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ORIGINAL ARTICLE

Involvement of the mesenteric ganglia on androstenedione, noradrenaline and nitrite release using a testis ex vivo system

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Abstract The autonomic nerve fibres converge to the testis along two major pathways, the superior spermatic nerve (SSN) and the inferior spermatic nerve (ISN). The object of this work was to evaluate whether the addition of noradrenaline (NA) in the ganglionic compartment of two ex vivo systems: superior mesenteric ganglion (SMG)–SSN–testis, inferior mesenteric ganglion (IMG)–ISN–testis modulate androstenedione (A_2), NA and nitrite release and to determine whether there are secretory differences between the right and the left testis. Each gonad with its respective ganglion was transferred into a cuvette with two compartments and incubated in a Dubnoff metabolic shaker. The testis incubation liquids were collected and analysed for NA by HPLC, A_2 by RIA and nitrites by the Griess method. When NA is added to the IMG, A_2 and NA release diminishes and nitrite increases in the left testis, while in the right gonad, A_2 and NA increase and nitrite decreases. When NA was administered to the SMG, A_2 and NA increase and nitrite diminishes in the left gonad, but they show opposite fluctuations in the right testis. These ex vivo systems appear to be excellent models for studying the sympathetic ganglionic control of the testis though A_2, NA and nitrite release from the male gonad. It is evident that a better knowledge about the role of catecholamines and nitric oxide in the testis physiology may facilitate the understanding of some reproductive diseases.

Keywords Testis · Mesenteric ganglia · Nitric oxide · Catecholamine · Peripheral nervous system

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Introduction

Previous studies have indicated that blood testosterone levels in males are not only controlled by hypothalamic gonadotrophin-releasing hormones through pituitary hormones but also by a multisynaptic pathway connecting the sympathetic ganglia to the testis. In adult male rats, only left-sided deafferentation combined with left orchidectomy resulted in decreased testosterone production, while in prepubertal male rats, only right-sided brain surgery plus left orchidectomy resulted in a significant decrease in basal testosterone secretion of the remaining testis [1]. Lee et al. [2] have demonstrated that a neural hypothalamic testicular pathway interferes with Leydig cell function independently of the pituitary. Chow et al. [3] examined whether disruption of neural input to the testis contributes to the cascade that leads to the regression of spermatogenesis. In the rat, regression of spermatogenesis during the chronic stages of spinal cord injury occurs in the presence of a normal function of the pituitary–testis axis, suggesting that a non endocrine mechanism might be involved. There is evidence that the peripheral nervous system also controls the hormonal release from the testis through a catecholaminergic pathway [4–6].

Pharmacological and denervation experiments have documented the importance of autonomic innervation, especially of the adrenergic tone in the maintenance and

development of the reproductive organs, both in males [6–8] and in females [9–11]. Several types of receptors for catecholamines exist in the gonads in vascular or endocrine cells, and their activation can alter blood flow, steroidogenesis and gene expression, depending on the target cells [7]. Mhaouty-Kodja et al. [12] found that adrenoreceptor α1-signalling plays a critical role in the control of male fertility, spermatogenesis and steroidogenic capacity of Leydig cells. Also adrenergic β-receptors have been described in these cells [13].

On the other hand, Andric et al. [14] have found that testosterone up-regulates nitric oxide (NO) signalling via increasing nitric oxide synthase (NOS) expression and contributes to the down-regulation of the cGMP signalling pathway and androgenesis in the rat Leydig cells.

Nitric oxide is synthesized by NOS. Three traditional NOS, specified as endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS), and one testis-specific nNOS (TnNOS) are found in the testis. Of these, eNOS and iNOS have been recently shown to have putative function regulation properties. The NOS/NO relationship participates in controlling the levels of cytokines and hormones in the testis [15].

The autonomic nerve fibres converge to the testis along two major pathways, the superior spermatic nerve (SSN) and the inferior spermatic nerve (ISN). The first one reaches the testis from the superior mesenteric ganglion (SMG) and the renal plexus along the testicular artery, whereas the ISN originates in the pelvic or inferior mesenteric ganglion (IMG), accompanies the vas deferens and penetrates into the epididymis and the testis [16–18]. In the rat, this innervation presents neurons that originate in the sympathetic prevertebral ganglionic chain. Postganglionic catecholaminergic fibres have been described around the testicular capsule and blood vessels [19]; however, there is little available information about the participation of the superior or the inferior mesenteric ganglia in testicular steroidogenesis and liberation of NA and nitrites.

In order to further explore these topics, in this work, we have evaluated whether the addition of NA in the ganglionic compartment of the two studied ex vivo systems modulates A_2, a testosterone precursor in its biosynthesis, NA and nitrite release. Also, we have evaluated possible differences in the secretion of these substances between the right and the left gonad.

Materials and methods

Animals

Adult male rats of the Wistar strain, 90–100-days-old, weighing 300 ± 50 g, bred in our laboratory were used throughout this protocol. They were housed under controlled light (lights-on from 07:00 to 19:00 h) and temperature (24 ± 2°C) conditions; water and food were provided ad libitum. Animals were handled according to the procedures approved in the UFAW Handbook on the Care and Management of Laboratory and other Research Animals [20] and the Committee for the Use and Handling of Animal for Research and Education of the Medical School of Cuyo National University, Mendoza, Argentina.

Chemicals reagents

The following drugs: L-noradrenaline bitartrate (NA), ascorbic acid, bovine serum albumin fraction V (BSA), sulphanilamide and *N*-1-naphthyl-ethylenediamine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other reagents and chemicals were of analytical grade. 1,2,6,7-[^{3}H] androst 4-ene-3,17-dione (115.0 Ci/mmol) direct solid phase radioimmunoassay (Coat-A-Count kit) was purchased from DPC Inc. (Los Angeles, CA, USA).

Experimental procedures

Groups of six adult animals were used in each experimental group. Each system, formed by the corresponding plexus, identified by its anatomical position, and the corresponding testis, was carefully removed with the assistance of a stereomicroscope. Briefly, the tissues were removed under an atmosphere of ether anaesthesia under a bell. Surgical operations and standardization of incubation conditions of the SMG–SSN–testis and IMG–ISN–testis systems were performed as previously reported [6]. Surgery was carried out between 14:00 and 15:00 h. After cleaning with the incubation medium, the organs were transferred into a cuvette with two compartments, one for the ganglion and the other for the testis, and incubated in a Dubnoff metabolic shaker in an atmosphere of 5% CO_2 in 95% O_2 at 37°C. Each testis compartment contained 20 ml of Krebs–Ringer bicarbonate buffer, pH 7.4, with the addition of glucose (0.1 mg/ml) and albumin (0.1 mg/ml). The ganglionic compartment contained the same solution but only in 2 ml, as described for incubation in other ex vitro systems [21, 6].

For stabilization of the systems, ganglia were pre-incubated for 15 min. The end of the preincubation period was considered as incubation time 0. At this time, the buffer was changed in both compartments and ascorbic acid (0.1 mg/ml in Krebs–Ringer) was added as an antioxidant agent to the ganglionic compartment [22]. Periodical extractions (500 µl) were made in the testis compartment for determination of A_2, 500 µl for catecholamines and 50 µl for nitrites at 15, 30, 60, 90, 120 and 180 min.

The values of A_2, NA and nitrites released under these conditions were considered as control (control groups). For the experimental groups, the noradrenergic agent was added to the ganglionic compartment. NA was dissolved (10^{-6} M) in the Krebs–Ringer's solution plus ascorbic acid. The samples of liquid removed for A_2 and nitrites assays from the testis compartment were stored in a freezer at −20°C until determinations and NA was stored at −80°C.

Assay procedures

Androstenedione assay

For measurement of A_2, a direct solid phase radioimmunoassay (Coat-A-Count kit) purchased from DPC Inc. (Los Angeles, CA, USA) was used. Androstenedione- antiserum cross–reactivity with 5α-dihydrotestosterone was less than 4% and less than 0.6% with testosterone and other natural steroids. Neither lipemia, nor bilirubin or haemolysis interfered with the assay. The inter assay was 6–11% and the intra assay 4.5–10%, respectively, for the low dose point. The results were expressed as ng of A_2 per mg of testicular tissue per ml of the incubation medium (A_2 ng/mg testis/ml) against time of incubation. The corresponding corrections were made in all cases, taking into consideration the volume extracted from the testis compartment in each tested period.

Catecholamine assay

For NA measurements, 20 µl aliquots from the testis compartment were partially purified by batch alumina extraction, separated by reverse-phase high pressure liquid chromatography (HPLC) using a 4.6 mm × 250 mm Zorbax RxC18 column (Du Pont, USA) and quantified by a current procedure upon exposure of the column effluent to oxidising and then reducing potentials in series using a triple-electrode system (Coulochem II, ESA, Bedford, MA) [23]. Recovery through the alumina extraction step averaged 70 and 80% for catecholamines. Catechol concentrations, in each sample, were corrected for the recovery of an internal standard dihydroxybenzylamine. The results were expressed as nanograms of catecholamine per milligram of testis tissue per ml (NA ng/mg testis/ml). The detection limit of the assay was about 15 ng per volume assayed. The electrochemical response was linear ($r = 0.99$) for amounts of NA or from 50 to 2,000 pg. The inter-assay variation coefficients were 14 and 15% and intra-assay variation coefficient was 10% for NA incubation and NA in ng of catecholamines per milligrams of testis tissue per ml (ng/mg testis/ml).

Nitrite assay

For determination of nitrites, a water-soluble metabolite of nitric oxide was measured spectrophotometrically by the Griess method [24] and expressed in nanomols of nitrites per milligrams of testis tissue per ml (nmol/mg/ml). Samples (50 µl) were immediately mixed with Griess reagent (sulphanilamide with *N*-1-naphthylethylenediamine/HCl). After a 10-min incubation period at room temperature, they were read for absorbance at 540 nm, and the nanomols of nitrite were determined using a standard curve. The assay sensitivity was less than 2.5 nmol/ml. The intra-assay coefficients variation for all the assays was less than 10.0%.

Statistical analysis

The concentrations of A_2, NA and nitrites were corrected according to the sampling volume in each point. Comparisons between the means of the two groups, control and experimental were carried out using the Student's *t* test. A value of $P < 0.05$ was accepted as being statistically significant [25].

Results

Androstenedione, NA and nitrites (soluble metabolite of nitric oxide) were released in the testis compartment by the NA stimulus (10^{-6} M) in the ganglionic compartment and assessed in the liquid obtained from the testis compartment.

Androstenedione, NA and nitrite concentrations obtained from the testis compartment after NA administration in the IMG compartment using the IMG–ISN–left or right testis system

In the left testis, A_2 diminished in all the determinations ($^*P < 0.001$) (Fig. 1, left top). In the right gonad, results were in general opposite to the ones in the left testis: A_2 increased at 30, 60, 90 and 180 min ($^*P < 0.001$) and at 120 min ($^{**}P < 0.01$) (Fig. 1, right top).

In the left testis, NA diminished at 15, 120 and 180 min. ($^*P < 0.001$) and at 30, 60 and 90 min ($^{**}P < 0.01$) (Fig. 1, left bottom), while in right testis it increased at all studied times ($^*P < 0.001$) (Fig. 1, right bottom).

When nitrites were evaluated in the IMG–ISN–left testis, they increased at 15, 120 and 180 min ($^*P < 0.001$) and at 30 min ($^{**}P < 0.01$) (Fig. 3, left top) and decreased at 15, 30, 60, 120 and 180 min ($^*P < 0.001$) in the right testis (Fig. 3, right top).

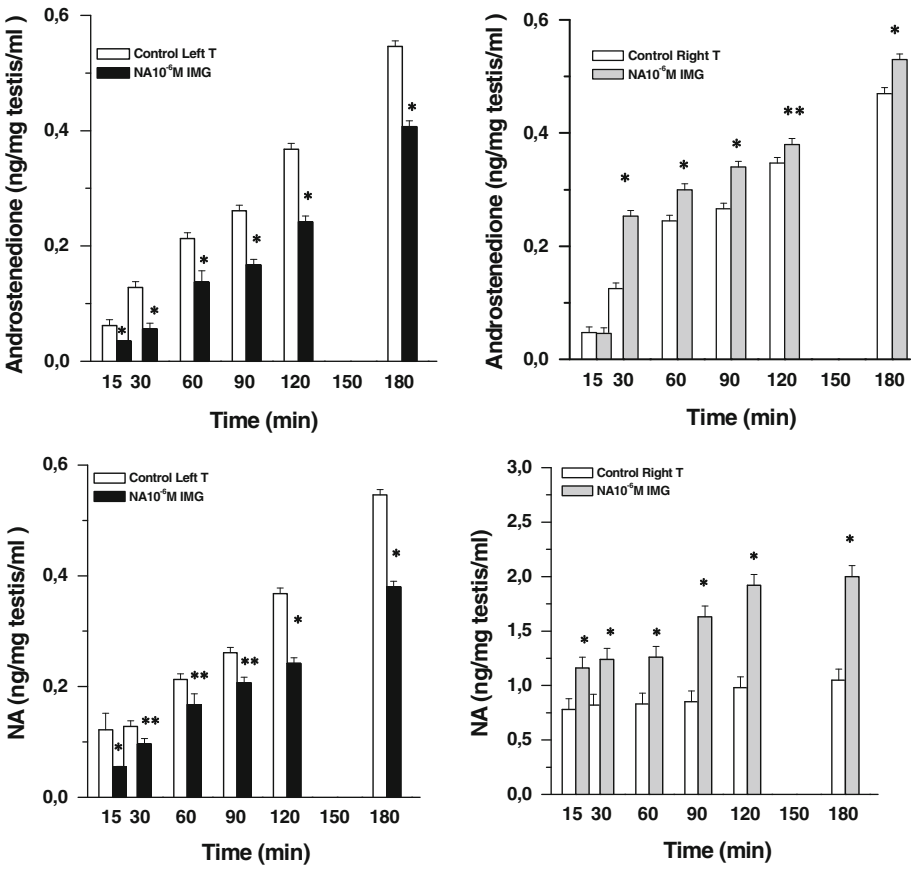

Fig. 1 Effect of noradrenaline (NA) administered in the ganglionic compartment on androstenedione and NA released in the incubation liquid of the testis compartment in the inferior mesenteric ganglion–ISN–left testis system (*left*) and right testis system (*right*). *Bar* represents the mean ± SEM of six animals per experimental group. $*P < 0.001$; $**P < 0.01$ (Student's *t* test)

Androstenedione, NA and nitrite concentrations obtained from the testis compartment after NA administration in the SMG compartment using the SMG–SSN–left or right testis system

Androstenedione increased at 90 and 180 min ($*P < 0.001$) and at 120 min ($**P < 0.01$) in the left testis (Fig. 2, left top). In the right gonad, results were in general opposite to those found in the left testis: A_2 decreased at 15, 30, 120 and 180 min ($*P < 0.001$) and at 60 min ($**P < 0.01$) (Fig. 2, right top).

NA increased at 15, 60 and 180 min ($*P < 0.001$) and at 30 and 90 min ($**P < 0.01$) in left testis (Fig. 2, left bottom) and decreased at 15, 30 and 120 min ($*P < 0.001$) and at 60 min ($**P < 0.01$) in the right testis (Fig. 2, right bottom).

In the SMG–SSN–left testis system, nitrites decreased at 120 and 180 min ($*P < 0.001$) (Fig. 3, left bottom) and increased ($*P < 0.001$) at all studied times in the right gonad (Fig. 3, right bottom).

Discussion

The hypothalamic–pituitary–gonadal axis is crucial for the physiological function of the gonads but non-endocrine regulatory influences, such as neurotransmitters and nitric oxide found within the gonads, also exert an essential role [14, 26, 27]. All these studies suggest that steroid secretion does not only depend on gonadotrophin secretion but is also regulated by neural inputs from the peripheral nervous system.

This study provides a physiological demonstration of changes in the release of androstenedione, a precursor in the biosynthetic pathway of testosterone from the testis, and of NA and nitrite in the left or right testis compartment after the addition of NA in the ganglionic compartment of two ex vivo systems, SMG–SSN–testis and IMG–ISN–testis.

These two ganglia are innervated, among others, by fibres of adrenergic nature that come from the spinal medulla and pre-aortic ganglia [28, 29]. Secondary neurons, such as the small intense fluorescence cells, liberate intraganglionar NA and other neurotransmitters that are co-localised in these inter-neurons [30–35]. Results clearly indicate that this response depends not only on the stimulated ganglion but also on the gonadal side localization.

In our research group, similar changes have been previously observed in adult female rats on progesterone release [9–11, 36]. Moran et al. [37] showed an apparent asymmetry in the activity of neural connections between

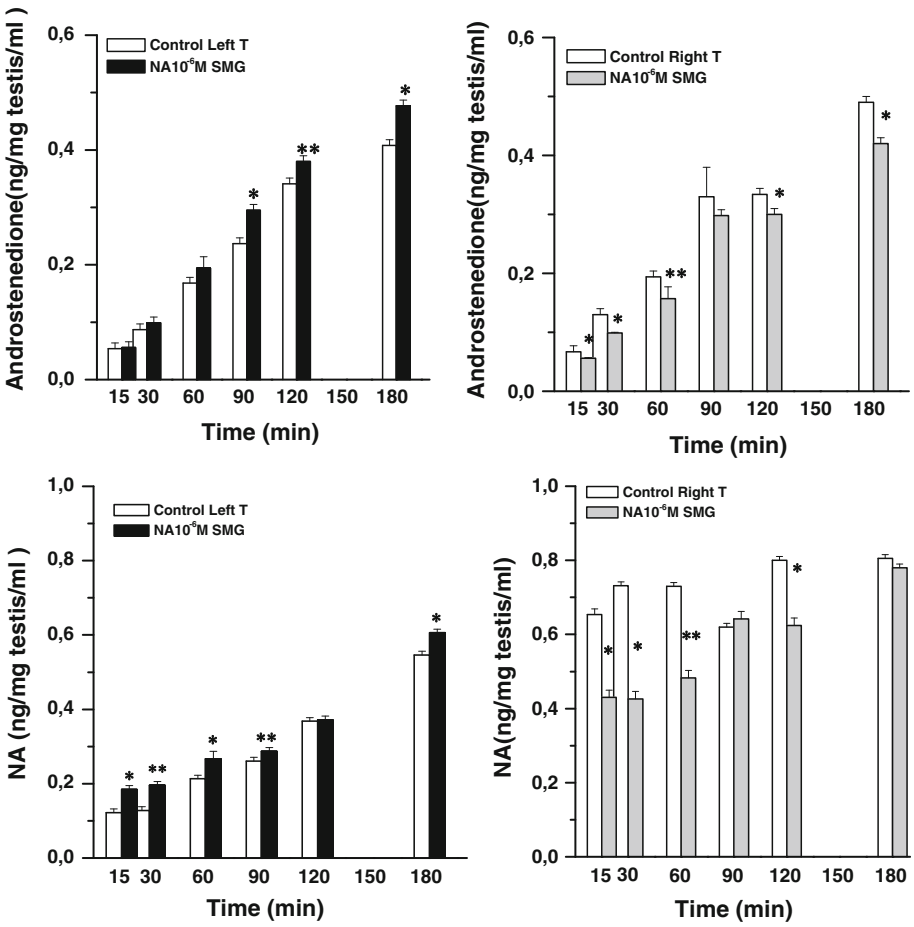

Fig. 2 Effect of noradrenaline (NA) administered in the ganglionic compartment on androstenedione and NA released in the incubation liquid of the testis compartment in the superior mesenteric ganglion–SSN–left testis system (*left*) and right testis system (*right*). *Bar* represents the mean ± SEM of six animals per experimental group. $*P < 0.001$; $**P < 0.01$ (Student's *t* test)

ovaries and the prevertebral coeliac–superior mesenteric ganglia. Morales et al. [38] suggested an asymmetric regulation of steroid hormones secretion by the vagal innervations in animals with unilateral ovariectomy.

On the other hand, Selvage et al. [39] and De Bortoli et al. [40] have reported anatomical and functional evidence for a direct, inhibitory neural pathway that regulates testosterone or progesterone secretion independently of the pituitary in male [39] and female rats [40]. They found that this pathway is activated by the intracerebroventricular (icv) administration of NA and found a decrease of progesterone release in female rats [40]. The fast effect of the icv-administered NA on the A_2 secretion suggested that this response was dependent on a neural pathway rather than a gonadotrophic effect.

In a previous contribution we reported that α and β adrenoceptor antagonists in ganglion modified testosterone release in the SMG–SSN–testis system [6]. We should clarify that it was testosterone, rather than A_2, that changed under the antagonists influence. Such finding provided evidence that the mesenteric–coeliac ganglionic complex possesses both types of receptors in the male rats, as it has been previously described for other sympathetic ganglia [41, 42].

Our results show that when NA is added to the different ganglia, the responses differ, this to say, in the IMG, A_2 and NA release diminishes and nitrite increases in the left testis, while in the right gonad, A_2 and NA increase and nitrite decreases. When NA was administered to the SMG, A_2 and NA increase and nitrite diminishes in the left gonad, but they show opposite fluctuations in both right testis. It is surprising that in the right testis catecholamine release is higher than in the left one. These results are consistent with other studies where it was shown that the electric stimulation of the SSN increased testosterone and NA concentrations in the spermatic vein blood [43]. Most probably the testosterone increment was due to the effect of NA released from the ganglionic terminals on Leydig cells [44]. In this study, we show changes in NA and A_2 release. We postulate that A_2 changes are due to the release of NA in the testis. Mayerhofer et al. [44] have informed that α and β adrenergic receptors have been reported in Leydig cells. It is well known that Leydig cells are the endocrine cells in the testis that synthesise and release not only testosterone

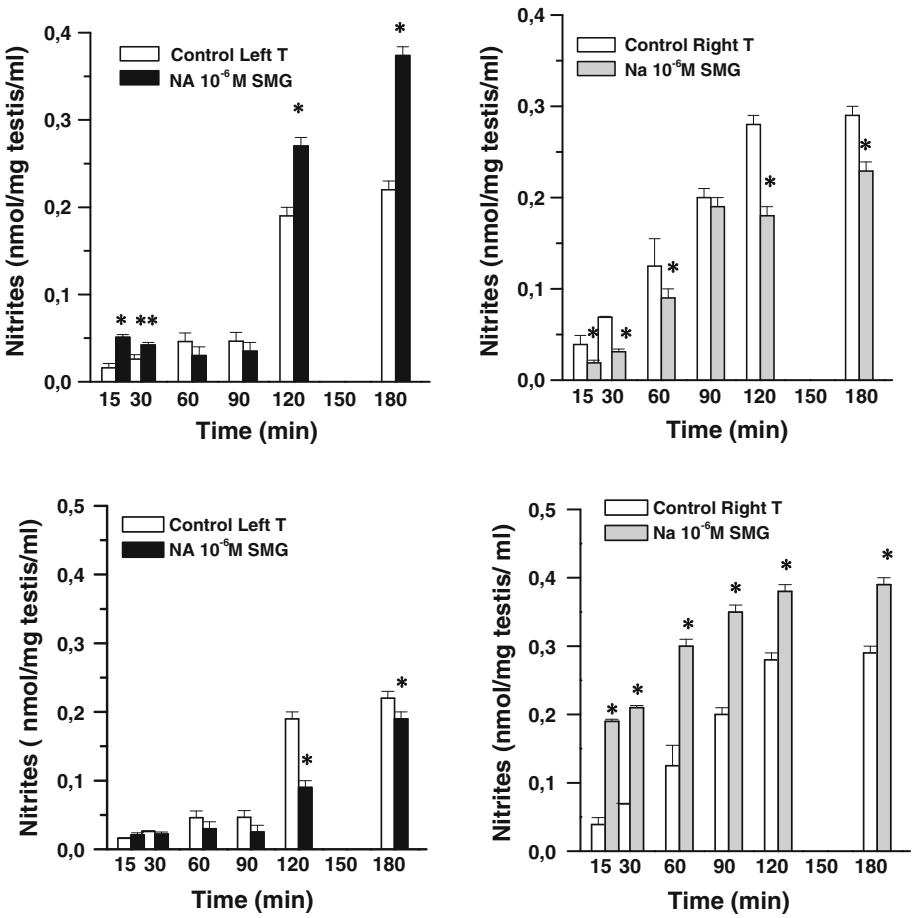

Fig. 3 Effect of noradrenaline (NA) administered in the ganglionic compartment on nitrites released in the incubation liquid of the testis compartment in the inferior mesenteric ganglion–ISN–testis (*top*) and superior mesenteric ganglion–SSN–testis system (*bottom*) in the left and right testis. *Bar* represents the mean ± SEM of six animals per experimental group. $^{*}P < 0.001$; $^{**}P < 0.01$ (Student's *t* test)

but also A_2, a testosterone precursor, due to NA stimulus. In vitro experiments have demonstrated the ability of NA in increasing testicular steroidogenesis [13, 44–46].

The mechanism by which NA influences androstenedione release in the testis is yet unknown although it has been proposed that the intraganglionar neurons may generate signals in the spermatic nerve terminals that eventually release NA in the testis [4, 8]. In fact, Frungieri et al. [26] confirmed that the testicular innervation in monkey includes two components, innervation through intrinsic neuron-like cells and extrinsic fibres, both of which are catecholaminergic or peptidergic in nature. Nerve fibres represent mainly the extrinsic innervation and were observed at all ages although they become more prominent after puberty. An additional indication that supports the neural hypothesis that was established by Chow et al. [3].

According to the above-mentioned observations, the A_2 changes in the testis compartment could be due to other stimulatory or inhibitory neurotransmitters such as NO or others. These neurotransmitters, including NO, have been found in ganglia [32–35]. In addition, NOS, responsible for NO synthesis, which has been found in Leydig cells [14, 15] and ganglia [35, 47, 48] might indicate its participation in steroidogenic events of the testis in the same way as occurs in the ovary [47, 48]. NO synthesis decreases and catecholamines increase were observed in interstitial cell preparations from rats exposed to stress. These results indicate both stimulatory and inhibitory effects on NO production [27]. Coincidently, our results also indicate NA increase and nitrite reduction. These apparent contradictions could partly explain the opposite behaviour observed in the left or the right testis herein explored. Our present results also indicate a differential response between the SMG and the IMG. A similar differential response in the female gonad has been previously reported by our research group and others [36, 49].

In conclusion, these ex vivo systems appear to be excellent models for studying the sympathetic ganglionic control of the testis through catecholaminergic stimulus in the male gonad. The testis showed a clear differential response according to the stimulated neural connections. We also report an asymmetric behaviour between the left and the right testis.

Mayerhofer et al. [44] found by immunohistochemical methods neuron-like catecholaminergic cells in adult men testicular biopsies without testicular pathologies and from infertile patients with either Sertoli cell only syndrome or severe hypospermatogenesis and germ cell arrest. The

authors detected in the mentioned pathologies significantly increased immunoreactive cell bodies and nerve fibres. They concluded that this neural type may complement and act in concert with the testicular sympathetic innervation. Our results, also related to catecholaminergic findings, reaffirm the importance to continue this research line using new approaches and searching for other neurotransmitters or including ex vivo systems.

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