC; and fibroblast nuclei, fb) can be seen. Only the satellite cell (arrow) is labeled. Occasional fibroblasts and endothelial cells (not shown) were also labeled although at a much lower frequency than satellite cells. Abbreviation: (mf, myofiber). Bar: 2.5 μm. (c) At higher magnification (X100,000) a clear boundary (see arrows) can be seen to separate the satellite cell (sc) from the closely apposed muscle fiber (mf). The satellite cell has a large nucleus and is enveloped by the basal lamina (bl, arrow) of the adjacent muscle fiber. Bar: 2 μm.

The proliferative response to androgens is not seen in adults. Autoradiograms of adult larynges, prepared according to the protocol followed for juveniles, revealed little or no labeling (labeling index <1%; n = 2 males, n = 2 females). It may be argued that since these studies were carried out on intact (nongonadectomized) adults, the testosterone would be competed away by endogenous hormones, in particular estrogens in the female. Thus, we also carried out experiments on six adult females that were ovariecotimized before androgen treatment and injected with [3H]thymidine every day for 2 weeks. Although the larynges double in size and weight after such prolonged treatment, autoradiographic examination showed no difference in percent labeled nuclei compared with controls (labeling index <1%; n = 4 females). These two observations, androgen-induced cell proliferation in sexually immature frogs and the lack of a proliferative response in adults, suggest that males, which are normally exposed to testicular androgen, have bigger larynges than females due to androgen-induced proliferation during the juvenile period and that this proliferative ability is lost later in development.

What accounts for the loss of mitotic responsiveness of laryngeal muscle to androgen at sexual maturity? One explanation might involve an age-dependent decline in myogenic cells as noted by Schultz and others (Schultz, 1974; and reviewed in Campion, 1984) who have observed that satellite cells decrease in relative as well as absolute numbers during postnatal growth in mice. An alternative hypothesis is that an androgen-responsive subpopulation of myoblasts loses hormone sensitivity or undergoes selective cell death by the time of sexual maturity.

The proliferative response to androgens in the developing larynx of juvenile Xenopus laevis and that one source of new myonuclei is muscle satellite cells. The proliferative response to androgens is not seen in adults. Autoradiograms of adult larynges, prepared according to the protocol followed for juveniles, revealed little or no labeling (labeling index <1%; n = 2 males, n = 2 females). It may be argued that since these studies were carried out on intact (nongonadectomized) adults, the testosterone would be competed away by endogenous hormones, in particular estrogens in the female. Thus, we also carried out experiments on six adult females that were ovariecotimized before androgen treatment and injected with [3H]thymidine every day for 2 weeks. Although the larynges double in size and weight after such prolonged treatment, autoradiographic examination showed no difference in percent labeled nuclei compared with controls (labeling index <1%; n = 4 females). These two observations, androgen-induced cell proliferation in sexually immature frogs and the lack of a proliferative response in adults, suggest that males, which are normally exposed to testicular androgen, have bigger larynges than females due to androgen-induced proliferation during the juvenile period and that this proliferative ability is lost later in development.

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The sex difference in size of the larynx of X laevis is most probably the result of male-specific androgen regulation of cell number and cell size. As demonstrated here, during juvenile stages androgen induces proliferation of chondroblasts and of myoblasts, which are incorporated into maturing cartilage and muscle. We do not exclude the possibility that androgens also act to
Fig. 4. Identification of [3H]thymidine-labeled cell types after 3 weeks of testosterone stimulation. Animals were injected with [3H]thymidine on Days 4 and 5 of the hormone treatment. Two injections were given to assure adequate labeling of myogenic precursors. Method for cell identification was the same as in Fig. 3. (a) Light microscopic autoradiogram of a labeled cell in a 0.5-μm plastic section. Label is concentrated over the nucleus (arrow). Bar: 10 μm. (b) Adjacent EM section (×100). The nucleus (mn) is associated with a myofiber containing numerous myofilaments and mitochondria. Bar: 10 μm. (c) At higher magnification, the nucleus is clearly located within the cytoplasm, underneath the myofiber cell membrane (arrow). The labeled cell nucleus is thus identified as a myonucleus. Bar: 7.5 μm.

promote survival of cells during juvenile stages. Such a mechanism, prevention of cell death, has been shown to be responsible for sex differences in rat levator ani (Ci-hak et al., 1970). The possibility that androgen-induced myogenesis also plays a role in the masculinization of the levator ani remains to be examined. In addition, an-
drosis-induced hypertrophy of existing cells in adulthood would further magnify the male–female difference in laryngeal size without, however, concomitant cell division.

We do not know whether laryngeal cell proliferation is a direct effect of testosterone or an indirect effect exerted through androgen action on other tissue types. Androgen-sensitive muscles, including rat levator ani, bird syrinx, and X laevis laryngeal muscle, have high concentrations of androgen receptor as compared with other striate muscle (Jung and Baulieu, 1972; Lieberburg and Nottebohm, 1979; Segil et al., 1983) which suggests that androgens can act directly on muscle tissue. Androgen-target muscles are innervated by androgen-concentrating motor neurons (Kelley et al., 1975; Kelley, 1980; Breedlove and Arnold, 1980; Arnold et al., 1976) whose activity could mediate or induce proliferation via a trophic signal.

An important observation related to the control of muscle development is that muscle size can influence the survival of the motor neuron population that provides innervation (Hamburger, 1939; Oppenheim, 1981; McLennan, 1982). The motor neuron pools that innervate the rat levator ani and the frog larynx contain more neurons in males than in females (Breedlove and Arnold, 1981; Hannigan and Kelley, 1981). Evidence that androgens regulate motor neuron pool size comes from the following observations: (1) Androgen treatment during the perinatal period increases the number of motor neurons innervating the levator ani in female rats (Breedlove and Arnold, 1983b) and (2) androgens decrease motor neuron cell number in males (Breedlove and Arnold, 1983a). Thus, one possible scenario for the establishment of CNS sex differences in motor neuron number could involve a "rescue" of motor neurons from ontogenetic cell death by an increase in muscle size. Androgen-induced myogenesis may underlie such a process during normal development of sexually dimorphic neuromuscular systems.

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