Localization of Endothelin Receptor Subtype-A in the Testis of Rats, Dogs, and Monkeys

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ABSTRACT. In order to evaluate the physiological roles of the testicular endothelin (Edn) signaling via Edn receptor subtype-A (Ednra) in mammals, the localization of Ednra was investigated by *in situ* hybridization and immunohistochemistry in the testis of rats, dogs, and monkeys. For *in situ* hybridization, a rat *Ednra* RNA probe which is highly homologous to the subcloned canine and monkey *Ednra* (88.7% and 87.9% identical, respectively) was used. Both *Ednra* mRNA and protein were detected in interstitial cells and cells in the basal compartment of the seminiferous tubules, mainly Sertoli cells, as well as spermatogonia and some early spermatocytes, but not spermatids. The localization pattern of *Ednra* was exhibited in a same manner among species, indicating that the physiological role of Edn signaling throughout *Ednra* was maintained in the mammalian testis.

KEY WORDS: *Ednra*, endothelin, testis.

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The endothelins (Edns) consist of three isopeptides of Edn (Edn1, Edn2, and Edn3). Edn1, a 21 amino acid peptide, was first described as a potent vasoconstrictor produced by endothelial cells [15]. Edns bind specifically to two types of receptors, Edn receptor subtypes-A and -B (*Ednra* and *Ednrb*), which belong to the G protein-coupled receptor family. *Ednra* has specific affinity for Edn1, whereas *Ednrb* has similar affinity for all three Edns [2].

A variety of physiological actions, including reproductive functions, have been attributed to Edns since either the peptides themselves or their receptors are distributed widely in mammalian tissues [4, 10]. In testicular cells, autocrine and paracrine roles of Edns have been discussed. However, in laboratory animals, the localization of Edn receptors have been evaluated only indirectly by ¹²⁵I-labeled Edn1 binding assays, which potentially detect both *Ednra* and *Ednrb*, *in vivo* and *in vitro* [3, 5, 7, 8, 13]. In this study, therefore, to understand the specific role of *Ednra* in the testis, the *in vivo* localization of *Ednra* was investigated using *in situ* hybridization and immunohistochemistry in the rat, canine, and monkey testis.

Animals and tissue preparation: Male adult Sprague-Dawley rats (16 weeks of age), beagle dogs (11 months of age), and cynomolgus monkeys (7-8 years of age) were maintained and used in accordance with the institutional guidelines for the care and use of laboratory animals. Testes were removed from animals sacrificed under anesthesia. They were then frozen with liquid nitrogen or fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, overnight at 4°C, for total RNA isolation and *in situ* hybridization (ISH), or immunohistochemistry (IHC), respectively. The OTC-compound-embedded frozen sections (10-μm

thick) and paraffin-embedded sections (4-μm thick) were prepared using general procedures for ISH and IHC, respectively.

Hybridization probes: For northern blot hybridization and ISH, digoxigenin (DIG)-labeled rat *Ednra* riboprobes were used. Sense or antisense probe template cDNAs were amplified from Rat *Ednra* cDNA (obtained from Dr. Sakurai) using rat *Ednra* primers (forward 217-236: 5'-(T7)-CCTGCCTAGCAATGGCTCAA-3', reverse 519-538: 5'-(T7)-CTGCAAAAAGGGGAACAGCT-3', GenBank Accession No. M60786) containing a T7 promoter sequence in the 5'-region of the forward and reverse primers, respectively. Hybridization probes, which are 322 bp in size and recognize a sequence containing the first hydrophobic transmembrane domain of *Ednra* [11], were synthesized with T7 RNA polymerase (Invitrogen Corp., Carlsbad, CA) from probe template cDNAs.

Subcloning of canine and monkey *Ednra*: To evaluate nucleotide sequence homology between rat *Ednra* probe, and canine and monkey *Ednra*, the total RNAs (1 μg) isolated from canine and monkey testes using Trizol (Invitrogen) were reverse-transcribed using SuperScript II RNase H⁻ reverse transcriptase (Invitrogen) with oligo(dT)₁₂₋₁₈ primer. They were then amplified with rat *Ednra* primers without the T7 sequence and Taq DNA polymerase (Invitrogen; 95°C for 30 sec, 62°C for 30 sec, and 72°C for 60 sec, repeated for 33 cycles). Purified PCR products were subcloned into the pCR2.1 cloning vector, transformed into TOP10 cells (Invitrogen), and sequenced.

Northern blot hybridization: Poly(A)⁺ RNA (1 μg) isolated using MessageMaker (Invitrogen) was electrophoresed in 1% formaldehyde-agarose gel and transferred to nylon membranes (Hybond N⁺; Amersham Biosciences Corp., Piscataway, NJ). Following prehybridization for 2 hr at 55°C in DIG Easy Hyb (Invitrogen), hybridization was carried out for 16 hr at 55°C in DIG Easy Hyb containing

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Fig. 3. *Ednra* mRNA localization in rat testis (A), dog (B), and monkey (C) detected by ISH. Hybridization signals were abolished by incubating with sense probes in rat (D), canine (E), and monkey (F) testis. Arrows and arrow heads in higher magnifications in the rat (G), canine (H), and monkey (I) testis hybridized with antisense probes represent positive *Ednra* signals in seminiferous tubule and interstitial cells, respectively. Bar: 20 μ m in A-F, 40 μ m in G-I.

anti-human *Ednra* antibody (IBL, Gunma, Japan) at a dilution of 1:50 or PBS. Following incubation, sections were treated with biotinylated goat anti-rabbit IgG (Dako LSAB Kit; Dako Corporation, Carpinteria, CA) for 1 hr at RT. Tissue sections were incubated with streptavidin-conjugated horseradish peroxidase (Dako LSAB Kit) for 1 hr at RT. Finally, sections were treated for 10 min at RT with 0.01% 3,3'-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan) in 0.05 M Tris-HCl (pH 7.6) with 0.068% imidazole (Sigma, St. Louis, MO) and 0.02% H_2O_2 . All sections were lightly counter-stained with Mayer's hematoxylin.

Hybridization probe specificity: Canine and monkey *Ednra* had a high homology (88.7% and 87.9%, respectively) to rat *Ednra* in the hybridization probe region (Fig. 1), and was detected by Northern blot hybridization using rat *Ednra* probe (Fig. 2). Also, in the other region from the subcloned one in this study, canine *Ednra* was highly homologous to human *Ednra* nucleotide sequence (93%

identical) [12]. Rat, canine, and monkey *Ednra* mRNA exhibited 4.2 and 5.2-kb fragments (Fig. 2), as previously reported in various rat tissues [9], showing the specificity of the rat *Ednra* probe for canine and monkey *Ednra*. Previous studies demonstrated that the two bands might represent differential splicing of the same transcript, since both bands were seen under high stringency washing conditions [9, 11]. In monkey tissues, however, major 4.2 and minor 3.0-kb fragments were reported [1]. The cause of the difference was thought to be differences in the hybridization probe or the mRNA purification procedure.

***Ednra* localization:** *Ednra* mRNA was detected in the cytoplasm of the interstitial cells and cells in the basal compartment of the seminiferous tubule in rats, dogs, and monkeys (Fig. 3). However, *Ednra* mRNA expression was not observed in elongating spermatids, and it was difficult to identify Sertoli cells morphologically because of degradation of frozen sections. Positive hybridization signals were completely abolished by using the sense probe (Fig. 3 D-F).

Fig. 4. Immunolocalization of Ednra in rat testis (A), dog (B), and monkey (C). Ednra immunostainings were abolished by incubating with PBS instead of anti-Ednra antibody in rat (D), canine (E), and monkey (F) testis. Arrows and arrow heads represent positive Ednra signals in seminiferous tubule and interstitial cells, respectively. Bar: 20 μ m.

At the protein level, cytoplasmic Ednra immunostaining was consistently present in the interstitial cells including Leydig cells, and Sertoli cells and some of the cells in the basal compartment of the seminiferous tubule, possibly spermatogonia, early spermatocytes, as well as myoid cells in rats, dogs, and monkeys (Fig. 4 A-C). Ednra immunostaining was abolished by incubation with PBS instead of the primary antibody (Fig. 4 D-F).

Localization of 125 I-Edn1 binding sites in rat testis was reported in interstitial areas including Leydig cells and around the seminiferous tubules, but not in germ and Sertoli cells [3]. However, Fantoni *et al.* [7] reported that 125 I-Edn1 binding sites were present in rat interstitial and seminiferous tubular cells, including Leydig and Sertoli cells, respectively. Additionally, 125 I-Edn1 binding was found in cultured rat myoid cells [8]. In human testis, immunohistochemistry revealed Ednra expression in Leydig cells, Sertoli cells, and spermatids [6]. In the present study, both Ednra mRNA and protein were expressed in Leydig and Sertoli cells, and some of spermatogonia, early spermatocytes, as well as myoid cells. These differences between Edn1 binding and Ednra expression in testicular cell types may be explained by species differences, the age of the animals or culture-dependent changes.

Edn1 production was also demonstrated in cultured rat Sertoli cells and human testis [6, 7]. Fantoni *et al.* [7] demonstrated that follicle-stimulating hormone inhibited Edn1 production from cultured rat Sertoli cells, suggesting that Edn1 may be a paracrine factor involved in the regulation of Leydig cell activity. Indeed, Edn1 enhanced testosterone

secretion of dispersed rat Leydig cells, and the response was suppressed by Ednra selective antagonist, but not by Ednrb selective antagonist [3]. The murine Leydig tumor cell line, MA-10, possessed high affinity binding sites for Edn1 and stimulated progesterone production by Edn1 [5]. While, Edn1 has been reported to influence the intracellular calcium level of rat Sertoli cells [14], in which Ednra expressed [6, 7]. However, Edn1 binding sites and Ednra expression in Sertoli cells were lower than those in Leydig cells [6, 7], and the functional role of Sertoli Ednra remains to be determined. Taken together, these observations demonstrate the physiological importance of Edn1 signaling via Ednra for the function of the mammalian testis.

In conclusion, Ednra localization examined by ISH and IHC showed interstitial and peripheral Ednra expression in rat, canine, and monkey testis. This study serves information fundamental for understanding the physiological role of the Ednra signaling in the mammalian testis.

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