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D-Serine modulates neurogenic relaxation in rat corpus cavernosum

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ABSTRACT

D-Serine, an endogenous co-agonist for the N-methyl-D-aspartate (NMDA) receptor in mammals, is synthesized from L-serine by serine racemase. Although much attention has been focused on the role of D-serine within the central nervous system, the physiological role of D-serine in peripheral nerves such as corpus cavernosal nerves has not been investigated. The present study was aimed to study the expression, cellular localization and function of serine racemase/D-serine system in isolated rat corpus cavernosum. Reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis showed the expression of serine racemase in rat corpus cavernosum. Immunogold electron microscopy demonstrated the cellular localization of serine racemase in the cavernosal nerves' membrane of the tissue. The organ bath studies on isolated rat corpus cavernosum showed that D-serine increases the non-adrenergic non-cholinergic neurogenic relaxation of isolated rat corpus cavernosum *in vitro*. This effect of D-serine was inhibited by a variety of NMDA receptor antagonists (ketamine, MK 801 and ifenprodil), suggesting that NMDA receptors are involved in the effects of D-serine on the neurogenic relaxation of corporal tissue strips. These observations provide the first evidence for the role of D-serine in modulating the neurogenic relaxation of rat corpus cavernosum, and may open new therapeutic avenues for the treatment of impotence.

1. Introduction

The discovery that mammalian brain contains high levels of D-serine overturned the dogma that only L-isomers of amino acids occur in mammalian tissues [1–3]. It is now well accepted that D-serine is a neurotransmitter within the mammalian central nervous system which selectively binds to a co-agonist site at the N-methyl-D-aspartate (NMDA) receptors and, along with glutamate, mediates several important physiological processes [3].

D-Serine is synthesized from L-serine by a pyridoxal-phosphate-dependent enzyme, serine racemase [4], and is rapidly degraded by a variety of enzymes including D-amino acid oxidase (DAAO) [5]. The expression of serine racemase has been demonstrated in astrocytes as well as neurons in various areas of mammalian central nervous system [4,6–8]. Although much attention has been focused on D-serine within the central nervous system, the expression of serine racemase has also been shown in the peripheral tissues including retinal ganglion cells [9], Schwann cells [10], epineural fibroblasts [10] and chondrocytes [11]. However, the physiological role of D-serine in peripheral nervous system remains to be investigated.

Penile tumescence (erection) and de-tumescence are regulated by a complex neurophysiological process of relaxation and contraction, respectively, of the corpus cavernosum [12,13]. There is consensus among scientists that the neural control of erection via cavernosal nerve stimulation involves chemical mediators that are described as non-adrenergic non-cholinergic (NANC) transmitters [14–16]. Nitric oxide is considered to be the main NANC transmitter which mediates the relaxation of corpus cavernosum [15–17]. Apart from nitric oxide, other NANC transmitters are also involved in the relaxation of corpus cavernosum which include purines (e.g., ATP and ADP), eicosanoids, calcitonin-gene-related peptide and anandamide [14,18–20].

It is well known that sexual responses such as penile erection are controlled by neural circuits in the brain and spinal cord that are stimulated by activation of post-synaptic NMDA receptors [21,22]. There is several convincing evidence that central NMDA receptors play a role in modulation of penile erection [23]. Moreover, the pioneering studies by Gonzalez-Cadavid et al. demonstrated that peripheral NMDA receptors may play a role in the relaxation of corpus cavernosum and thereby on erectile function [24,25]. The expression of NMDA receptor subunits has been shown in rat and human corpus cavernosum [24,25].

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Gonzalez-Cadavid et al. also demonstrated that NMDA receptor blockers at sub millimolar concentrations are able to induce relaxation of corpus cavernosum *in vitro* [24]. However, it is yet unclear to what extent these observations with high concentration of these chemicals result from NMDA receptor blockade, or from effects on non-NMDA ion channels in cavernosal smooth muscle [24].

NMDA receptors play a pivotal role as an excitatory neurotransmission which leads to the influx of Na^+ and Ca^{2+} in postsynaptic membrane. Since Ca^{2+} entry through NMDA receptors activates neuronal nitric oxide synthase (NOS) by a Ca^{2+}/calmodulin-dependent mechanism, NMDA receptors are considered as an important modulator of neuronal NOS activity in a variety of model systems [26–27]. Magee et al. showed the co-localization of NMDA receptors with neuronal NOS in the rat and mouse cavernosal nerve [25]. However, the role of such interaction is not well understood in context of corpus cavernosal function.

Since D-serine is involved in modulation of NMDA receptors in a variety of biological systems, we initially wished to confirm that serine racemase is expressed in rat corpus cavernosum and act as a modulator of NANC relaxation *in vitro*. The present study reports our investigations on the expression, cellular localization and function of glutamate/D-serine system in isolated rat corpus cavernosum.

2. Methods and materials

2.1. Reagents

Phenylephrine hydrochloride, guanethidine sulfate, atropine sulfate, ifenprodil tartrate, N^G-L-nitro-arginine methyl ester (L-NAME), dizocilpine (MK 801) and ketamine were purchased from Sigma (Sigma–Aldrich, Bristol, UK). All drugs were freshly dissolved in distilled water. All reagents and enzymes used for PCR and Western blotting were purchased from Promega (Promega, Madison, USA).

2.2. Animals

Male Sprague–Dawley rats (body weight 200–250 g) were obtained from the Comparative Biology Unit at the UCL Medical School (Royal Free Campus, UCL, London, UK). The animals were housed in a light-controlled room with a 12 h day/night cycle and were given free access to food and water. All animal procedures were in accordance with Home Office (UK) recommendations. Each separate experimental group consisted of six animals.

2.3. 2.3. mRNA extraction & reverse transcription polymerase chain reaction (RT-PCR) procedure

Corpus cavernosum, liver and brain were obtained from rats and immediately immersed in liquid nitrogen. Total RNA was extracted from tissue homogenate using RNeasy Fibrous Tissue mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Further elimination of genomic DNA was performed by DNase after RNA extraction. First strand cDNA was then generated by using 1 μg of deoxyribonuclease treated RNA, 1 μl of random hexamer primer (p(dN)6), and ribonuclease free water, heated at 70 °C for 5 min, and then placed on ice. RNasin (ribonuclease inhibitor), 100 unit of Moloney murine leukaemia virus reverse transcriptase, Moloney murine leukaemia virus buffer, and 0.4 mM deoxynucleoside triphosphates were added, and the mix was incubated at 42 °C for 1 h. Oligonucleotide primers used for PCR amplification of rat serine racemase, DAAO and β-actin were as follows:

a. Rat serine racemase (NM 198757), PCR product: 295 bp
   Sense: 5′-ATTGCAAGAAACTGGCCATC
   Anti-sense: 5′-TCAGCAGCGTACACCTTCAC
b. Rat DAAO (NM 053626), PCR product: 460 bp
   Sense: 5′-TTCGAAGAGGTGGTGAGAGGA
   Anti-sense: 5′-AGTGAATCGTGAGCCCGTAAC
c. Rat β-actin (NM 031144), PCR product: 453 bp
   Sense: 5′-AGAGGGAAATCGTGCGTGACA
   Anti-sense: 5′-ACATCTGCTGGAAGGTGGACA

PCR reactions comprised of 1 μl of cDNA template, 100 ng each of sense and anti-sense oligonucleotide primers, 2.5 μl of optimized TaqPCR buffer, 0.4 mM dNTP mixture, and 2 U of Taq polymerase in a total reaction volume of 25 μl. After initial 5 min incubation at 94 °C, PCRs were performed using a 1 min annealing step, followed by a 1 min elongation step at 72 °C and a 45 s denaturation step at 94 °C. Forty PCR cycles were performed for amplification of serine racemase and DAAO cDNAs, 25 cycles for β-actin cDNA, followed by a final elongation for 10 min at 72.0 °C. PCR products were separated by electrophoresis through a 1% agarose gel and detected by ethidium bromide staining.

2.4. Western blotting

Corpus cavernosum and liver (positive control) were obtained from rat and were immediately frozen in liquid nitrogen. Snap frozen tissues were homogenized in ice-cold RIPA buffer containing protease inhibitors (protease inhibitor mixture from Roche, Mannheim, Germany), 50 mM Tris (pH 8), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS. Homogenates were then sonicated followed by centrifugation at 10,000 × *g* for 5 min at 4 °C. After determining the protein concentrations of the supernatants (Bradford assay with bovine serum albumin as standard), 10 μg protein of each sample was fractionated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with Tris buffered saline (10 mM Tris, 100 mM NaCl) containing 0.1% Tween-20 for 1 h, the membranes were incubated overnight with rabbit anti-serine racemase antibody (1:500 rabbit polyclonal antibody from Santa Cruz Biotechnology, Santa Cruz, CA, USA). After rigorous washing, the membranes were incubated with anti-rabbit IgG alkaline phosphatase-linked antibody (1:5000 dilution, Perbio Science Ltd., Northumberland, UK). Alkaline phosphatase was detected using a BCIP/NBT developing kit.

2.5. Immunogold electron microscopy

Freshly isolated corporal tissues from rats were excised and immersed in isotonic fixative (4% paraformaldhyde, 0.5% glutaraldehyde, in 0.1 M phosphate buffer, pH 7.4, with 0.1 M sucrose) for electron microscopic immunocytochemistry as has been previously described ($n = 2$). Tissues were then infiltrated and embedded in LR white resin. Thin sections (70–90 nm) were cut and mounted on coated nickel grids. The grids were then blocked (0.1% bovine serum antigen, 0.1 M glycine in PBS) for 30 min and incubated for 2 h with rabbit polyclonal antibody against serine racemase (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Following a series of washes, grids were incubated for 1 h with 10 nm Immunogold-linked, EM grade, goat anti-rabbit IgG (1:50 dilution). Following another series of washes, grids were successively stained with uranyl acetate and Reynold's lead citrate before visualization with a transmission electron microscope. Electron micrographs were scanned using a digital imaging system.