Expression of aromatase and steroidogenic factor 1 in the lung of the urodele amphibian Pleurodeles waltl

Sandra Kuntz, Dominique Chardard, Amand Chesnel, Mariette Ducatez, Martine Callier, Stéphane Flament

0013-7227/04/$15.00/0
Printed in U.S.A.

Endocrinology 145(7):3111–3114

doi: 10.1210/en.2004-0245

Expression of Aromatase and Steroidogenic Factor 1 in the Lung of the Urodele Amphibian *Pleurodeles waltl*

SANDRA KUNTZ, DOMINIQUE CHARDARD, AMAND CHESNEL, MARIETTE DUCATEZ, MARTINE CALLIER, AND STEPHANE FLAMENT

EA 3442 Génétique, Signalisation, Différenciation, Université Henri Poincaré, Nancy I. Faculté des Sciences, BP 239, 54506 Vandoeuvre-lès-Nancy Cedex, France

We report here the results of the analysis of aromatase and steroidogenic factor 1 (Sf1) expression in adult lung of the urodele amphibian *Pleurodeles waltl*. Using RT-PCR experiments, we show the expression of the estrogen-synthesizing enzyme, aromatase, in this organ. In the lung, no significant difference between males and females was observed in the level of aromatase mRNAs. Aromatase mRNA levels were also identical to those found in the brain or the testis, but the levels were 2-fold lower than in the ovary. Aromatase activity measurements revealed the presence of an active form of aromatase in the lung, which was similar in males and females. There was no difference in the level of aromatase activity between lung, brain, and testis, but a higher activity was measured in the ovary (13.7-fold compared with testis). Therefore, the differences in aromatase mRNA level between the ovary and the other organs did not mirror the differences in aromatase activity, suggesting the involvement of posttranslational events. Aromatase was also expressed in the lung of the anuran amphibian *Xenopus laevis*. In *Pleurodeles* lung, Sf1 mRNAs were also detected. There was no difference between males and females in the level of these mRNAs. The Sf1 mRNA levels were not significantly different from those measured in the brain, but a significant 2.1-fold higher level of expression was found in the gonads. These results demonstrate clearly the expression of steroidogenic markers in the adult lung of amphibians, but the biological significance of this remains to be determined. (*Endocrinology* 145: 3111–3114, 2004)

MANY PHYSIOLOGICAL FUNCTIONS, including metabolism, homeostasis, growth, and reproduction, are under hormonal control or, at least, are influenced by hormones (1). Sex steroids play a key role in reproductive function among all vertebrate species and in sex differentiation of nonmammalian vertebrates. Estrogens are synthesized by a microsomal enzyme complex termed aromatase, which is comprised of aromatase cytochrome P450 and a ubiquitous reduced nicotinamide adenine dinucleotide phosphate-cytochrome P450 reductase (2). Aromatase cytochrome P450 is responsible for binding the C19 steroid substrate (androstenedione and testosterone) and catalyzing the rate-limiting reaction in estrogen biosynthesis, which leads to the phenolic A ring typical of estrogens. In most vertebrate species that have been studied, aromatase expression occurs exclusively in the gonads and brain. In adults of humans and other primates, aromatase is also found in the placenta and in adipose tissue. In these mammals, in fetal tissues, the distribution of aromatase is widespread (3–7). Thus, in addition to the endocrine action of estrogens, the local production of estrogens acting in an autocrine or a paracrine way is also important.

One of the known regulators of aromatase expression is steroidogenic factor 1 (Sf1) (8, 9). Sf1 encodes a nuclear orphan receptor belonging to the nuclear receptor superfamily (10). The sequence and function of this gene are highly conserved among many taxa. It is expressed in several tissues involved in steroidogenesis and/or reproduction, including the adrenal gland in mammals and its corresponding organ in other species (*e.g.* interrenal organ in amphibians), the brain, and the gonad (11).

In the context of sex differentiation, we have performed several studies on the urodele amphibian *Pleurodeles waltl.* Aromatase activity measurements in the gonads of the larvae revealed a higher level in females than in males (12). We have cloned the P_{450}-aromatase gene in that species (GenBank accession no. AY135485) and observed that the differences in aromatase activity might be explained at least in part by differences at the translational level (13). We have recently cloned the Sf1 gene in *P. waltl* (GenBank accession no. AY540336) to determine whether the differences in aromatase expression could be related to differences in Sf1 expression.

During the study of adult tissues, we observed an expression of both aromatase and Sf1 in the lung of *Pleurodeles waltl*. We report here the results of a more detailed analysis of this organ. Our results showing the expression of active P_{450}-aromatase and Sf1 in the lung of *Pleurodeles waltl,* as well as aromatase expression in the lung of *Xenopus laevis*, suggest that this organ could have a steroidogenic function in some amphibian species.

Materials and Methods

Animals

Adults of *P. waltl* and *X. laevis* were reared in fresh water at 20 ± 2 C in our laboratory. Animals were anesthetized in a solution of benzocaine (0.03%), and several organs, such as brain, testis, ovary, heart,

Abbreviations: EF1-α, Elongation factor 1-α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Sf1, steroidogenic factor 1.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

liver, intestine, kidney, spleen, and lung, were dissected and checked for aromatase and Sf1 expression.

RT-PCR analyses

The detailed protocol for reverse transcription has previously been described (13). Total RNA was extracted from 50–100 mg of tissue using 1 ml of TRIzol reagent (Invitrogen Corp., Carlsbad, CA) and quantified. Total RNA (3 μg) was reverse transcribed using hexamer primers and 100 U Moloney murine leukemia virus reverse transcriptase (Invitrogen) in a total volume of 25 μl. A 2-μl aliquot of resultant cDNA was used for PCR. The amplification was performed with 0.1 U *Taq* DNA Polymerase (Invitrogen) in PCR buffer containing 25 mM of each deoxynucleotide triphosphate, 2.5 mM $MgCl_2$, and 0.4 pM of each primer in a total volume of 25 μl.

Specific primers for *P. waltl* P_{450}-aromatase (GenBank accession no. AY135485) were 5′-ATTGCAGCACCTGACACGAT-3′ and 5′-TTGTTCTGTACATTCTCTAA-3′ and led to a PCR product of 510 bp in length. For *X. laevis* aromatase, the primers were deduced from the sequence published by Miyashita *et al.* (14); the primers were 5′-CATTTCTTATGGCCGTTT-3′ and 5′-TTGCATAAGTCCTGGACCAGAC-3′ and led to a 300-bp PCR product. The primers for Sf1 were 5′-GGGTACACCTACCCACACTT-3′ and 5′-TCTTGGCCTGTGACCAGGAG-3′. They were deduced from the sequence of *P. waltl* Sf1 cDNA that we recently cloned (GenBank accession no. AY540336). The resulting PCR product was 462 bp in length. All the PCR products encompass two exon/intron boundaries based on other vertebrate aromatase and Sf1 genes.

As a control to ensure that the RNA was not degraded or to prevent poor transcription quality, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used in the case of *P. waltl* (primers: 5′-ACTACAAAGGACTAGGTCAGG-3′ and 5′-CCGGTATTGCACTCAACGACC-3′, leading to a 290-bp fragment), whereas elongation factor 1-α (EF1-α) was chosen in the case of *X. laevis* (primers: 5′-CCTGAATCACCCAGGCCAGATTGGTG-3′ and 5′-GAGGGTAGTCTGAGAAGCTCTCCACG-3′, leading to a 222-bp fragment). The PCR conditions were 1 min denaturation at 94 C, 1 min annealing, and 1 min elongation at 72 C. Annealing temperatures were 52 C for *Pleurodeles* aromatase and GAPDH, 60 C for Sf1, and 57 C for *Xenopus* aromatase and EF1-α. We performed 35 cycles for qualitative analyses. For semiquantitative analyses, a 26-cycle amplification was performed because, in that case, the amplification takes place in the linear phase of the PCR.

PCR products (10 μl) were run in a 1% agarose gel and either observed directly or transferred to nylon membrane (Hybond-N; Amersham Biosciences, Piscataway, NJ) before hybridization with digoxigenin-labeled probes and detection by chemiluminescence using standard conditions (13). The intensity of the hybridization signal was measured by using the GelDoc 2000 (Bio-Rad Laboratories, Hercules, CA) and a software package (Quantity One version 4.3.1, Bio-Rad Laboratories). The relative amounts of either P_{450}-aromatase or Sf1 mRNA were normalized to GAPDH for *Pleurodeles* and to EF1-α for *Xenopus*.

The PCR products were sequenced to verify that they had the expected nucleotide sequences for both aromatase and Sf1 (Genome Express, Paris, France).

Aromatase activity measurement

The activity of the enzyme was determined by the use of the tritiated water method as previously described (12). The radioactivity from tritiated water released by aromatization was measured, and enzymatic activity was calculated and expressed in femtomoles of estrone produced per hour per milligram of tissue.

Statistical analysis

Aromatase activity, aromatase mRNA, and Sf1 mRNA were quantified one time per organ for each animal. The results are expressed as mean ± SE of several measurements performed on different animals as indicated in the text. Data were analyzed by means of ANOVA and Student-Newman-Keuls multiple comparison procedures using the SPSS 11.5 software (SPSS Inc., Chicago, IL).

Results and Discussion

Qualitative RT-PCR analyses (Fig. 1) revealed the presence of aromatase mRNA in the lung of adult *Pleurodeles*. The sequence of the PCR product showed 100% identity with the cDNA sequence (data not shown), demonstrating that there was no doubt about the nature of the signal detected. Aromatase expression was also found in gonads and brain, whereas no signal was observed in other organs such as heart, liver, intestine, kidney, or spleen. Semiquantitative measurements (Fig. 1) revealed that the amount of mRNA was not significantly different between males and females in different organs. In the lung, the aromatase to GAPDH mRNA ratio appeared similar to that observed in the brain (males and females grouped: 0.59 ± 0.19 in lung *vs.* 0.6 ± 0.24 in brain) but was significantly lower than the ratio measured in the gonads (males and females grouped: 1.2 ± 0.19; $P < 0.05$). In *P. waltl*, in both sexes, the lung is physically linked to the gonad. Thus, we verified that the presence of aromatase mRNA in this organ was not the result of a gonadal component. A lung was divided into three pieces, posterior, medium, and anterior, and the most posterior and the most anterior ones were devoid of any link with the gonad. Aromatase mRNAs were found in equal amounts in all pieces

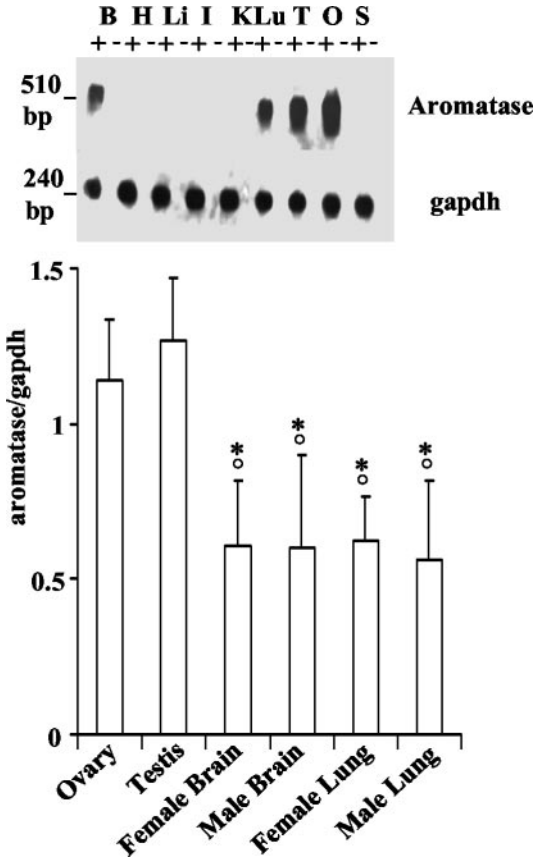

FIG. 1. Aromatase expression in different organs of *P. waltl*. *Top*, Hybridization signal after qualitative RT-PCR. For brain (B), heart (H), liver (Li), intestine (I), kidney (K), lung (Lu), testis (T), ovary (O), and spleen (S), two experiments with (+) or without (−) reverse transcriptase were performed. *Bottom*, The results of semiquantitative analyses (means ± SEs of four replicates; one measure per organ per animal). *, $P < 0.05$ *vs.* ovary and °, $P < 0.05$ *vs.* testis.

(data not shown), and this discarded the possibility of a gonadal contaminant.

To determine whether these mRNAs were subjected to translation, aromatase activity measurements were performed (Fig. 2). The strongest activity was found in the ovary in which the enzyme was able to aromatize 3.15 ± 1.9 fmol of (1β-^{3}H)androstenedione/h·mg of tissue. The activity in the testis was 13.7-fold lower (0.23 ± 0.08 fmol/h·mg of tissue; $P < 0.05$). The enzymatic activity in the lung was not significantly different from that measured in the testis (0.26 ± 0.12 and 0.23 ± 0.08 fmol of (1β-^{3}H)androstenedione/h·mg of tissue in female and male lungs, respectively). These activities were not significantly different from those of female or male brain.

It is interesting to note that the level of aromatase mRNAs is not correlated to the level of activity of the enzyme. Thus, a similar level of transcription seems to exist in testis and ovary, whereas the activity is 13-fold higher in the ovary. This indicates that posttranscriptional modifications are involved and that they are important for the final status of aromatase activity. This might be the result of a different concentration of the protein in the organs, although studies of the hypothalamus of the female frog *Rana esculenta* revealed changes in aromatase activity throughout the reproductive cycle without changes in aromatase concentration (15). Posttranslational modifications, such as phosphorylation, could also be involved because kinase activators or kinase inhibitors are able to induce fast changes in cerebral aromatase activity in birds (16).

To our knowledge, the presence of aromatase in vertebrate lung has only been found previously in the case of human and monkey fetuses. This was observed by enzyme activity measurements (3, 4) as well as by RT-PCR amplification (5, 6) and more recently by real-time RT-PCR (7). In these experiments, no sex differences in aromatase levels were evident in the lung that could be in accordance with our results. Nevertheless, a widespread distribution was observed and the level of aromatase expression or activity in fetal lung was very low. Our results on adult *P. waltl* are different because we did not observe a ubiquitous distribution (no expression was observed in heart, liver, intestine, kidney, or spleen). Moreover, the level of aromatase expression or activity in the lung was as high as in the testis where aromatase is supposed to play a physiological role as in mammals (17).

Because the presence of active aromatase was demonstrated in *Pleurodeles* lung, we studied the expression of Sf1. Indeed, the product of this gene is known to be a regulator of aromatase expression (8, 9). The primers that we designed allowed us to amplify a cDNA fragment of 462 bp in ovary, testis, kidney, brain, spleen, and lung (Fig. 3). No expression was observed in heart, liver, and intestine. The sequence of the PCR product obtained from lung mRNAs showed 100% identity with the cDNA sequence (data not shown), demonstrating that there could be no doubt about the nature of the signal detected. Semiquantitative RT-PCR analyses revealed no significant difference between male and female Sf1 levels in all of the organs. There were no significant differences in the ratio of Sf1 to GAPDH between the brain and the lungs. A significant 2.1-fold higher ratio was found in the gonads compared with lungs (males and females grouped: 1.03 ± 0.29 *vs.* 0.49 ± 0.14).

So, Sf1 was expressed in *Pleurodeles* lung. The expression of Sf1 in an organ is not always associated with a steroidogenic function for this organ. For instance, in *Pleurodeles*, we have found Sf1 expression in the spleen, which is not known as a steroidogenic organ. However, in *Pleurodeles* lung, the expression of Sf1 was also accompanied by the expression of aromatase, and this could indicate a steroidogenic function.

To determine whether our findings about the lung of *P. waltl* might reflect a feature common to other amphibians, we studied aromatase expression in the anuran *X. laevis*, whose P_{450}-aromatase gene has already been cloned (14). A study of adult organs in that species has been performed previously, but it did not include the lung (14). Our RT-PCR analysis revealed aromatase expression in *X. laevis* lung too (Fig. 4). The PCR product had the expected sequence (data not shown). It is interesting to note that, in that species, there is no connection between the lung and the gonad. To our

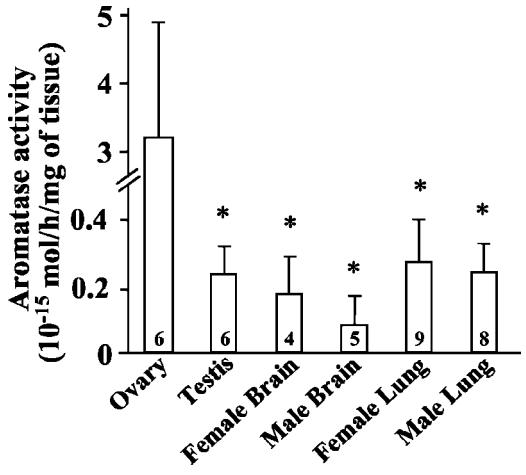

FIG. 2. Aromatase activity in several organs of *P. waltl*. Ovaries, testes, brains, and lungs were submitted to aromatase activity measurement by the tritiated water method. Values are means ± SEs of several replicates (one measure per organ per animal) whose number is indicated. *, $P < 0.05$ *vs.* ovary.

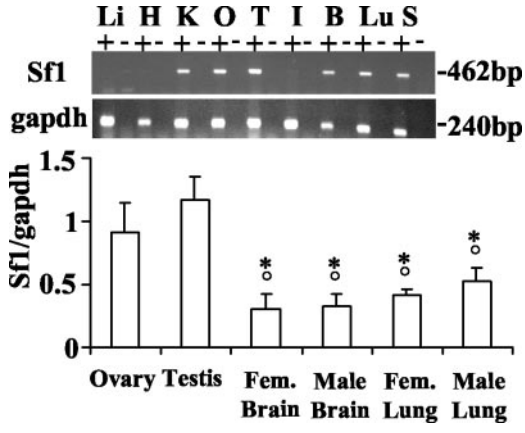

FIG. 3. Sf1 expression in different organs of *P. waltl*. *Top*, Typical result of a qualitative RT-PCR after ethidium bromide staining. *Bottom*, Results of semiquantitative analyses (means ± SEs of four replicates; one measure per organ per animal). *, $P < 0.05$ *vs.* ovary; °, $P < 0.05$ *vs.* testis.

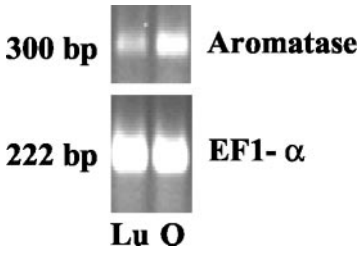

FIG. 4. Aromatase expression in the lung and ovary of *X. laevis*. The PCR products were run on an agarose gel and stained with ethidium bromide. EF1-α was used as a control.

knowledge, in other amphibian species, no study of aromatase expression has been performed in adult tissues.

In *Rana rugosa*, Northern blot analysis of Sf1 revealed only a faint band in testis samples, whereas RT-PCR revealed a wider distribution (testis, brain, spleen, and adrenal/kidney) (18). No expression was found in the lung, and surprisingly, no expression was detected in the ovary, which is in contrast to what is observed in other vertebrate species. In *Rana catesbeiana*, Sf1 was studied at the protein level using an immunserum raised against the mouse antigen (19). In the adult frog, the protein was found in different tissues including gonads, brain, interrenal, liver, and intestine, but unfortunately, the search for the protein in the lung does not seem to have been performed.

What could be the reason for such an expression of steroidogenic markers, especially aromatase, in the lung of *P. waltl* and *X. laevis*? One might expect that the local production of estrogens could regulate some aspects of lung physiology. Several roles for estrogens in the lung have been illustrated in mammals. For instance, estrogens, via their receptors α and β, seem to be involved in the proliferation of lung-derived cell lines, suggesting a potential role in normal and malignant proliferation processes (20, 21). In addition, in rats, 17β-estradiol attenuates chronic hypoxia-induced pulmonary hypertension by decreasing pulmonary expression of the vasoactive and mitogenic peptide endothelin-1 (22). Nevertheless, our knowledge of amphibian lung physiology is very poor, and further studies are required to determine the exact role for aromatase and Sf1 expressions in this organ.

Acknowledgments

We thank Dr. J. Magdalou and M.-H. Piet for their help in radioactivity measurements, Dr. H. Schroeder for statistical analysis, and Drs. C. Rickman and F. Kuepper for reading the manuscript. We also thank Jean-Charles Olry and Alain Iurétig for rearing the animals and Christiane Tankosic for her technical support.

Received February 25, 2004. Accepted April 1, 2004.

Address all correspondence and requests for reprints to: Pr. S. Flament, Université Henri Poincaré-Nancy I. Faculté des Sciences, EA3442, Entrée 1B, 9ème étage, Boulevard des Aiguillettes, BP 239, 54506 Vandoeuvre-lès-Nancy Cedex, France. E-mail: stephane.flament@scbiol.uhp-nancy.fr.

This work was supported by grants of the Université Henri Poincaré (BQR), of the Ligue Contre le Cancer, comités de la Meurthe et Moselle, de la Meuse et des Vosges, of the Association pour la Recherche sur le Cancer, and of the Conseil Régional de Lorraine. S.K. is a recipient of a grant from the French "Ministère de la Recherche."

References

1. **Schmidt-Nielsen K** 1997 Animal physiology. Cambridge, UK: Cambridge University Press
2. **Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorence S, Amarneh B, Ito Y, Fisher CR, Michael MD, Mendelson CR, Bulun SE** 1994 Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr Rev 15:342–355
3. **Doody KJ, Carr BR** 1989 Aromatase in human fetal tissues. Am J Obstet Gynecol 161:1694–1697
4. **Sholl SA, Goy RW, Kim KL** 1989 5α-Reductase, aromatase, and androgen receptor levels in the monkey brain during fetal development. Endocrinology 124:627–634
5. **Price T, Aitken J, Simpson ER** 1992 Relative expression of aromatase cytochrome P450 in human fetal tissues as determined by competitive polymerase chain reaction amplification. J Clin Endocrinol Metab 74:879–883
6. **Toda K, Simpson ER, Mendelson CR, Shizuta Y, Kilgore MW** 1994 Expression of the gene encoding aromatase cytochrome P450 (CYP19) in fetal tissues. Mol Endocrinol 8:210–217
7. **Pezzi V, Mathis JM, Rainey WE, Carr BR** 2003 Profiling transcript levels for steroidogenic enzymes in fetal tissues. J Steroid Biochem Mol Biol 87:181–189
8. **Michael MD, Kilgore MW, Morohashi K, Simpson ER** 1995 Ad4BP/SF-1 regulates cyclic AMP-induced transcription from the proximal promoter (PII) of the human aromatase P450 (CYP19) gene in the ovary. J Biol Chem 270: 13561–13566
9. **Carlone DL, Richards JS** 1997 Functional interactions, phosphorylation, and levels of 3′,5′-cyclic adenosine monophosphate-regulatory element binding protein and steroidogenic factor-1 mediate hormone-regulated and constitutive expression of aromatase in gonadal cells. Mol Endocrinol 11:292–304
10. **Whitfield GK, Jurutka PW, Haussler CA, Haussler MR** 1999 Steroid hormone receptors: evolution, ligands, and molecular basis of biologic function. J Cell Biochem 32–33(Suppl):110–122
11. **Val P, Lefrancois-Martinez AM, Veyssiere G, Martinez A** 2003 SF-1 a key player in the development and differentiation of steroidogenic tissues. Nucl Recept 1:8–31
12. **Chardard D, Desvages G, Pieau C, Dournon C** 1995 Aromatase activity in larval gonads of *Pleurodeles waltl* (Urodele Amphibia) during normal sex differentiation and during sex reversal by thermal treatment effect. Gen Comp Endocrinol 99:100–107
13. **Kuntz S, Chesnel A, Duterque-Coquillaud M, Grillier-Vuissoz I, Callier M, Dournon C, Flament S, Chardard D** 2003 Differential expression of P450 aromatase during gonadal sex differentiation and sex reversal of the newt *Pleurodeles waltl*. J Steroid Biochem Mol Biol 84:89–100
14. **Miyashita K, Shimizu N, Osanai S, Miyata S** 2000 Sequence analysis and expression of the P450 aromatase and estrogen receptor genes in the *Xenopus* ovary. J Steroid Biochem Mol Biol 75:101–107
15. **Guerriero G, Roselli CE, Paolucci M, Botte V, Ciarcia G** 2000 Estrogen receptors and aromatase activity in the hypothalamus of the female frog, *Rana esculenta*. Fluctuations throughout the reproductive cycle. Brain Res 880:92–101
16. **Balthazart J, Baillien M, Ball GF** 2001 Phosphorylation processes mediate rapid changes of brain aromatase activity. J Steroid Biochem Mol Biol 79:261–277
17. **Carreau S, Lambard S, Delalande C, Denis-Galeraud I, Bilinska B, Bourguiba S** 2003 Aromatase expression and role of estrogens in male gonad: a review. Reprod Biol Endocrinol 1:35–40
18. **Kawano K, Miura I, Morohashi K, Takase M, Nakamura M** 1998 Molecular cloning and expression of the SF-1/Ad4BP gene in the frog, *Rana rugosa*. Gene 222:169–176
19. **Mayer LP, Overstreet SL, Dyer CA, Propper CR** 2002 Sexually dimorphic expression of steroidogenic factor 1 (SF-1) in developing gonads of the American bullfrog, *Rana catesbeiana*. Gen Comp Endocrinol 127:40–47
20. **Mollerup S, Jorgensen K, Berge G, Haugen A** 2002 Expression of estrogen receptors α and β in human lung tissue and cell lines. Lung Cancer 37:153–159
21. **Zang EA, Wynder EL** 1996 Differences in lung cancer risk between men and women: examination of the evidence. J Natl Cancer Inst 88:183–192
22. **Earley S, Resta TC** 2002 Estradiol attenuates hypoxia-induced pulmonary endothelin-1 gene expression. Am J Physiol Lung Cell Mol Physiol 283:86–93