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Expression of Constitutive Endothelial, Neuronal and Inducible Nitric Oxide Synthase in the Testis and Epididymis of Horse

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ABSTRACT. The expression of three isoforms of nitric oxide synthase (NOS) were examined in the testis and epididymis of a thoroughbred horse. Immunohistochemical studies demonstrated the presence of eNOS immunostaining in some germ cells in the seminiferous tubules and in vascular endothelial cells in the interstitial tissues. Interstitial cells, most likely Leydig cells, were also intensely immunopositive for eNOS. The pattern of immunostaining for nNOS was similar to that for eNOS in the testes. Weak expression of iNOS was detected in the seminiferous tubules of the testis, but intense expression was found in interstitial cells. Inducible NOS was also strongly detected in stereocilia, sperm, epithelium and connective tissue of the epididymis of normal horses. These findings suggest that three isoforms of NOS are expressed in the testes and epididymis of horse and that they play important roles in the biology of interstitial cells that produce testosterone, as well as in spermatogenesis in the seminiferous tubules.

KEY WORDS: epididymis, equine, nitric oxide synthase, testis.

Nitric oxide (NO) is a short-lived free radical with biological functions in the nervous, cardiovascular, and immune systems [2, 14]. NO is synthesized from L-arginine by activation of the enzyme nitric oxide synthase (NOS). NOS exists in two forms: (1) constitutive, Ca2+ -dependent forms that are rapidly activated by agonists that elevate intracellular free Ca2+, including neuronal NOS (nNOS) and endothelial NOS (eNOS); and (2) a Ca2+-independent inducible form (iNOS) [14, 27]. Neuronal NOS has been shown to be constitutively expressed in a variety of cell types including testicular interstitial cells [24] and neuronal cells. Like nNOS, eNOS is expressed in many cell types in addition to the vascular endothelium in which it was first identified, and is also known to be associated with a variety of inflammatory processes [4]. These phenomena imply that both constitutive nNOS and eNOS are normally expressed in cells other than the neuronal and vascular endothelial cells in which they were respectively first detected. In contrast to the low level of NO generated by constitutive NOS, iNOS produces high levels (nanomolar quantities) of NO in various cell types when expression is activated [27]. NOS is known to play both beneficial and detrimental roles, depending on the cell activation status. It is generally accepted that excess NO production, induced mainly by iNOS, causes tissue damage, whereas constitutive nNOS and eNOS function in normal physiological events, such as regulation of the microcirculation, synaptic plasticity, and neuroprotective processes [5, 11].

Some reports indicate that NOS may be involved in the normal biosynthesis and secretion of the steroid hormones in the male reproductive system, demonstrating direct effects on the function of interstitial cells in the testes [21, 25]. On the other hand, excessive NOS may induce the production of large amounts of NO metabolites in response to a variety of stressors, possibly reducing the survival rate and motility of sperm cells [18, 20]. High levels of NO may also reduce testosterone secretion, as indicated by the suppression of testosterone secretion in male rats treated with the NO donor, isosorbide dinitrate (ISDN) [1]. In addition, it has been reported that normal human spermatozoa exhibit eNOS immunostaining that correlates with sperm motility [16], and nNOS immunoreactivity has also been detected in normal rat epididymis [8]. The finding that iNOS was expressed in normal tissue as well as inflamed tissue in the rat testis suggests a unique role for NOS in the male reproductive organs [17].

Thus, although NOS is known to be involved in the physiology of the reproductive system, little is known about the expression patterns of the three isoforms of NOS found in the testis and epididymis of the horse. This study used immunohistochemistry to examine the expression of constitutive eNOS, nNOS, and iNOS in the testis and epididymis of the horse.

MATERIALS AND METHODS

Animals: Seven horses (2-year-old thoroughbreds) were kindly supplied by the stud farm of the Korean Racing Association (Jeju, Korea). The testes of each horse were surgically removed under local anesthesia.

Antibodies: The following polyclonal antisera and monoclonal antibodies (mAb) were used in this study: mouse anti-eNOS, anti-nNOS, and anti-iNOS antibodies (Transduction Laboratories, Lexington, KY), and rabbit polyclonal anti-eNOS, polyclonal anti-nNOS, and polyclonal ant i-iNOS (Sigma, St. Louis, MO). The immunohistochemical
results using monoclonal antibodies (anti-eNOS, anti-nNOS, and anti-iNOS) were similar to those obtained with polyclonal antisera, and representative results for monoclonal antibodies are shown in this study.

**Tissue sampling:** The horses were castrated by surgical removal of the testes and epididymides. The tissues were dissected and samples were frozen at –70°C for protein analysis. Additional samples were processed for paraffin embedding after fixation in 10% buffered formalin for 48 hr to prepare for histological examination.

**Immunohistochemistry:** Deparaffinized tissue sections were treated with 0.3% hydrogen peroxide in deionized water for 20 min to block endogenous peroxidase. After three washes with PBS, the sections were exposed to 10% normal horse serum, and then incubated with primary antibodies, either mouse monoclonal anti-eNOS, mouse monoclonal anti-nNOS, or mouse monoclonal anti-iNOS antibodies (diluted 1:200), for 1 hr at RT. After three washes, the appropriate biotinylated secondary antibody and the avidin-biotin-peroxidase complex (ABC) from the Elite kit (Vector, Burlingame, CA) were added sequentially. Immunostaining was developed with diaminobenzidine (DAB) substrate kit (Vector). The sections were counterstained with hematoxylin before being mounted.

**RESULTS**

Immunohistochemical localization of eNOS, nNOS, and iNOS in the testis and epididymis of normal horses: Histological examination confirmed that all testes from normal horses showed no pathological changes, and these were used for further study. There was no inflammation in the testis and epididymis. Immunostaining for three isoforms of NOS was detected in the reproductive system of normal male thoroughbred horses. The histochemical reactions of the constitutive neuronal, endothelial, and inducible NOS in the testis and epididymis are summarized in Table 1.

In the testis, immunoreactivity for eNOS was intense in round spermatids, spermatocytes, and in the seminiferous tubules (Fig. 1A). In contrast, only weak immunostaining for eNOS was found in some Sertoli cells and elongate spermatids (Fig. 1A).

In the caput epididymis, eNOS was strongly detected in the stereocilia, sperm and basal cells, while connective tissue showed weak staining (Fig. 1B). Stereocilia and sperm of the corpus epididymis of normal horses also stained strongly for eNOS. The epithelium and connective tissue of the corpus epididymis stained weakly for eNOS, but basal cells were negative (Fig. 1C). In the cauda epididymis, expression of eNOS was strongly detected in connective tissue and in stereocilia, with weaker staining of epithelium and sperm, while basal cells were again negative (Fig. 1D).

Intense immunoreactivity for nNOS was detected in interstitial cells and round spermatids of the testis. Neuronal NOS was also weakly detected in elongate spermatids, but not in spermatocytes and Sertoli cells (Fig. 2A).

In the caput epididymis, nNOS was strongly detected in stereocilia and epithelium, and weakly detected in basal cells and connective tissues (Fig. 2B). In the corpus epididymis, nNOS was strongly detected in epithelium and sperm, but only weakly detected in stereocilia and not detected at all in basal cells or connective tissues (Fig. 2C). In the cauda epididymis, nNOS was strongly detected in epithelium, weakly detected in stereocilia, sperm, and connec-

| Table 1. Histochemical staining pattern for three NOS isoforms in various cell types in the reproductive system of normal two-year-old thoroughbred horses (−, negative; +, weak; ++, moderate; ++++, intense) |
|-----------------|-----------------|-----------------|
| **Cell Type**   | **eNOS**        | **nNOS**        | **iNOS**       |
| Testis          |                 |                 |                |
| Elongate spermatid | +               | +               | –              |
| Round spermatid  | ++              | ++              | –              |
| Spermatocyte     | ++              | –               | –              |
| Sertoli cell     | +               | –               | –              |
| Interstitial cell | +++             | +++             | +++            |
| Caput epididymis |                 |                 |                |
| Stereocilia      | ++              | ++              | +              |
| Epithelium       | –               | +               | –              |
| Basal cell       | +               | +               | –              |
| Sperm            | +               | ++              | +              |
| Connective tissue| +               | +               | +              |
| Corpus epididymis|                 |                 |                |
| Stereocilia      | ++              | +               | +              |
| Epithelium       | +               | ++              | +              |
| Basal cell       | –               | –               | ++             |
| Sperm            | ++              | ++              | +              |
| Connective tissue| +               | –               | +              |
| Cauda epididymis |                 |                 |                |
| Stereocilia      | +               | +               | +              |
| Epithelium       | +               | ++              | +              |
| Basal cell       | –               | –               | –              |
| Sperm            | +               | +               | –              |
| Connective tissue| ++              | +               | +              |
NITRIC OXIDE SYNTHASE IN TESTIS OF HORSE

Immunoreactivity of iNOS was intense only in the interstitial cells of the testis of normal horses (Fig. 3A). Spermatocytes and Sertoli cells were both negative for iNOS. In the caput epididymis, iNOS was detected in the stereocilia and connective tissue and weakly detected in sperm, but not detected in epithelial or basal cells (Fig. 3B). Sperm in the corpus epididymis showed strong iNOS immunostaining, with somewhat less intense staining present in the stereocilia and epithelium (Fig. 3C). In the cauda epididymis, expression of iNOS was detected in connective tissue, sperm, and stereocilia, but not in epithelium or basal cells (Fig. 3D).

DISCUSSION

This is the first report to show that constitutive eNOS, nNOS, and inducible NOS are differentially expressed in the seminiferous tubules of normal thoroughbred horses. In the testis, constitutive NOS was strongly detected in the interstitial cells, while in the epididymis both eNOS and nNOS expression were detected mainly in sperms and epithelium. Although it has been reported that iNOS is overexpressed in ischemia-reperfusion injuries of the testis [18], this is the first report of iNOS expression in the interstitial cells of the testis, as well as in basal cells and sperm in the epididymis of the normal horse.

Recent in vitro studies have indicated that the inflammatory vasodilator NO is capable of inhibiting steroidogenesis by Leydig cells [1]. It has also been reported that aluminum chloride decreases production of testicular testosterone in mice [10]. Elevated aluminum levels in spermatozoa and seminal plasma were previously shown to be correlated with decreased sperm motility [6]; however in a later study, NO
levels were also shown to be increased in the related tissue of mice [10].

Interstitial macrophages in the testis are an important source of nitric oxide produced by iNOS. It is well known that macrophages express iNOS upon activation and release NO in cultured cell systems. In a previous study, a majority of the macrophages that expressed ED1 and all Leydig cells were immunopositive for iNOS in both control and LPS-treated rat testes [9]. It is thus likely that the iNOS-immunoreactive cell populations detected in the interstitial tissue of horse testes in this study were comprised of Leydig cells and macrophages. When the testis is inflamed during adjuvant-induced orchitis, there are many iNOS-positive cells in the testicular interstitium, possibly suggesting that adjuvant-induced orchitis is in part mediated by an increased number of iNOS-positive cells (data not shown). However, possible involvement of constitutive eNOS and nNOS in the macrophages is not excluded, because these two isoforms are also involved in tissue injury in other model systems [22].

There are many reports that eNOS is associated with cell death in certain cell types. It is postulated that over-activation of eNOS mediates the cell death program through the generation of NO, and may play a role in the cell-selection process that occurs in the seminiferous tubules. Spermatogenesis in the tubules is essentially a result of ongoing cell proliferation, cell selection, and cell death [7, 19].

The functional role of NOS in interstitial cells and Leydig cells remains to be elucidated. In the present study, three isoforms of NOS were intensely immunostained in the interstitial cells of the testis, implying that NOS is involved in Leydig cell biology. If this is the case, it is possible to postulate that protein kinase C (PKC), most likely PKC theta (PKCθ) [23], is intimately associated with NOS regulation in interstitial cells. There is much evidence that PKC and
NOS, among other signaling molecules, are expressed in the interstitial tissue, including in Leydig cells [12, 13, 23]. In one study, differential expression of PKCδ and PKCθ was identified in the horse testis [12]. Differential expression of PKCθ in interstitial cells (probably Leydig cells) and PKCδ in spermatids suggests that PKCθ and PKCδ play distinct roles in the regulation of testosterone synthesis and spermatid development, respectively. Additional evidence indicates that PKC subtypes are associated with NOS activation derived from in vitro systems. It has been shown that lipopolysaccharide stimulation increases intracellular calcium and activates PKC in cultured cells, thus inducing iNOS gene expression, which leads to production of high levels of NO, demonstrating that a significant signaling role for NO is septic shock [3, 15].

There is little information on the role of NOS in the epididymis. We postulate that both cNOS and iNOS in epithelial cells help to explain epididymal function: sperm storage, passage, and maturation. Excessive epididymal NO production may also play a role in inflammation and male infertility [26, 28].

The findings of the present study suggest that three isoforms of NOS are expressed in interstitial cells of the testis of the horse, and play important roles in the biology of testosterone-producing interstitial cells, as well as in spermatogenesis in the seminiferous tubules.

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