RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES
PRINT ISSN 2221-1896, ONLINE ISSN 2223-0343
www.roavs.com

Transforming growth factor β1 mRNA expression in mouse gonads and the effect of serine/threonine or glycine on its expression

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Abstract

The objective of this study was to show transforming growth factor-β1 (TGF-β1) mRNA expression in mouse gonads and to show the changes in TGF-β1 mRNA expression after the injection of serine/thraonine mixture or glycine. Male and female mouse (19-21 days old, CD-1 strain, n=60) were divided into six groups and intraperitonealy injected for 5 days with saline (control), a mixture of serine/threonine or glycine (tests). After five days, the mice were killed and gonads were removed under RNAase free conditions. Total RNA was extracted and transferred on to a nylon membrane and hybridized with ^{32}P-labelled cDNA probes. In both testis and ovary, a weakly expressed 5.2 kb spice of mRNA was detected and its expression was tended to increase by the injection of serine/threonine in ovaries and testes. The reason for increased TGF-β1 mRNA expression might be caused by increased intracellular concentration of glucose due to the serine/threonine or glycine injection.

Key words: Mouse, mRNA, Ovary, Testis, TGF-β1,

Introduction

Transforming growth factor-β subfamily (including TGF-βs, growth differentiation factor-9, mullerian inhibiting substance, bone morphogenetic proteins, inhibin and activin) is a member transforming growth factor-β superfamily. They are multifunctional proteins and are critically important in various physiological and developmental processes (Massague et al., 2000; Chang et al., 2002). In mammals, there are three isoforms of TGF-βs (TGF- β1, β2, and β3). They initiate signalling via the transmembrane type I (TGF-βRI) and type II (TGF-βRII) serine/threonine kinase receptors on the cell surface (Derynck, 1994; Kingsley, 1994). A third type of TGF-β receptor (TGF-βRIII), also called β-glycan, has been described as an accessory receptor, and it is known to facilitate the binding of TGF-β1 to TGF-βRII (Lopez-Casillas et al., 1993). Binding to TGF-βRII causes to RII recruit and transphosphorylate RI receptors, which is known as activin-like kinases, or ALKs. The phosphorylatin of RI receptors, on serine residues rich segment, leads to the activation of RI receptor linked TGF-β signal transuding smad2 proteins. Activated smad2 proteins are phosphorylated to bind to smad4 and to form smad2-smad4 complex. Then, this complex trans- located to cell nucleus and the transcriptions of TGF- β target genes are promoted (Massague, 1988; Kaivo-Oja et al., 2006).

By using reverse transcription–polymerase chain reaction, in situ hybridization, and immunohisto-chemistry, the expression of transforming growth factor-βI, βII, βIII and their receptors (TGF-βRI and TGF-βRII) have been shown in murine theca and granulosa cells, oocyte as well as in pre and post implantation embryos (Skinner et al., 1987; Feng et al., 1988; Teerds and Dorrington, 1993; Bernard et al., 1994; Gautier et al., 1994; Santamaria et al., 1995; Chow et al., 2001). In ovary, TBF-βs are predominately produced by theca cells (Skinner et al., 1987), but ıt is also produced by isolated granulosa cells in selected follicle stages (Christopher, 2000). They have critical roles in granulosa cell function and oocyte maturation (Knight et al., 2006). In testis, TGF-βs are produced by Leydig, Sertoli and peritubular cells and functioning paracrine and autocrine manner (Skinner et al., 2010).

Transforming growth factor-β1 (TGF-β1) is a 25 kD homodimeric polypeptide and first identified by its ability to induce a transformed phenotype in normal rat kidney fibroblast cells when grown in soft agar (Moses et al., 1981). It is an isoform of TGF-βs and it is expressed in theca, granulosa and luteal cells of mice ovary (Juneja et al., 1996). In mice, during the diestrus, it is weakly expressed in granulose cells of large healthy antral follicles, but not in large antral atretic follicles and also not in small and medium sized follicles (Christopher, 2000). Not enough information is available about the physiological action mode of TGF-

ßs in early folliculogenesis. Transforming growth factor-β1-null mice, maintained on an immunocom-promised genetic background, have a similar percentage of primordial, primary, and antral follicles as compared with controls (Ingman et al., 2006). While, an inhibitory role for TGF-β1 (in the presence of FSH) in preantral follicle development and progression, due to the increase in apoptosis at the primary and preantral stages of follicle development, was suggested in rat (Rosairo et al., 2008). In testis, TGF-β1 is expressed by the Sertoli cell (Skinner and Moses, 1989; Esposito et al., 1991) and its receptor is present in both somatic cells and germ cells (Teerds and Dorrington 1993; Caussanel et al., 1997; Olaso et al., 1998; Cupp et al., 1999). Therefore, it may also be important for spermatogenesis (Nargolwalla et al., 1990).

Serine and threonine amino acids are capable of phosphorylation in free form (Lauren and Friedmann, 1971) by enzymes. It might be possible that L-serine and L-threonine amino acids are the substrate for kinases *in vivo*, and this may change the activity of kinases leading to changes in TGF-β1 receptor activation and smad phosphorylation then changes in TGF-β1 mRNA expression and signalling. Therefore, the aim of this study was to show the presence of mRNA expression in mouse testis and ovary and to see the effect of serine/theronine or glycine injection on TGF-β1 mRNA expression.

Materials and Methods

Male and female mice (19-21 days old, CD-1 strain, n=60) were divided into six groups and daily injected (intra-peritoneal) 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/ 0.132 µg/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. The testes and overies were removed under RNAase free conditions and immediately stored in liquid nitrogen until the extraction of total RNA. Stored tissue was grounded in liquid nitrogen using a RNAase-free pestle and mortar. Then 100 mg of it was placed in 1ml trizol reagent (Cat; 15596-026,100 ml, Life Technologies. Paisley, Scotland), in 2 ml micro-centrifuge tube (Cat; 02-1420-2700, Fisher, Leicestershire, UK). The tubes containing the ground tissue and trizol were left 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 was shaken vigorously for 15 seconds then centrifuged at 1200g for 15 min at 4°C. The top colourless phase was transferred to a fresh tube. 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) and incubated at room temperature for 10 minutes, then centrifuged at 12000 g at 4°C for 10 minutes. The supernatant was removed, remaining 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 and then redissolved in 50 µl 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) and run in electrophoresis for the visualization of ribosomal bands (Figure 1).

Total RNA was converted to cDNA by reverse transcription by using Advantage RT-PCR kit (Cat; K1402-1, Colontech, Palo Alto, USA). Probes were obtained by Polimerase Chain Reaction (PCR)by using specific primers for TGF-β1 kindly provided from Dr. Gul ZAMAN (The Royal Veterinary Collage, Molecular Biology Unit, Royal Collage st., Camden Town, London). 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 loading buffer and run at 65V for 1.5 hour and then photographed under UV illumination (Figure 2).

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

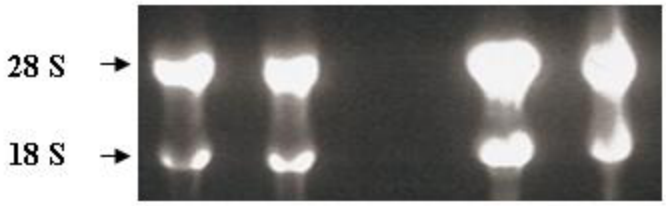

Figure 1. Fiveteen μg RNA run on gel containing 1.2% agarose in gel running buffer. The gel was run at 65V approximately 5 hours. During this time, ribosomal bands were spread.

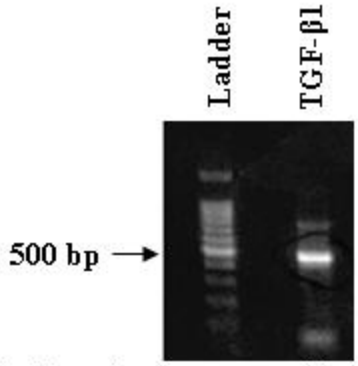

Figure 2. One μl primer was added to each tube and gaved approximately 480bp PCR product, which was extracted from the gel by using Quick gel extraction kit (Cat; 28704, Qiagen, Hiden, Germany). 100bp DNA ladder was used as a size marker.

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 16.27 μl of distilled and filtered water, denatured at 100°C for 5 minutes and then chilled on ice and added with 2.4 μl unlabelled dNTP, 3 μl reaction buffer, 3 μl primer solution, 3 μl [α-32P] dATP (3000CI/mmol),1.2 μl Klenow. To make a final volume of 30 μl 1.13 μl water was added to reaction tube. 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 minutes and then pouring the water down the sink. The membrane

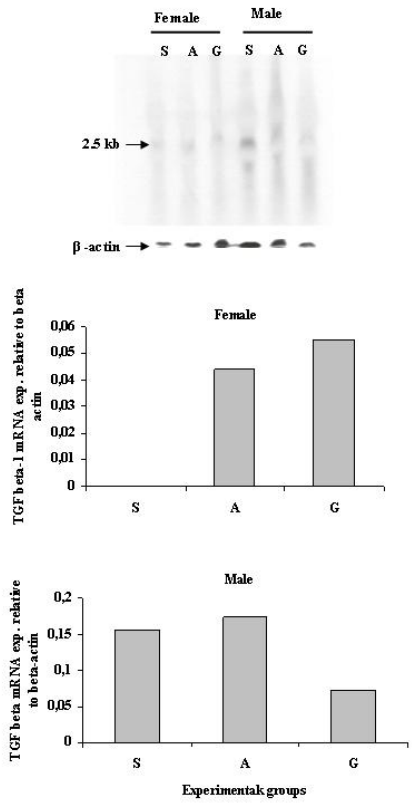

Figure 3. Male (M) and female (F) mouse were injected with saline (S), serine/threonine (A) or glycine (G). The injection of serine/threonine (A) or glycine (G) caused an increase in expression mRNA for TGF-β1 within the mouse ovary, while glycine injection reduced its expression in testis.

was then placed in 10 ml hybridisation buffer preheated to 68°C and incubated in hybridisation oven for 50 minutes. One ml hybridisation buffer was added to a screw cap tube, containing 30 µl labelled and denatured probe and 100 µl of denatured salmon sperm DNA (Cat; D-1626, Sigma, Steinheim, Germany). The tube was then mixed and the contents were transferred to a bottle and left to hybridise for 1hour. 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 100 ml 0.1XSSC and 0.1XSDS for 10 min at 37°C and washed again with 100 ml 0.1XSSC and 0.1XSDS at 60°C for 20 minutes. 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 24 hours. After 24hours, 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.

Results

The results show that TGF-β_1 mRNA is weakly expressed in both mouse testis and ovary. Especially in the ovary, very weak TGF-β_1 mRNA expression was measured (Figure 3) and only 5.2 kb spice of mRNA was detected. The expression of mRNA for TGF-β_1 was tended to increase by the injection of serine/threonine in both male and female mice.

Discussion

Transforming growth factor-β is synthesized, in a latent form, in tissues of the different organs including the gonads (Graycar et al., 1989). It is known to inhibit cell growth, proliferation and leading to apoptosis (Yamamura et al., 2000).

The data shows that TGF-β1 mRNA is expressed in mouse ovary and testes. Its expression in ovary was much weaker than the expression in testes. Here, injection of serine/theronine or glycine, to female mouse, increased TGF-β1 mRNA expression. The reason for this increase might be caused by elevated extracellular concentration of glucose due to the serine/threonine or glycine injection. Because serine, threonine and glycine amino acids are all glucogenic. Serine is catabolised by serine/threonine dehydratase to glycolytic intermediate, to 3-phosphoglycerate or to pyruvate. Threonine is catabolised by threonine aldolase to acetyl-CoA and glycine. Threonine aldolase also converts glycine to serine. Glycine is classified as a glycogenic amino acid, since it can be converted to serine by serine hydroxymethyltransferase.

Studies conducted in human have revealed that elevated extracellular D-glucose concentration increases transforming growth factor-β1 (TGF- β1) release from umbilical vein endothelium. In diabetic patents, an elevated plasma level of transforming growth factor-β1 is associated with a high extracellular D-glucose concentration (McGinn et al., 2003; Flores et al., 2004). It has been reported that, TGF-β1 synthesis, from human peritoneal mesothelial cells, was induced by D-glucose (Chan et al., 2003). The reason for elevated TGF-β1 synthesis is linked to the stimulation of TGF-β1 gene promoter by D-glucose (Phillips et al., 1995).

Briefly serine/threonine or glycine increase TGF-β mRNA expression as a result of the increase in intracellular concentration of glucose. Also serine/threonine amino acids block TGF-β1 signalling by depressing the phosphorylation of smad2 by activated TGF-βRI. Serine and threonine amino acids

are free substrate for serinelthreonine kinases. 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). Serine and theronine also block the phosphorylation of activator protein 1 (AP-1) complex of TGF-β1 promoter. Therefore, the synthesis of TGF-β1 was increased by serine/threonine or glycine injection, but the apoptotic effect of TGF-β1 was block by serine/threonine injection.

Conclusions

Serine/threonine or glycine injection may cause an increase in intracellular concentration of glucose, leading to the increase in TGF-β1 mRNA expression. At the same time, serine and threonine amino acids block the apoptotic effect of TGF-β1 by interrupting the psophorylation of smad2 and AP-1. Because, serine and threonine are substrate to serine threonine kinases. It is possible that, these kinases tend to prepare to phosphorylate free serine threonine rather to phsphorylate smad2 and AP-1.

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