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Endothelial and inducible nitric oxide synthase (NOS) immunoreactivity and NOS-associated NADPH-diaphorase histochemistry in the domestic cat (*Felis catus*) testis

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ABSTRACT

In this study, the cellular localization of nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) and the endothelial (eNOS) and inducible (iNOS) forms of nitric oxide (NO) synthase in the cat testis were studied using enzyme histochemical and immunohistochemical techniques. Stage-dependent nuclear and cytoplasmic eNOS/iNOS immunoreactivity and cytoplasmic NADPH-d reactivity were found in all germ cells, including spermatogonia, primary spermatocytes (preleptotene, zygotene, and pachytene spermatocytes), and round (Sa, Sb1) and elongating spermatids (Sb2, Sc) of the seminiferous epithelium. The pachytene spermatocytes exhibited strong positive reactions at all spermatogenic stage. Interestingly, in elongated spermatids (Sd1) at stages VI to VII, eNOS and iNOS immunostainings was observed only in the cytoplasm but not in the nuclei. eNOS and iNOS immunolabeling was observed in the acrosomal vesicle of some round spermatids (Sb1) at stages I, VII, and VIII, and in the acrosomal cap of elongating spermatids (Sb2) at stage II. Furthermore, eNOS, iNOS, and NADPH-d reactions in elongated spermatids (Sd2) just before spermiation at stage VIII were restricted only to the middle and principal pieces of the tail. Positive reactions were also observed in the Sertoli and Leydig cells as well as in other tissues including vascular endothelial and smooth muscle cells and peritubular myoid cells. These results suggest that NO may play an important role in chromatin condensation, spermatid shaping, and the final release of sperm from the spermatogenic epithelium. Furthermore, NO may also be involved in spermiogenesis, steroidogenesis, and apoptotic cell death.

1. Introduction

Multiple proteins and/or molecules are known to be involved in the regulation of many cellular processes in the testes, including the initiation of the spermatogenic wave and many other aspects of germ cell development. These molecules include the well-characterized cytokines interleukin-1a (IL-1a), IL-6, and tumor necrosis factor-α, but also nonproteinaceous mediators of inflammation and immunity, such as nitric oxide (NO) [1,2]. NO is highly effective in these processes because of its small molecular size and diffusible nature; thus, its sites of action may be distant from its production sites. At low levels (<1 μM), NO acts as a regulatory molecule, but at high levels, NO causes damage to DNA, proteins, and lipids through free radical generation [3]. NO is an inorganic free radical gas synthesized by a family of enzymes referred to as the nitric oxide synthases (NOS). Three different isoforms constitute the NOS family: neuronal

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NOS (nNOS), endothelial NOS (eNOS), and inducible or macrophageal NOS (iNOS) [4]. Interestingly, a testis-specific subclass of nNOS, known as the truncated form of nNOS (TnNOS), has been recently identified as a major contributor to the formation of NO. TnNOS has been found to be localized solely in the Leydig cells of the testes and not in the Sertoli and germ cells, thereby suggesting its involvement in steroidogenesis [5]. Conversely, these NOS isoforms are functionally categorized into two groups based on their intrinsic NO production efficacy. Both nNOS and eNOS are constitutively expressed enzymes, the activity of which is regulated through a calcium-calmodulin mediated mechanism [6,7]. In contrast, iNOS is a constitutively active form of NOS that is induced in response to inflammatory stimuli such as pro-inflammatory cytokines (for example, IL-1, tumor necrosis factor-α, and interferon-γ) and bacterial lipopolysaccharide [8].

All of the NOS isoforms are modular enzymes comprised of an N-terminal oxygenase domain that contains heme, 6*R*-tetrahydrobiopterin, and an L-arginine binding site, and a C-terminal flavoprotein domain (reductase domain of NOS) that contains a flavin mononucleotide, flavin adenine dinucleotide, and a nicotinamide adenine dinucleotide phosphate (NADPH) binding site [9]. These isoforms catalyze the same two-step reaction sequence and appear to share the same catalytic mechanism. First, the isoforms oxidize L-arginine to the stable intermediate *N*-hydroxy-L-arginine and subsequently oxidize this intermediate to NO and L-citrulline. Both steps in this sequence are NADPH- and O_2-dependent, and NADPH is the reductant of the NOS reactions [9,10]. During NO synthesis, the NOS flavoprotein domain transfers NADPH-derived electrons in a linear sequence to the oxidized forms of flavin mononucleotide or flavin adenine dinucleotide and later to a heme located in the N-terminal oxygenase domain (reviewed elsewhere [11]). The ability of the reductase domain, located at the C-terminal sequence of NOS, to transfer electrons from the coenzyme NADPH to other substrates, including tetrazolium salts, leads to the so-called NADPH-diaphorase (d) activity [12]. This NADPH-d reaction, by which soluble nitroblue tetrazolium salts are converted to insoluble visible formazan in the presence of NADPH, is considered an indicator of the presence of NOS [12]. NADPH-d colocalizes with all known NOS isoforms, but NADPH-d histochemistry does not distinguish among the different isoforms of NOS [13–15]. Tracey et al. [16] have suggested that NOS reactivity represents only a fraction of total cellular NADPH-d activity and that these activities are not always colocalized.

In the testes, NO/NOS has been shown to regulate an array of functions, including spermatogenesis [17–21], sperm motility, and maturation [17,22] as well as Leydig cell steroidogenesis [23–26]. A physiological level of NO is required for successful fertilization. At the moment of contact, release of NO by the acrosome of the sperm activates the egg to complete meiosis II and other steps of fertilization [27].

NOS/NO contributes to the testicular production of hormones and cytokines, and the bidirectional relationship manifested by NOS/NO and the hormone/cytokine level is crucial in maintaining the physiological functions of the testes. NOS/NO is also one of the important regulators of the adherens junction and tight junction dynamic in the seminiferous epithelium (reviewed elsewhere [21]).

NO/NOS also regulates germ cell apoptosis in the seminiferous epithelium during spermatogenesis. Excessive NO levels can directly trigger germ cell apoptosis. This effect is clearly described in the artificial spermatic vessel ligation model [28] and in the mouse model of congenital cryptorchidism [29]. The normal aging process also illustrates the association of increased NO and germ cell apoptosis [30]. Both iNOS and eNOS are postulated to participate in germ cell apoptosis in the testes. Specifically, eNOS is highly expressed in degenerating germ cells in comparison to other germ cell types [17,31]. In addition, iNOS plays a determining role in restricting germ cell numbers, particularly the numbers of pachytene spermatocytes and round spermatids [32]. NO/NOS also play a role in the relaxation of seminiferous tubules and blood vessels to modulate sperm transport and testicular blood flow, respectively [33,34]. Unbalanced levels of NO or the dysregulation of NOS in the testes contribute to testicular defects. For instance, excessive NO levels in human varicoceles can be harmful for sperm motility [35]. eNOS protein levels are reduced in patients with idiopathic azoospermia versus patients with obstructive azoospermia or varicocele and healthy individuals [36]. In addition, several studies indicate that iNOS is the primary source of NO generation in varicocele cases [37–39].

The presence and possible functions of NOS isoforms have been demonstrated using immunohistochemical and histochemical methods in the testes of several species, including rats [18,33,40–43], humans [17,23,25,34], monkeys [20,44], mice [32,45,46], pigs [47,48], and horses [49]. However, the expression of NOS in the cat testes has not been studied.

Although cats are maintained by man as pets, companions, and entertainers, little is known regarding the structure and biology of the reproductive system of the domestic cat. In this regard, the domestic cat is a neglected species. In the past several years, considerable attention has been devoted to the endocrinology of the feline reproductive cycle and the mechanism involved in the ovulatory process of the female domestic cat. The male cat has not yet received the same attention. Consequently, little is known about its reproductive system. Reproductive studies in cats have involved investigations of gamete biology, endocrinology, and cryobiology [50–56]. Knowledge of male reproductive functions in the domestic cat is important because this species serves as a valuable model for examining the structure and physiology of the reproductive system in felines. Considering that most wild felines are listed as threatened or endangered species, these studies must be performed on domestic cats [57].

Therefore, we analyzed the distribution of both NADPH-d activity and NOS immunoreactivity in the cat testes to answer the following questions: (1) What is the localization of NADPH-d and NOS isoforms in the testes? (2) Is there a relationship between NADPH-d histochemical activity and immunoreactivity against NOS isoforms?