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Estrogen regulation of the male reproductive tract in the frog, *Rana esculenta*: A role in Fra-1 activation in peritubular myoid cells and in sperm release

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Abstract

Endogenous and environmental estrogens have been proved to affect male reproduction in vertebrates. Both positive and negative effects in the regulation of the reproductive tract have been described. Since it is well known that amphibians represent a useful model to study several aspects concerning reproductive activity, we have taken advantage of the frog, *Rana esculenta*, to study the involvement of estrogens in sperm release. We show here that pituitary hormones increased the number of peritubular myoid cells (PMCs) expressing Fra-1 and induced testicular morphological changes related to sperm release. The estrogen antagonist ICI182-780 counteracted the hypophysis driven effects. *In vivo* and *in vitro* experiments demonstrated that 17β-Estradiol acted directly on the testis to switch-on Fra-1 in PMCs. Furthermore, impairment of estrogen activity significantly reduced sperm release mainly affecting the detachment of spermatozoa from Sertoli cells (spermiation). Therefore, estrogens can be considered a new entry in the list of substances involved in spermiation.

Keywords: Sperm release; Spermiation; Sperm transport; Fra-1; Estrogens; Peritubular myoid cells; Male reproductive tract

1. Introduction

Spermatogenesis is the process whereby immature germ cells (spermatogonia, SPG) develop into spermatids (SPT) which elongate through spermiogenesis. This process occurs among the somatic Sertoli cells in the seminiferous epithelium of the testis and is dependent on endocrine, paracrine and autocrine communications. When elongated spermatids undergo their final maturation to become spermatozoa (SPZ), they are released from Sertoli cells into the tubule lumen via a process termed spermiation (Hess, 1999). Simultaneously, peritubular myoid cells (PMCs) surrounding seminiferous tubules propel SPZ through tubules and ducts via rhythmic contractile activity: this process is known as sperm transport (Hargrove et al., 1977; Ellis et al., 1981; Maekawa et al., 1996). Spermiation and sperm transport are closely related as far as the timing is concerned. They contribute together to sperm output from the testis. Hence, in the present paper, we collectively refer to them as "sperm release". Few data are available on signals responsible for sperm release.

It is well known that androgens are critical for male reproduction whereas a definite role for estrogens remain unclear (Hess et al., 1997). Recent observations have unequivocally demonstrated in vertebrates that estrogens regulate the physiology of the male reproductive tract as well as the fertility process (Hess et al., 1997; O'Donnell et al., 2001; Pierantoni et al., 2003). Indeed, estrogens and estrogen receptors (ERs) are produced in the testis (O'Donnell et al., 2001; Pierantoni et al., 2003).

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Furthermore, infertility affects knock out male mice for alpha ER (αERKO) or aromatase (ArKO) genes (Lubahn et al., 1993; Eddy et al., 1996; Robertson et al., 1999). αERKO males are characterized by low sperm count and defective sperm function (Eddy et al., 1996). The exposure to environmental estrogens has been related to the decrease of sperm count observed in humans over the past 50 years (Sharpe and Skakkebaek, 1993; Auger et al., 1995; Toppari et al., 1996). Estrogen administration impairs sperm production and maturation in mice (Steinberger and Duckett, 1965; Meistricth et al., 1975).

In amphibians, estrogens regulate testicular activity (Cobellis et al., 2003a,b; Pierantoni et al., 2003). In particular, estrogens act on the frog, *Rana esculenta*, testis to induce SPG proliferation through c-Fos (Cobellis et al., 1999, 2002, 2003a,b) and induce Fra-1 expression (Cobellis et al., 2002). c-Fos and Fra-1 are Fos family proteins that require heterodimerization with one of the Jun family members to be functional as AP1 transcription factor (Karin et al., 1997). Recently, we have reported the presence of Fra-1 in PMCs of *R. esculenta* and rats (Cobellis et al., 2005a). In the frog, Fra-1 cyclically appears in March–April period, when mating occurs (Cobellis et al., 2005a,b). Besides possessing contractile activity, PMCs secrete a number of substances, including extracellular matrix components and growth factors, some of which are known to regulate Sertoli cells in a paracrine fashion (Maekawa et al., 1996). Although PMCs have morphologically and biochemically been characterized (Tung and Fritz, 1990; Galdieri and Ricci, 1998) and recent reports describe paracrine factors regulating their functions (Tripiciano et al., 1996, 1999), very few data are available concerning PMC activity during sperm transport (Tripiciano et al., 1996, 1999).

Due to morphological and physiological features, lower vertebrates have greatly contributed to give insight into the role of estrogens on the regulation of testicular activity (Pierantoni et al., 2002). Thus, using the frog, *R. esculenta*, we have already demonstrated that hypophyseal hormones regulate sperm release (spermiation and sperm transport) (Minucci et al., 1989; Cobellis et al., 2005a) and simultaneously increase the number of PMCs expressing Fra-1 (Cobellis et al., 2005a,b). Our scope is now to establish how the hypophyseal activity switches-on Fra-1 expression in peritubular compartment. We strongly suppose that the hypophysis acts on sperm transport through estrogens which in turn regulate PMC activity. Several information support our hypothesis: (1) in mammals, FSH regulates estrogen production (Pierantoni et al., 2003); (2) in *R. esculenta* both hormones are available in March–April period (Polzonetti-Magni et al., 1998), when mating as well as sperm release and appearance of Fra-1 in PMCs occur (Polzonetti-Magni et al., 1998; Cobellis et al., 2005a,b); (3) in frogs, hypophyseal hormones regulate sperm release (Pierantoni et al., 2003; Cobellis et al., 2005a) and simultaneously increase the number of PMCs expressing Fra-1 (Cobellis et al., 2005a,b). Therefore, we have planned experiments in order: (I) to confirm whether pituitary activity induces Fra-1 expression in testes; (II) to establish whether estrogens have a role in the regulation of the pituitary-induced expression of Fra-1 in PMCs and (III) to establish whether estrogens are involved in sperm release (spermiation and/or sperm transport).

Here we provide new direct evidences about an additional emerging physiological role of estrogens in male fertility (O'Donnell et al., 2001).

2. Materials and methods

2.1. Animals

Male frogs (*R. esculenta*) were captured near Napoli (Italy) early in the afternoon during March–April period. Animals for *in vivo* treatments were maintained in plastic tanks ($50 \times 25 \times 17$ cm) with food (mealworms) and water *ad libitum*. Anaesthesia was carried out with MS222 (Sigma–Aldrich Corp., St. Louis, MO). Animals were sacrificed and testes were immediately fixed or processed for nuclear protein preparation.

This research has been approved by the Italian Ministry of Health.

2.2. 17β-Estradiol (E_2) treatments

All the doses used for *in vivo* and *in vitro* experiments have been chosen on the basis of dose–response experiments carried out in previous studies (Pierantoni et al., 1986).

2.2.1. In vivo experiments: multiple injections

Animals ($n = 30$), collected during March, were divided into 3 experimental groups as follows: frogs ($n = 10$ control group) treated with 100 μl of amphibian Krebs Ringer Buffer (aKRB), pH 7.6; frogs treated with 100 μl of aKRB containing 10^{-5} M E_2 ($n = 10$, E_2 treated group) or in combination with the estrogen antagonist ICI182-780 ($n = 10$, E_2 + ICI treated group). The ICI, injected 1 h before E_2, was used at 10^{-4} M. Injections were carried out in the dorsal sac for two weeks on alternate days. Two hours after the last injection, animals were anaesthetised and sacrificed by decapitation. Testes were removed and immediately processed for nuclear protein preparation ($n = 14$/group) or Fra-1 immunolocalization ($n = 6$/group). The choice of ICI has been carried out on the basis of the antagonist properties (e.g., specificity for both α and β estrogen receptors—particularly for α receptor—lack of agonistic effects) (Stygar et al., 2003).

Multiple injections using 10^{-5} M E_2 were carried out in order to achieve a consistent amount of the protein to be assayed after a chronic treatment.

Counting of PMCs expressing Fra-1 was carried out on 3 randomly chosen sections/testis from 3 animals. Values are expressed as Fra-1 immunopositive PMC/total tubules/section × 100 (21).

2.2.2. In vivo experiments: single injection

Animals ($n = 30$), collected during March, were divided into 3 experimental groups and injected in the dorsal sac as follows: 100 μl of aKRB ($n = 10$ controls); 100 μl of aKRB containing 10^{-4} M E_2 ($n = 10$ E_2 10^{-4} treated group) or 10^{-5} M E_2 ($n = 10$ E_2 10^{-5} treated group). Two hours after the injection, SPZ and testes were collected and used for sperm release evaluation. We have used 10^{-5} M E_2 (already tested in the multiple injection experiment) and 10^{-4} M E_2 in order to achieve an effect on spermiation using an acute treatment.

2.2.3. In vitro experiments

Testes of animals ($n = 15$) collected in March were rapidly removed, washed and then incubated in a shaking water bath for 1 h at 22 °C in 10 ml aKRB ($n = 10$, control group), or 10^{-6} M E_2 ($n = 10$, E_2 treated