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Evidence for nitrite reductase activity in intact mouse Leydig tumor cells

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ABSTRACT

Nitric oxide (NO) supposedly derived via L-arginine–NO synthase (NOS) pathway has been implicated in inhibiting steroidogenesis by binding the heme moiety of steroidogenic enzymes. Previously, nitrite, and to a lesser extent nitrate ions inhibited steroidogenesis via NO by hitherto unknown reduction mechanism. Recently, a putative mammalian nitrite reductase activity ascribed to complex III of mitochondrial respiratory chain complexes (MRCC) has been reported, where MRCC inhibitors reduced NO production from nitrite variably. We thus studied the effects of MRCC inhibitors on testosterone production in mouse Leydig tumor cells (MLTC-1) without (basal) or with human chorionic gonadotropin (hCG) stimulation. In stimulated MLTC-1, MRCC inhibitors decreased testosterone production, order being: complex III (antimycin A and myxothiazol) > complex I (rotenone) > complex II (thenoyltrifluoroacetone), while cAMP production increased inversely. In unstimulated MLTC-1, MRCC inhibitors in same order, increased basal testosterone production, which correlated inversely with the percentage inhibition of NO production, with one exception; while antimycin A did not inhibit NO production in the nitrite reductase study mentioned above, it increased basal testosterone production in the present study. While MLTC-1 expressed mRNA for endothelial and neuronal, but not inducible NOS, various stimulators and inhibitors of L-arginine–NOS pathway had no effect on basal testosterone production in MLTC-1 or fresh Balb/c Leydig cells. Moreover, hCG increased nitrate uptake into MLTC-1, which suggests the gonadotropin aids nitrite and nitrate ions in their steroidogenesis inhibitory activity. In conclusion, this study supports the existence of a surrogate mammalian nitrite reductase and the dormancy of L-arginine–NOS pathway in MLTC-1.

1. Introduction

Quite accidentally, it was found that a buffer containing nitrate ions inhibited steroidogenesis in mouse Leydig tumor cells (MLTC-1) *in vitro* [1]. On equimolar parity, nitrite ions were nearly six-fold more potent than nitrate, and rats fed these ions in drinking water for 4 weeks had decreased levels of steroid hormones [2]. The search for a possible inhibitory mechanism led to nitric oxide (NO), the renowned vasodilatatory molecule, which had been reported to inhibit steroidogenesis by binding to heme moiety of cytochrome P450 steroidogenic enzymes. The inclusion of NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxy-3-oxide (c-PTIO) increased the yield of testosterone, thus confirming that NO indeed was the inhibiting agent [2]. Although NO synthesized from L-arginine by nitric oxide synthase (NOS) has been ascribed a modulatory role in steroidogenesis (for review see ref. [3]) the studies reporting its involvement in Leydig cells either used NOS inhibitors in cellular preparations contaminated with other cells, including

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macrophages [4], which are well endowed with NOS especially inducible NOS (iNOS), or used NO donors [5], which operate via NOS-independent mechanisms.

Nitrate and nitrite ions are reversibly converted to NO and vice versa *in vivo* in mammalian species [6]. The conversion to NO can occur non-enzymatically under pathological situations of ischemia and tissue necrosis where acidic conditions prevail [7]. The conversions also occur enzymatically. In plants, fungi and lower organisms specific nitrate and nitrite reductases are abundant. Nitrate reductase in *Neurospora* is a flavoprotein containing molybdenum and cytochrome b, and employs NADPH as an electron donor [8]. In *Escherichia coli* nitrite reductase converts nitrite to ammonia, and in green plants the reaction involves ferredoxin as a reductant [9]. Xanthine oxidoreductase catalyses the reduction of therapeutic organic nitrate, nitroglycerin, and inorganic nitrate and nitrite to NO under hypoxic conditions in the presence of NADH [10]. Heme containing proteins such as deoxyhemoglobin, myoglobin, soluble guanylyl cyclase, cytochrome P450 or mitochondrial cytochromes can also perform the reduction function [11]. For example, deoxyhemoglobin reduces nitrite as follows: NO_2^- (nitrite) + $HbFe^{2+}$ (deoxyhemoglobin) + $H^+ \rightarrow HbFe^{3+}$ (methemoglobin) + NO + OH^- followed by NO + $HbFe^{2+}$ (deoxyhemoglobin) → $HbFe^{2+}NO$ (iron-nitrosylated hemoglobin) [11]. Recently a putative mammalian nitrite reductase activity was ascribed to the oxidant site of ubiquinol at the cytochrome bc1 of complex III in the mitochondrial respiratory chain [12]. Employing submitochondrial fractions under cell free conditions, the authors found, rotenone, thenoyltrifluoroacetone (TTFA) and myxothiazol, which, respectively, inhibit complex I, II and III of the mitochondrial respiratory chain, caused 60%, 40% and >90% inhibition of NO production from nitrite ions under anaerobic conditions.

If the respiratory chain inhibitors (RCI) indeed interfere with the mitochondrial nitrite reductase and lower cellular NO production, then based on our previous studies [1,2], they may be expected to increase testosterone synthesis in Leydig cells. The premise that testosterone synthesis is sensitive to intracellular NO provides a unique opportunity to shed more light on the two NO production pathways. We have studied the effects of various stimulants and/or inhibitors of L-arginine/NOS or nitrite reductase pathways on the testosterone production in Leydig cells. Our tentative results particularly with regard to the basal testosterone production in MLTC-1 cells support the existence of an autonomous NO synthesizing mechanism, which may be due to mitochondrial nitrite reductase. Moreover, despite expressing the mRNAs for endothelial NOS (eNOS) and neuronal NOS (nNOS), but not inducible NOS (iNOS), L-arginine–NOS pathway was essentially inactive in MLTC-1 cells, as has recently been reported for rat Leydig cells [13], and further answers some reservations we expressed against the pathway [1,2].

2. Materials and methods

Fetal bovine serum (FBS), RPMI-1640, L-glutamine, penicillin, streptomycin, and trypsin-EDTA were obtained from Invitrogen (Gaithersburg, MD, USA). MLTC-1 and RAW (mouse macrophage) cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Tissue culture wares were obtained from IWAKI (Tokyo, Japan). Balb/c mice were obtained from a colony maintained in the Laboratory Animal Services Centre at our institution. Bovine serum albumin (BSA), human chorionic gonadotropin (hCG—3000 IU/mg, purified from pregnancy urine), Hanks balanced salt solution (HBSS) with or without calcium and magnesium, sodium salt of cAMP, various nitrite salts, antimycin A, myxothiazol, rotenone, TTFA, aminoguanidine (AG) bicarbonate salt, L-arginine, *asym*-dimethylarginine (ADMA), N^G-monomethyl-L-arginine acetate salt (NMMA), lipopolysaccharides (LPS) from *Escherichia coli* and collagenase Type I were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Other analytical grade chemicals, diethyl ether, hydrochloric acid, and ethanol were obtained from Merck (Darmstadt, Germany) or Sigma Chemical Co. [1,2,6,7-^{3}H]-Testosterone (specific activity 94 Ci/mmol) and [8-^{3}H]-cAMP, ammonium salt (specific activity 29 Ci/mmol) were obtained from Amersham International plc (Amersham, UK). The Division of Steroid Endocrinology of the University of Leeds (Leeds, UK) provided the testosterone antiserum for radioimmunoassay. The cAMP binding protein was purified from fresh bovine muscle obtained locally. The RNeasy Mini total RNA isolation kit was obtained from Qiagen (Valencia, CA, USA). Primers for the RT-PCR experiment were synthesized by Operon Biotechnologies Inc. (Huntsville, AL, USA). The primer sequences for the three mouse NOS isoforms and their expected product sizes in base pairs (in parenthesis) were: nNOS forward: 5′-TCATGCTGCCATCCCATCAC-3′, reverse: 5′-TGCTTGGCGCCATAGATGAG-3′ (217 bp) [14]; iNOS forward: 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′, reverse: 5′-GGCTGTCAGAGCCTCGTGGCTTTGG-3′ (497 bp) [15]; eNOS forward: 5′-TTCCGGCTGCCACCTGATCCTAA-3′, reverse: 5′-AACATATGTCCTTGCTCAAGGCA-3′ (340 bp) [16]; L19 forward: 5′-GAAATCGCCAATGCCAACTC-3′, reverse: 5′-TCTTAGACCTGCGAGCCTCA-3′ (395 bp) [17]. Access One-Step RT-PCR system and thermocycler were obtained from Promega (Madison, WI, USA) and MJ Research (Watertown, MA, USA), respectively. 6-Methoxy-*N*-(3-sulfopropyl) quinolinium (SPQ) was obtained from Molecular Probes Inc. (Eugene, OR, USA). The DeltaScan Illumination System and the Felix™ software for monitoring real-time cellular chloride/nitrate flux were obtained from Photon Technology International (PTI) (Monmouth Junction, NJ, USA). The epifluorescence microscope fitted with a SPQ dichroic cube (obtained from PTI) and the stage adapter was from Nikon Corporation (Tokyo, Japan). The temperature controlled perfusion system was obtained from Warner Instrument Inc. (Hamden, CT, USA).

2.1. Buffers

The 145 mM chloride ion buffer HEPES-phosphate-buffered Ringer (HPBR) contained 135 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.83 mM Na_2HPO_4, 3.33 mM NaH_2PO_4, 5 mM HEPES, 10 mM glucose, and 0.5% BSA (pH 7.4). A 145 mM nitrite ion buffer was prepared by substituting the chloride ions in HPBR for similar cationic nitrite salts. The 10 mM nitrite ion buffer was prepared by mixing appropriate volumes of the aforementioned buffers.