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Aromatase enzyme ($P450_{arom}$) mRNA expression in mouse gonads and the effect of intra-peritoneal amino acid injection

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Abstract

The aim of this study was show the presence of ($P450_{arom}$) mRNA expressions in mouse gonads and to measure the effect of intra-peritoneal serine/threonine or glycine injections on the expression of aromatase enzyme $P450_{arom}$ mRNA. Three different (1.4, 2.4 and 4.4kb) $P450_{arom}$ mRNA species were indentified in mouse testis and the ovary. Relative to saline injected group (S), all the species of $P450_{arom}$ enzyme mRNA decreased in serine/threonine (A) or glycine (G) injected female mice, while all of them were increased in male. Aromatase enzyme mRNA expression is differently regulated in male and female mice.

Key words: Aromatase Enzyme, Amino Acid, Gonad, mRNA, Mouse

Introduction

Aromatase enzyme (P450arom) encoded by the single-copy CYP19 gene and converts C19 androgens to C18 estrogens. Therefore, it is the rate-limiting enzyme in oestrogen biosynthesis (Bulun et al., 2003). Its expression in the gonads has been extensively studied over a number of years. However, there is not enough information available about the regulation of gonadal aromatase enzyme expression. Expression of the gene encoding aromatase is controlled by tissue-specific promoters, which modulate expression in diverse tissues including testes and ovary (Lanzino et al., 2001). A promoter proximal to the translation start site of aromatase gene, called promoter PII, regulates P450arom expression in ovary and testis (O'Donnell et al., 2001; Lanzino et al., 2001). Promoter PII activity is regulated by cAMP and requires the transcription factors cAMP response element binding protein (CREB), cAMP response-element modulator (CREM), and also steroidogenic factor-1 (SF-1) (Simpson, 2000). Differences in aromatase anzyme, in female mice, cause cessation of the antrum formation, ovulation and the increases in atresia (Fisher et al., 1998), while deficiency disrupts the spermatogenesis in male mice (Robertson et al., 1999).

Cyclic AMP response element binding protein (CREB), CREM and SF-1 are transcription factors and bind to cyclic AMP responsive elements in regulatory portion of aromatase gene (Montminy, 1997). The N-terminal half of CREB and CREM contains phosphorylation box also known as the kinase-inducible domain, which contains a cluster of sites phosphorylated by various kinases that regulate the transcription of aromatase gene (Gonzalez et al., 1991; De Cesare et al., 1999). Expression of aromatase gene is depending on the phosphorylation of CREB, CREM and SF-1 by kinases. The phosphorylation of these transcription factors occurs mainly on serine residues with less than 5% on threonine and none on tyrosine residues (Chua and Ferro, 2005).

Free serine and threonine amino acids are capable of phosphorylation (Lauren and Friedmann, 1971) by enzymes. Serine and threonine amino acids are phosphorilated by kinases, although non a specific kinas has been specified. Also, serine and threonine amino acids are taken up by intracellular proteins. In a study, non-phosphorylatable analogs of the serine and threonine used to determine the role of phos-phorylation in the acute regulation of steroidogenesis in MA-10 mouse Leydig tumour cells. According to their results, substitution of the threonine analogue into protein results in inhibition of hormone stimulated steroid production in these cells (Stocco and Clark, 1993).

Therefore, the aim of this study was to show the expression of P450arom enzyme in mouse testis and ovary and to monitor the changes in P450arom enzyme mRNA levels after intra-peritonal Injection of serine/ threonine mixture or glycine.

Materials and Methods

Nineteen and twenty one day old male (n=30) and female (n=30) mice (CD-1 strain) were injected (intra-peritoneal) daily between 10:00 and 12:00, for five days. With saline, as a control or a mixture of Serine/Threonine (Containing 0.264 µg/200µl L-Serine, Cat; 21101-019, Life Technologies, Paisley, Scotland/ 0.132µg/200µl L-Threonine, Cat; 103053, ICN, Ohio, USA) or 38 ng Glycine (Cat; G-8898, Sigma, Steinheim, Germany) in 0.2 ml saline. After five days, the mice were killed by cervical dislocation 60 minutes after the last injection then testes and overies were removed.

Removed testes and the ovaries were grounded in liquid nitrogen usuing a RNAase-free pestle and mortar. Ground tissue (100mg) was placed in 1ml Trizol reagent (Cat; 15596-026,100 ml, Life Technologies. Paisley, Scotland), in 2ml micro-centrifuge tube (Cat; 02-1420-2700, Fisher, Leicestershire, UK) The tubes containing the ground tissue and Trizol were left at room at room temperature for 5 minutes to permit complete dissociation of the nucleoprotein complexes. Then 0.2 ml of chloroform (0.2ml/1ml Trizol used) was added and the tube and shaken vigorously for 15 seconds then centrifuged at 1200g for 15 min at 4°C. The top colourless phase was transferred to a fresh eppendorf. The contents of each tube were mixed with 0.5 ml, per ml of Trizol, isopropyl alcohol (Cat; 405-7, Sigma. St. Louis, MO, USA), incubated at room temperature for 10 min. and centrifuged at 12000 g at 4°C for 10 min. The supernatant was removed and the RNA pellet washed with 1 ml of 75% ethanol (Cat; 200-578-x, Hyman Limited. Essex, England) and RNA recovered by centrifuging again at 7500g for 5 min at 4°C. Finally, the supernatant was removed, the RNA pellet dried briefly and then redissolved in 50µl nuclease free water which was prepared by adding 1ml diethyl pyrocarbonate (DEPC; Cat; D-5758, Sigma, Steinheim, Germany) to 1L dH2O.

Total RNA was quantified by using a RNA/DNA spectrophotometer (Mod; 80-2103-98, Ser; 66884, Pharmacia Biotech. Cambridge, England).Total RNA was converted to cDNA by reverse transcription by using Avantage RT-PCR kit (Cat; K1402-1, Colontech, Palo Alto, USA).

Synthesized cDNA was amplified by using specific primers for cytocrom P450arom. Probes were obtained by Polimerase Chain Reaction (PCR), two tubes and two step procedures were aplied. Primers for cytocrom P450arom kindly provided by Prof. PJ O'Shaughnessy (University of Glasgow Veterinary School Bearsden Rd, Glasgow G61 1QH).

Samples were run in 1% ultra pure agarose (Cat; 15510-019, Life Technologies. Paisley, Scotland). One-gram agarose was placed in a 500ml-glass flask and 100ml TEA buffer was added then placed in an oven to dissolve the agarose and allowed to boil until the agarose was completely dissolved. Then, 5µl of a 10mg/ml solution of Ethidium bromide (Cat; E-7637, Sigma, St Louis, MO, USA) was added, mixed gently and cooled down and then poured into an electrophoresis tank. When the gel was solid, it was placed in 1XTris ethylene acetate (TEA) buffer made up to 50X by adding 252 g Tris base (Cat; T-1503, Sigma. St Louis, MO, USA), 57.1ml Glacial Acetic Acid (Cat; 100001 8Q, BDH. Poole, England) and 100 ml 0.5 M EDTA (Cat; E-4884, Sigma. St Louis, MO, USA) then autoclaved. The DNA ladder, size marker, 100bp (Cat; H0595, Perkin Elmer, Germany) was made up by mixing 60µl ladder with 10 µl gel loading solution (Cat; G-2526, Sigma, Steinheim, Germany) from this 5µl was loaded. Both PCR product and DNA size marker were diluted in lauding buffer and run at 65V for 1.5h and then photographed under UV illumination. Probes were extracted by using quick gel extraction kit (Cat; 28704, Qiagen, Hilden, Germany).

Fifteen µg of total RNA from each experimental group was placed in 0.2 ml nuclease free micro tubes (Cat; AB-0337, Advanced Biotechnologies) and the volume made up to 15µl with sample buffer. The tubes, containing RNA plus sample buffer, were placed in a water bath at 61°C for 5 min and then immediately placed on ice, 1µl ethidium bromide (0.1mg/ml solution) was added. Fifteen µl was then loaded into the wells of the gel and run at 65V for 5h at room temperature. The gel was placed in saran film and photographed under UV illumination. The gel was then inverted and placed in a NaOH solution (2g NaOH in 1L DEPC water) for 40 min to produce sharper bands.

A plastic platform was placed in a 25X15cm plastic nuclease free container, Then a piece of 3mm Whatman filter paper was cut to the size of plastic platform and and immersed in transfer buffer (10X SSC). The wet filter paper was laid on the platform and pressed gently and slowly so that there were no bubbles between the platform and the filter paper. The paper covered the edges of platform, but did not touch the bottom of buffer container so as to facilitate upward transfer. Another piece of wet filter paper was placed on the top of platform without bubbles. The gel was taken out of the NaOH solution and placed on the filter paper that wells of the gel facing the platform. The edges of gel were protected from direct contact with the buffer. A piece of nucleic acid transfer membrane, Hybond-N+(Cat; I6435, Amersham, Buckinghamshire, England) cut to the size of the gel was placed on the gel. Another piece of wet filter paper was placed on membrane. To facilitate upward transfer of the buffer through the gel, by capillary movement, dry paper

towels were stacked and a weight was placed on the gel and left approximately for 18 hours to transfer the RNA from the gel to the nylon membrane The membrane was sandwiched between Whatman filter paper and left at room temperature for 10 min to dry. The gel was taken to a dark room to visualise the transfer. The membrane was placed in an oven at 80°C for 2h to fix the RNA. The dried membranes were kept in a secure place for hybridisation.

Fifteen ng of a DNA probe (for labelling) was dissolved in 16.27µl of distilled and filtered water, denatured at 100°C for 5 min and then chilled on ice and added with 2.4µl unlabelled dNTP, 3µl reaction buffer, 3µl primer solution, 3µl [α-32P] dATP (3000CI/mmol),1.2µl Klenow. To make a final volume of 30µl 1.13µl water was added to reaction tübe. The tube was mixed gently by pipetting up and down and then centrifuged to concentrate the contents at the bottom of the tube. The tube placed in a water bath at 37°C, the water bath was then switched off and left overnight for labelling. Labelled probe was extracted by using spin column.

The membrane was removed from the filter cover and placed in a clear roller bottle, in de-ionised water (100 ml) to wash out the agarose and salts from the membrane before pre-hybridisation. The washing was done by rolling the bottle by hand for 1-2 min and then pouring the water down the sink. The membrane was then placed in 10 ml hybridisation buffer preheated to 68°C and incubated in hybridisation oven for 50 min. One ml hybridisation buffer was added to a screw cap tube, containing 30 µl labelled and denatured probe and an 100 µl of denatured salmon sperm DNA (Cat; D-1626, Sigma, Steinheim, Germany). The tube was then mixed and the contents was transferred to a bottle and left to hybridise for 1h. One hour later the oven was opened and hybridisation buffer poured away. The blot was rinsed with 100 ml 2XSSC and 0.1X SDS (w/w) and washed with a mixture of 100ml 0.1XSSC and 0.1XSDS for 10 min at 37 °C and washed again with 100ml 0.1XSSC and 0.1XSDS at 60°C for 20 min. After washing, the blot was wrapped with PVC and monitored for radioactivity and then placed in a case with the RNA side next to the film. The blot was exposed at -70°C for 24h. After 24h, the case was opened under the safe red light and developed using a Konica SRX-101 film processor (X-Ograph imaging systems, Gloucestershire, England).

Quantification was done by using molecular analyst software (Copyright 1992-1995, Bio-Rad Laboratories, Hercules, USA). For each RNA transcript, background optical density was dismissed and the pipetting errors were corrected by dividing each transcript by β-actin which was hybridised on the same membrane used for the hybridisation of LH-R and P450scc enzymes.

Results and Discussion

The expression of $P450_{arom}$ enzyme in gonads has been a subject of considerable interest for a number of years. In this study, three different (1.4, 2.4 and 4.4kb) P450 aromatase ($P450_{arom}$) enzyme mRNA species were indentified in mice testis and the ovary. Relative to saline injected group (S), all the species of $P450_{arom}$ enzyme mRNA decreased in serine/threonine (A) or glycine (G) injected female mice, while all of them were increased in the male (Figure 1). The result presented here is dimorphic that is because males have a greater amount of testosterone, as compared to female. And this probably causes increase in $P450_{arom}$ mRNA expression in testis (Roselli et al., 1996). Serine/threonine injection decreased ($P450_{arom}$) enzyme mRNA expression in females. This may be a result of the decrease in phosphorylation of the transcription factors (CREM, CREB and SF-1) due to the decrease in kinase activity or increase in activity of the phosphatases.

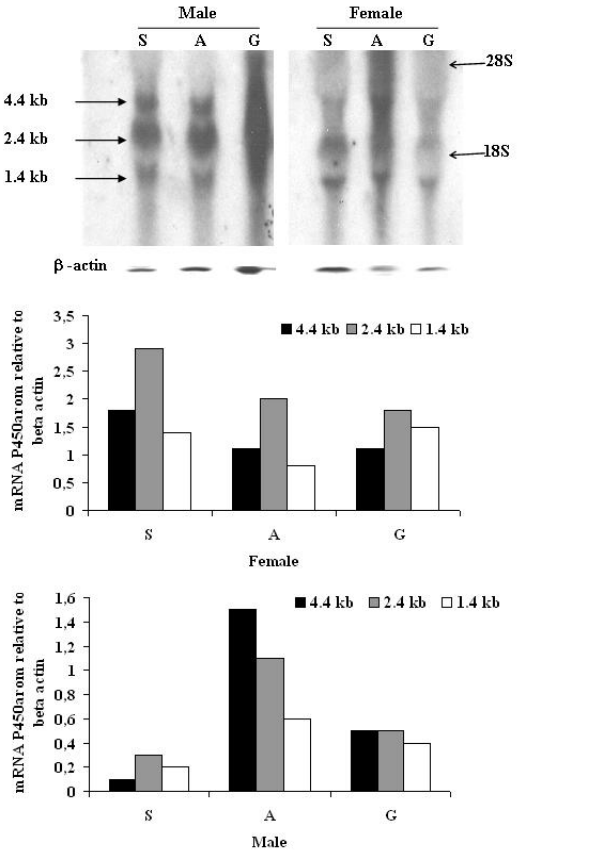

Figure 1. Three different $P450_{arom}$ mRNA species (1.4, 2.4 and 4.4kb) were identified in mice testis and ovary. All of mRNA species were decreased in female, while they all increased in male by serine/threonine or glycine injection.

A promoter proximal to the aromatase gene translation start site, known as promoter PII (Lazino et al., 2001; O'Donnel et al., 2001). The activity of PII is regulated by cAMP and requires the transcription factors CREB, CREM and SF-1 (Simpson, 2000). These transcription factors bears multiple serine and threonine residues for phosphorylation. Their functional activities, in aromatase gene expression, are dependent upon the phosphorylation.

Serine and threonine both are bearing an OH group on which they are both phosphorylated by enzymes. An enzyme in *Propionibacterium shermanii* which uses inorganic pyrophosphate to phosphorylate L-serine have been reported (Cagen and Friedmann, 1972). This indicates that free serine and threonine amino acids are also capable of phosphorylation by enzymes. Also Serine and threonine amino acids are taken up by intracellular proteins. In a study, non-phosphorylatable analogs of the serine and threonine used to determine the role of phosphorylation in the acute regulation of steroidogenesis in MA-10 mouse Leydig tumour cells. According to their results, substitution of the threonine analogue into protein results in inhibition of hormone stimulated steroid production in these cells (Stocco and Clark, 1993).

Conclusion

Regulation of aromatase gene expression was more complex than expected. It is differently regulated in male and female mice .Decrease in aromatase gene expressions, in female mice, probably due to the decrease in phosphorylation of free serine or threonine rather the phosphorylation of serine or threonine residues in transcription factors CREB, CREM and SF-1. Therefore, a study is required to measure the changes in phosphoserine, phosphothreonine as well as phosphatese concentrations after the intra-peritoneal injection of serine and threonine.

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